

A  
PHYSICAL  
THEORY  
OF THE  
LIVING  
STATE

GILBERT  
WENGBING LI





A PHYSICAL THEORY OF THE LIVING STATE:  
the Association-Induction Hypothesis

A BLAISDELL BOOK IN THE PURE AND APPLIED SCIENCES

*Consulting Editor*

P A U L R . G R O S S , *Brown University*

# A PHYSICAL THEORY OF THE LIVING STATE:

## the Association-Induction Hypothesis

with Considerations of the Mechanisms Involved  
in Ionic Specificity, Behavior of Proteins, Selective  
Accumulation of Ions and Nonelectrolytes, Cellular  
Electrical Potentials, Ionic Permeability and Diffu-  
sion, Excitation and Inhibition, Contractile Mech-  
anism, Enzyme Action, Drug and Hormone Action,  
Antibody-Antigen Reaction, Fertilization, Chemical  
Embryology, Growth, Differentiation, and Cancer

GILBERT NING LING



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*Dedicated to*

my father, Yen-tse Ling

凌宴池先生

my mother, Chi-lan Ling

凌启南夫人

and

my wife, Shirley W. Ling

凌本慎女士



# P R E F A C E

Over a dozen years ago, Gilbert Ling arrived at my laboratory at the University of Chicago as a Boxer Fellow from China to start his graduate studies. At that time, the capillary electrode was being developed and he entered vigorously into its improvement and use in measuring membrane potentials. Extensive experiments directed toward analysis of the metabolic events maintaining these potentials led to the conclusion that the frog *sartorius* muscle fiber retains a considerable membrane potential despite the blocking or suppression of all known metabolic processes. The potential and the asymmetric concentrations of ions inside and outside the muscle fiber with which the potential is associated must then be a static rather than a dynamic or flux type of equilibrium.

Ions, Ling reasoned, distribute themselves between the protoplasm and the intercellular phases in accordance with a pre-existing standard free-energy difference established by the architecturally maintained pattern of fixed charges. The protein skeleton of the cytoplasm, with its ionic sites, would bind free ions; on thermodynamic grounds, potassium would be the more firmly associated cation. This was the heart of Ling's "Fixed-Charge Hypothesis." It led to many experiments, successfully performed, which indeed indicated that no pump or semipermeable membrane is required to maintain the ionic inequalities inside and outside the cell. I attempted to summarize this phase of the theory for Ling at the Wisconsin Conference on "Metabolic Aspects of Transport Across Cell Membranes." In the intervening years, this relatively simple and static picture has been enriched in many directions. It has been applied in this monograph to the most varied phenomena of living systems.

The performance of living systems depends on their organized and dynamic heterogeneity, itself produced by the system's own early activity at the level of subordinate units in response to continuing environmental stimuli. The parallel-cylinder organization of muscle fibers, fibrils, and protein threads makes possible the functional shortening and thickening of contraction and the resultant forces

and movements of larger structures; but the organization of these fibers and fibrils is the result of previous activity of macromolecules, molecules, and ions, and demands the existence of an organized heterogeneity also in this micro domain. Biologists have advanced the understanding of structures in organisms, through organs, cells, and organelles, to the resolution limits of electron microscopy. Chemists have revealed the scaffolding from electron-shelled atoms, through simple complex molecules, to the great polymeric macromolecules.

It is in the realm between macromolecule and protoplasm that Ling's contribution falls. His formulation attempts to bring order into the confusion of phases within and without organized protoplasmic structures, much as W. Gibbs succeeded in bringing order out of the confusion of the behavior of solutes in multiphased simpler systems.

Life is, indeed, "a thing of watery salt," enmeshed in a macromolecular framework. Its rich play of behavior emerges from the inhomogeneities of its simplest components as these become magnified through interaction and combination, just as the full richness of language depends on the component letters and phonemes and their ordering. If the stuff of life is built from water, simple ions, molecules, and macromolecules, then particularities which seem trivial at the molecular level must magnify into singularities of great moment at higher levels of organization.

The courageous insight of Arrhenius gave us ions and the explanation of conductivity, of the reactions of acids and bases, of colligative properties, and of much more in physical chemistry. But "concentration" had to be corrected to "activity"; ions became hydrated; charges were no longer randomly distributed in a volume but achieved an electrostatic organization; the spatial distribution of charges within ionized or ionizable molecules became important; actual distances and shapes came to control interaction possibilities; and so on through the distinguished contributions of Bjerrum, Debye, Hückel, Linderstrøm-Lang, Kirkwood, and many others. Ling has taken a further step in examining the details of organization of the proteins and their binding sites and of the simple ions and their adsorption energies. His work builds on that of the physical chemists and lays a foundation for that of the biological morphologists. As ions determine collagen fiber pattern periods and these periods allow calcium precipitation only when of certain values, so do the fiber patterns determine cell growth and architecture and, indeed, also feed back to control ionic partition and action.

Ling derives such a wealth of biological phenomena from the interaction of proteins and ions that the question inevitably arises as to whether his theories are so general and unspecific that they would fit anything and are therefore of little value. That this is not the case is, however, obvious to the student of his presentation. In the great majority of cases, he has worked out the consequences of his formulation in a theoretically rigorous and numerically sharp manner and

has compared these expectations with extensive quantitative data in the literature as well as with his own critically designed experiments. Mostly the agreement is frighteningly good. Further, it is simple biological knowledge, since Ringer's solution, the Hofmeister series, and the like, that the simple salt ions do profoundly influence almost every attribute of living matter that has been examined. Moreover, if nucleic acids are concerned with synthesis and the proteins with function, as current research indicates, then the properties of proteins and the influence of differences in these properties upon salt ions must, indeed, be expected to underlie behavior.

Knowledge starts with the identification of material units and progresses to their dissection, or morphology, and their grouping, or taxonomy. Only later do functional units come to attention, and still later, developmental ones. This sequence is likely to repeat itself at each level of organization, from the individual up to the various sized groups and down to the organs, cells and molecules. Physiology has largely concerned itself with a description of the functional units associated with organs and, more recently, tissues and cells—gland secretion, muscle contraction, nerve conduction and, less centrally in traditional physiology, differentiation and growth and the like. Yet all cells and functions are no more than special cases of the generalized cell and protoplasm and of their properties. Thus, there must be some very comprehensive and basic principles at the molecular level that underlie and illuminate all the special manifestations of living systems. Ling offers no less than such a general molecular theory of life phenomena.

Finally, although Ling has introduced a number of concepts and assumptions, these are in no sense *ad hoc*, are not juggled to fit each case or exception, are mostly given a theoretical derivation and calculated precisely, and, once developed, account in each case for many varied phenomena.

Here, then, is a major synthesis, moving from the minor variables at the molecular level to the major properties of the cellular level. Whether or not entirely right, or still in need of major revisions, such an integrative and synthetic treatment cannot fail to give the whole field of biology a great forward impetus.

R. W. Gerard

Ann Arbor, Michigan

May, 1960



# FOREWORD

Sometime in the course of human history, man began to ask the questions: What is the world outside? What is man himself? These questions eventually led to the development of the sciences of physics and chemistry on the one hand, and the science of biology on the other.

With respect to external physical objects, man made the basic assumption that there is a universality in the principles governing their behavior. To elucidate these universal principles, he has evolved the method of choosing the simplest system possible as the subject of his study. Thus, whether he chooses the motion of planets in the solar system or the path of an electron beam in a magnetic field, the ultimate exactness of the physical insight achieved is matched by the degree of simplicity of the system under study.

On the other hand, scientists in general have not been certain that the universality of physical principles does extend into the realm of biology. For example, at one time the concept of "vitalism" was advanced; according to this view, living systems operate on a set of principles quite different from those governing the inanimate world. Vitalism, however, did not long survive its first proponents. The majority of biologists, in their endeavors to understand the uniquely complex living world, adopted methods similar to those employed by physicists and chemists, particularly the method of simplification. Thus, organs were studied instead of organisms; cells were studied instead of organs; fragments of cells were studied instead of cells; crystalline proteins were studied instead of cell fragments. Had this not been done, the rich collection of information about the properties specific to the fundamental units of living matter would be locked forever in secrecy within the intact organism, the intact organ, or the intact cell. However, simplification and analysis alone are not sufficient to complete the biologist's task, for the ultimate simplification leads to physics. The eventual goal of biology must be the understanding of intact living matter *per se*.

Thus, at some point in the development of biology, another approach must be made, an approach that involves conceptual synthesis of the principles derived from studies of simple inanimate systems on one hand, and from specific properties of isolated components of living matter on the other. The present theory is an attempt in this direction.

For the complete verification of any general theory of living matter, it is necessary to show that it is consistent with all physiological manifestations of living systems. Since this is obviously impossible, we have limited this discussion to a number of observations dealing with a variety of physiological activities. Discussion in two major areas, genetics and what is usually called "active transport," originally intended for publication as part of the present monograph, has, after much deliberation, been omitted. There are several reasons for this decision. First, it was our desire not to jeopardize the presentation of the main theme of the theory by including secondary hypotheses which, though important, are not directly related to the bulk of the material. Second, the wealth of information now being accumulated in the chemistry of genetics is so vast that, within the time and space at the author's command, it is difficult, if not impossible, to do justice to both these findings and the present theory. These subjects will be discussed in a later publication.

The scope of this monograph forced the author to choose for discussion only experimental evidence about whose validity he felt confident, either through his own experience with the experimental methods of the area in question or through the consensus of the opinions of authors of reviews. As the content of the monograph will indicate, there is a large body of facts which are consistent with the theory. Where discrepancies exist in the literature or are yet to be discovered, it is hoped that these will serve to guide further revision and refinement.

In writing this monograph, the author appreciates the unusual opportunity he has had to gather into a single volume examples of the major accomplishments in the various specialized fields discussed. To exploit this opportunity fully, he has included as many figures as is consistent with the purpose of the book. Because the average reader may not be familiar with the background material of all subjects discussed, a glossary at the end of the book and a general bibliography at the end of each chapter (not to be confused with the complete list of references at the end of the volume) have been provided. The former will primarily assist the reader in unfamiliar territory; the latter will provide him with other current views which may serve as a background for judicious evaluation of the present thesis. Such a general bibliography is bound to omit many important articles and give inadequate commentary on many of those included. It is hoped that the reader will point out such oversights so that corrections may be made in future editions.

The development of the theory and the completion of the book in its present form have encompassed more than ten years. To acknowledge each of the many

people who contributed to this work is, unfortunately, impossible. The author therefore, takes this occasion to thank only those who have been most closely involved in this venture.

To his professor in graduate school, Dr. Ralph W. Gerard, the author owes a debt of gratitude for his teaching and for providing a research environment of complete freedom for a number of years both at the University of Chicago and later at the Neuropsychiatric Institute of the University of Illinois, for his criticism of the author's work and for his suggestions, as well as for his constant friendship and encouragement. The author is also grateful to Dr. Stephen W. Kuffler for the three profitable years spent in his laboratory at the Wilmer Institute of Johns Hopkins University.

To Dr. Chen-ning Yang, the author owes a debt of gratitude for his indulgence in critically reading a book that is essentially biological and for freely giving of himself and his time in discussing the physical aspects of the theory. The author is also grateful to Dr. Tsung Dao Lee for many discussions through the years on aspects of physics related to the present theory.

Dr. Samuel B. Horowitz, the author's student and colleague, has devoted a considerable amount of his energy and time in the past five years to helping with the writing of the manuscript and participating in a countless number of valuable discussions. Other students and colleagues, Dr. Robert Fenichel and Mrs. Margaret Neville, although coming later to the author's laboratory, were indispensable to the completion of the book. The author would like to thank his present colleague, Dr. George Karreman who has taken great pains to go through the details of the mathematical calculations involved in the linear model presented in Chapter 4, pointing out several errors which have subsequently been corrected. Thanks are also due to Dr. George Eisenman and Mr. James U. Casby for many helpful discussions.

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Thanks are also due to Dr. Paul Gross, who, as consulting editor, introduced the manuscript to Blaisdell Publishing Co., and to members of the company, Mr. Warren Blaisdell, Mr. Joseph Byrne, Mr. James Gee and others, for their sympathy and cooperation.

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He also takes pleasure in expressing his appreciation to the two universities in which he was a faculty member during these periods for their support. The foundations of the present theory were laid at the Johns Hopkins Medical School, Baltimore, from 1951 to 1953; the better part of the theoretical calculations given in Chapter 4 and a considerable amount of experimental work were finished at the Neurophychiatric Institute of the University of Illinois at Chicago. The author would also like to acknowledge the financial support, of the Commonwealth of Pennsylvania, through the Eastern Pennsylvania Psychiatric Institute of the Department of Public Welfare.

Finally, the author wishes to take this opportunity to say a word of thanks to the many authors whose data and ideas he has either quoted or used, without directly quoting, as general background for the writing of this monograph. Their names are given in the References at the end of the book.

*Gilbert Ning Ling*

Philadelphia  
March, 1961

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# INTRODUCTION

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Being alive has more than one meaning. The biochemist Albert Szent-Györgyi (1951) stated that if reproduction is a unique qualification of life, two rabbits, a male and a female, are alive; one rabbit is not. For our purposes, a single rabbit and a pair of rabbits are all alive. Although animals can be described in terms of their gross physiology, they consist of more basic units of living material. We recognize as alive, for instance, the surviving cells of a long-dead rabbit. In a suitable environment such as tissue culture medium these cells and their descendants may survive almost indefinitely. But a slight alteration of this environment, such as a rise in temperature or a change in the ionic concentration of the medium, may bring about death. No change of environment can then restore life.

Surviving cells illustrate well the level of life that we shall discuss. This level includes a great diversity, ranging from free-living unicellular organisms to the highly specialized tissue cells of a human being. These diverse forms of life do not share an identical material composition, an identical environment, or an identical history. Rather, they share a common "state" that may be compared to the physical states (solid, liquid, and vapor) of inanimate substances such as water.

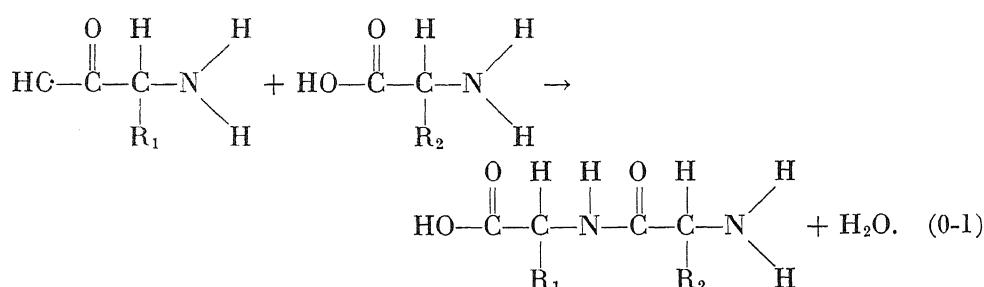
The existence of water in the solid state (ice) depends upon a particular environment, say  $-1^{\circ}\text{C}$ , at atmospheric pressure and upon a specific history of rapid cooling (since slow cooling may produce only a supercooled liquid). Similarly, some alkali metals and alkaline earths can be fused with alumina-silicate

compounds at high temperature; when cooled rapidly to room temperature, they enter the vitreous state and become glass. The proper ingredients, the proper ambient temperature, and the proper history are all necessary conditions determining this particular state. By analogy, we can refer to the state shared by surviving cells and living bacteria as the *living state*.

The solid state may be assumed by all matter; the vitreous state may be assumed by matter having certain specific properties. The living state, being even more specific, may be assumed by complex systems of material only if they share more stringent qualifications.

Figure 0.1 presents data on the gross composition of various living organisms ranging from viruses to humans. Although constituents such as nucleic acids and phospholipids may perform key functions and are thus indispensable to life, the chart indicates that proteins, water, and salts predominate in living matter. Present in relatively stable proportions, these compounds form the bulk and substance of all living systems.

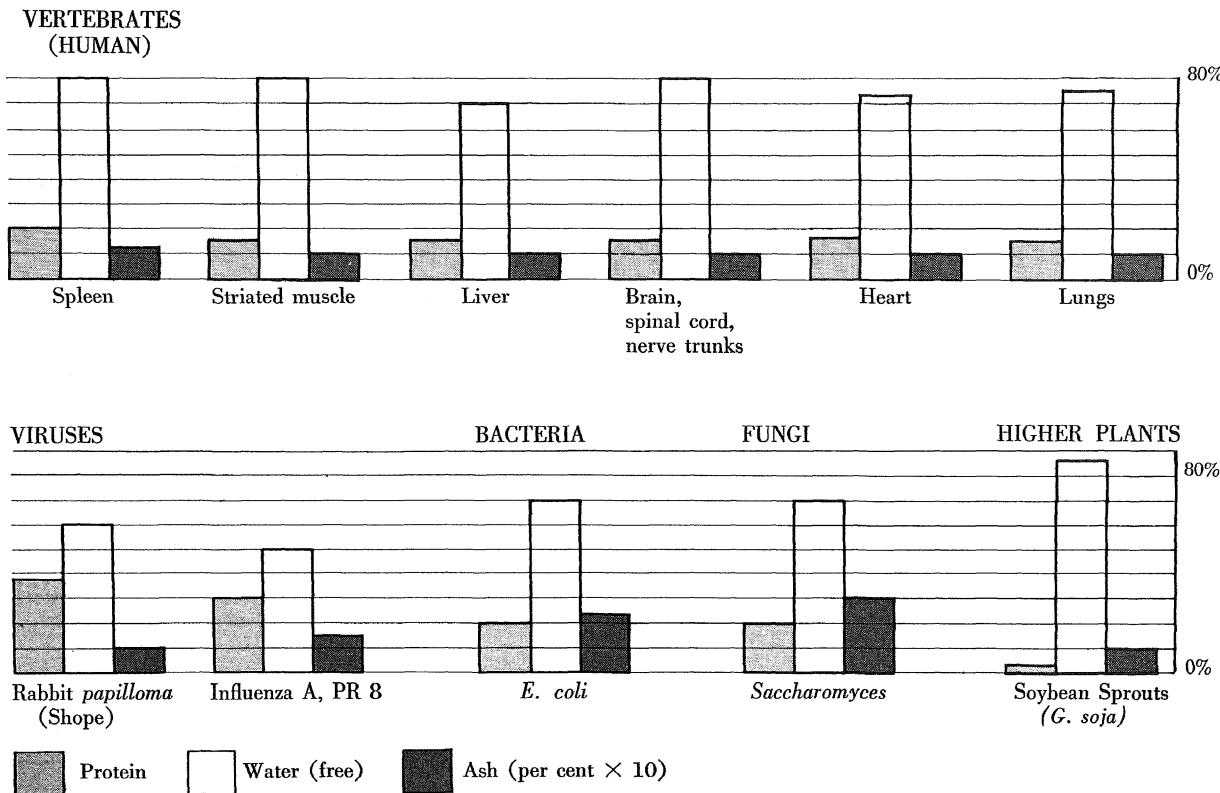
Salt and water are familiar objects in the nonliving world. Proteins, on the other hand, are produced exclusively by, and are found only in, living matter. These linear polymers are made up of about 23 different amino acids arranged in various proportions and sequences. Amino acids vary widely in their chemical structure (Table 0.1) but have in common the possession of an  $\alpha$ -amino and an  $\alpha$ -carboxyl group. When the  $\alpha$ -carboxyl group of one amino acid combines with the  $\alpha$ -amino group of another, one water molecule is lost and a peptide bond is formed:



Amino acids like glycine and alanine can be linked to form peptides having no residual charge.\* On the other hand, each residue of glutamic acid and aspartic acid lends one residual anionic charge to the peptide or protein, while each residue of lysine, arginine, and sometimes histidine, lends a cationic charge. Thus, proteins contain variable amounts of electric charge, depending upon the amount of these so-called "trifunctional" amino acids that they contain.

The amino-acid compositions of representative proteins are tabulated in Figure 0.2. In a general way the physiological activity appears to be related to

\* The part of the amino acid which does not participate in the peptide linkages is the "side-chain" and is represented by groups  $\text{R}_1$  and  $\text{R}_2$  in equation (0-1).

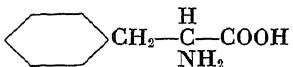
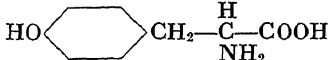
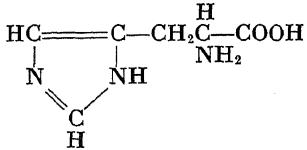
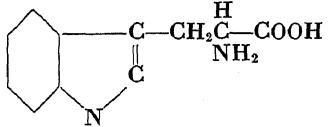
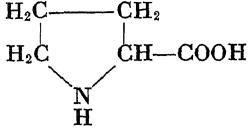
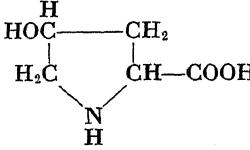


**Figure 0.1. THE COMPOSITION WITH REGARD TO PROTEIN, WATER, AND SALTS OF REPRESENTATIVE SPECIES FROM VARIOUS PHYLA.** [Data from Spector (1956): Viruses, bacteria, fungi, Table 70;

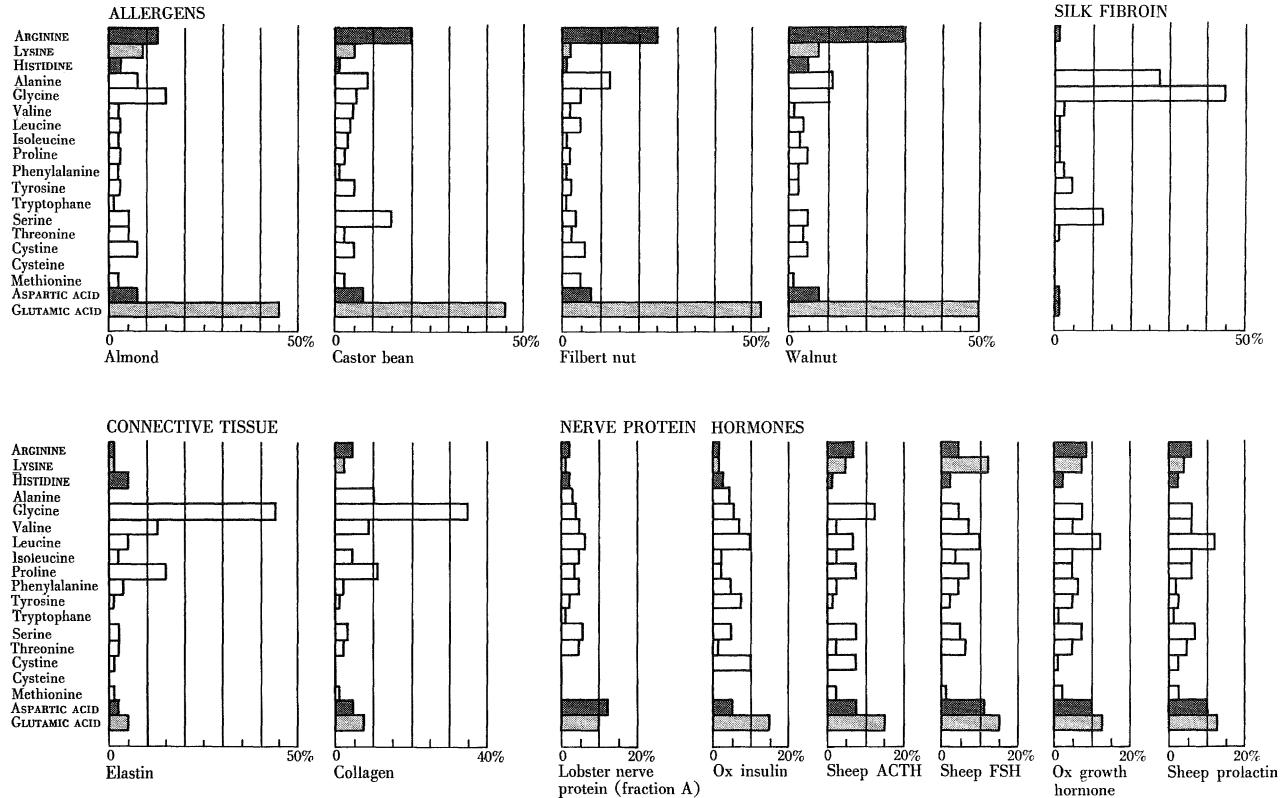
Higher plants, Table 156; from Mitchell *et al.* (1945): Chordates (humans).] Low protein and salt contents in higher plants are due to large water-filled vacuoles in their cells. (See footnote on p. 188.)

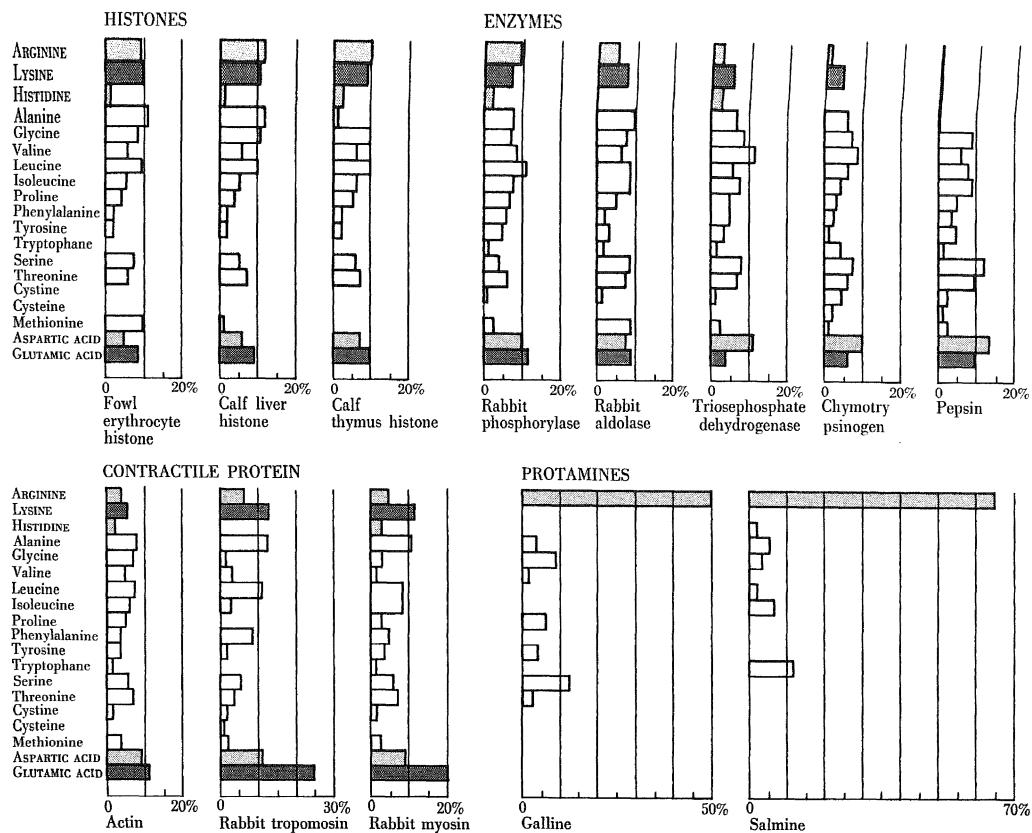
Name	Abbreviation	Formula
Glycine	Gly	$\begin{matrix} \text{H} \\   \\ \text{HC}-\text{COOH} \\   \\ \text{NH}_2 \end{matrix}$
Alanine	Ala	$\begin{matrix} \text{H} \\   \\ \text{CH}_3\text{C}-\text{COOH} \\   \\ \text{NH}_2 \end{matrix}$
Valine	Val	$\begin{matrix} \text{CH}_3 & & \\ & \diagdown & \\ & \text{CHC}-\text{COOH} & \\ & \diagup & \\ & \text{CH}_3 & \end{matrix}$
Norvaline	Nval	$\text{CH}_3-\text{CH}_2-\text{CH}-\begin{matrix} \text{H} \\   \\ \text{C}-\text{COOH} \\   \\ \text{NH}_2 \end{matrix}$
Leucine	Leu	$\begin{matrix} \text{CH}_3 & & \\ & \diagdown & \\ & \text{CHCH}_2\text{C}-\text{COOH} & \\ & \diagup & \\ & \text{CH}_3 & \end{matrix}$
Isoleucine	Ileu	$\begin{matrix} \text{CH}_3 & & \\ & \diagdown & \\ & \text{CHC}-\text{COOH} & \\ & \diagup & \\ & \text{C}_2\text{H}_5 & \end{matrix}$
Serine	Ser	$\begin{matrix} \text{H} & \text{H} \\   &   \\ \text{HC}-\text{C}-\text{COOH} \\   &   \\ \text{OH} & \text{NH}_2 \end{matrix}$
Threonine	Thr	$\begin{matrix} \text{H} & \text{H} \\   &   \\ \text{CH}_3\text{C}-\text{C}-\text{COOH} \\   &   \\ \text{OH} & \text{NH}_2 \end{matrix}$
Cysteine	(CySH)	$\begin{matrix} \text{H} & \text{H} \\   &   \\ \text{HC}-\text{C}-\text{COOH} \\   &   \\ \text{SH} & \text{NH}_2 \end{matrix}$
Cystine	(CyS—)	—S—S— Form of cysteine
Methionine	Met	$\begin{matrix} \text{H} & \text{H} \\   &   \\ \text{HC}-\text{CH}_2\text{C}-\text{COOH} \\   &   \\ \text{SCH}_3 & \text{NH}_2 \end{matrix}$
Aspartic acid	Asp	$\text{COOH}-\text{CH}_2\text{C}-\text{COOH}$ $\text{NH}_2$
Asparagine	(Asp—NH <sub>2</sub> )	$\text{CONH}_2-\text{CH}_2-\begin{matrix} \text{H} \\   \\ \text{C}-\text{COOH} \\   \\ \text{NH}_2 \end{matrix}$
Glutamic acid	Glu	$\text{COOH}-\text{CH}_2\text{CH}_2\text{C}-\text{COOH}$ $\text{NH}_2$

Table 0.1 (*Continued on facing page*)

Name	Abbreviation	Formula
Glutamine	(Glu—NH <sub>2</sub> )	$\text{CONH}_2-\text{CH}_2-\text{CH}_2-\overset{\text{H}}{\underset{\text{NH}_2}{\text{C}}}-\text{COOH}$
Lysine	Lys	$\overset{\text{H}}{\underset{\text{NH}_2}{\text{HC}}}-\text{CH}_2\text{CH}_2\text{CH}_2\overset{\text{H}}{\underset{\text{NH}_2}{\text{C}}}-\text{COOH}$
Arginine	Arg	$\text{NH}_2\overset{\text{H}}{\underset{\text{NH}}{\text{CN}}}\text{CH}_2\text{CH}_2\text{CH}_2\overset{\text{H}}{\underset{\text{NH}_2}{\text{C}}}-\text{COOH}$
Phenylalanine	Phe	
Tyrosine	Tyr	
Histidine	His	
Tryptophan	Try	
Proline	Pro	
Hydroxyproline	Hypro	

**Table 0.1. THE AMINO ACIDS COMMONLY FOUND IN PROTEIN HYDROLYSATES.** The abbreviations in column 2, which are conventionally employed to describe amino-acid sequences, are those originally proposed by Brand and Edsall (1947).





**Figure 0.2. THE AMINO-ACID COMPOSITION OF SOME REPRESENTATIVE PROTEINS.** Given as percentages of the total number of amino-acid residues. Silk fibroin and connective tissue are structural proteins manifesting little chemical activity. Hormones, allergens, and enzymes characteristically show profound physiological activity in low concentrations. The remaining proteins have specialized functions in contraction, impulse transmission, and protein reproduction. The amino acids containing a net charge when included in a peptide chain are the first three and the last two members of the list. Arginine, lysine, and histidine are positive;

aspartic and glutamic acids are negative. [Data from Tristram (1953): silk fibroin, connective tissue, hormones, enzymes, myosin, protamines, histones, Tables 22, 23, 15, 20, and 14, respectively; from Komlinz *et al.* (1954): contractile protein (except myosin), Tables I and II; from Koechlin and Parish (1953): nerve proteins,<sup>a</sup> Table I; and from Block and Weiss (1956): allergens, p. 332.]

<sup>a</sup> We are indebted to Drs. B. A. Koechlin and M. Maxfield for pointing out that Dr. Maxfield, who had isolated the lobster nerve protein, fraction A, is not entirely certain that it did not contain some contaminants from extraneous tissues around the nerve fibers.

the charges on the protein. For example, the inert protein, silk fibroin, which is secreted by silkworms to form a protective barrier, is almost entirely devoid of charged amino-acid residues, whereas all active proteins possess charged groups in varying proportions. It is thus reasonable that theories of proteins are concerned with the net charges on the protein molecules.

One major theory describing the behavior of proteins in a dilute water solution of salt ions was proposed by Linderstrøm-Lang (1924). This elegant theory, which has guided important biological research for the last four decades, is a useful point of reference. We must proceed considerably beyond it, however, because it deals only with proteins in dilute salt solution, while the present need is for a theory that can deal with these constituents as they exist within the living cell.

Linderstrøm-Lang's theory is built on three simplifying assumptions:

- (1) The protein molecule is a sphere with its net electric charges uniformly "smeared" over its surface.
- (2) Salt ions like  $K^+$  and  $Na^+$  are virtually identical particles constituting part of the total ionic strength; their entire interaction with the charged protein is through their presence in a diffuse ion cloud.
- (3) The aqueous medium is a continuous dielectric which determines the protein behavior only by virtue of its macroscopic dielectric constant.

While these assumptions are both necessary and valid for the formulation of a mathematical solution in the particular model Linderstrøm-Lang chose to study—namely, a protein in a *very dilute* salt solution—there is cause to suspect that such assumptions ignore the very factors that distinguish the mechanisms of life from the physics of simple dilute salt solutions. The following evidence supports this point of view:

(1) It has long been known that living organisms are very sensitive to the differences between such chemically similar ions as  $K^+$  and  $Na^+$  (Ringer, 1883). Moreover, even *in vitro* protein enzyme systems like pyruvate kinase, phosphotransacetylase, and tryptophanase readily distinguish  $K^+$  from  $Na^+$  in dilute salt solutions (20 millimole-per-liter concentration) (see Chapter 14).

(2) Heavy water ( $D_2O$ ) has a dielectric constant almost identical with that of ordinary water ( $D_{D_2O}/D_{H_2O} = 0.9963$  at  $25^\circ C$ , Wyman and Ingalls, 1938). Thus, if water affects protein interaction only through its macroscopic dielectric constant, substituting heavy water for normal water should have little biological effect. Actually, a large body of evidence collected since the pioneer work of G. N. Lewis (1934) and of Barbour and Dickerson (1939) shows that  $D_2O$  has profound influence on living processes *in vivo* as well as *in vitro*. (See also Flaumenhaft *et al.*, 1960; J. J. Katz, 1960; and Kritchevsky, 1960.)

(3) In the Linderstrøm-Lang theory, a protein molecule is characterized by both its molecular weight, which determines the radius of the hypothetically spherical molecule, and its *net* charge. These are not sufficient to describe the

physiological activity of a protein: (a) Biological function cannot be specified solely on the basis of the protein molecular weight. For example, the physiological function of adrenocorticotropic hormone (ACTH), a simple protein (molecular weight, 20,000), is retained in one of its hydrolytic products, a small peptide containing about seven amino-acid residues (molecular weight less than 1,000), see Li (1947). (b) The net charge on a protein is determined by the *pH* of the medium and the number of trifunctional amino-acid residues. Figure 0.2 makes it apparent that the trifunctional amino acid content differs very little among hormones with widely differing physiological actions. Since the internal milieu of living systems has virtually a constant *pH*, it is impossible for net charge alone to determine the functional characteristics of proteins.

It appears that the treatment of water as a continuous dielectric, ions as charged particles in diffuse ion clouds, and proteins as spheres bearing *net* charges does not lead to an adequate representation of the protein-salt-water system as it exists in the living state. Since protein, salt (ions), and water play the major roles in living phenomena, they must have other important attributes.

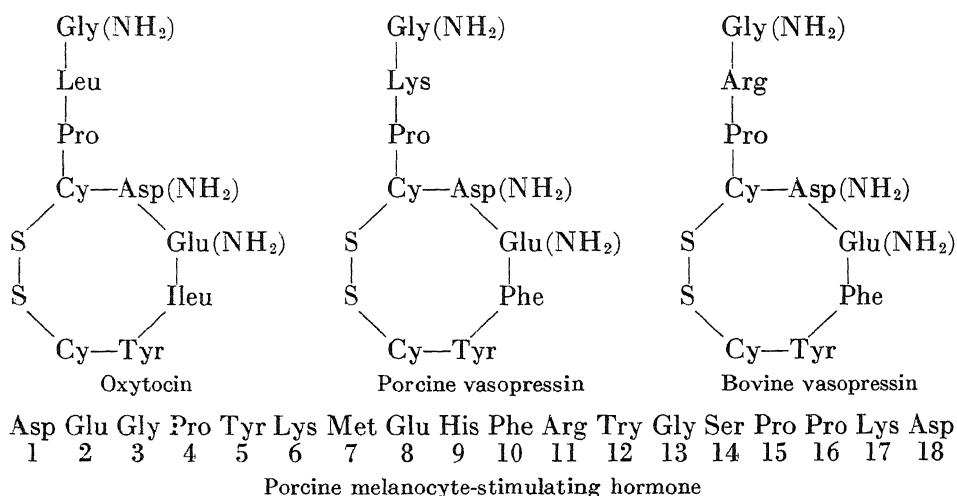
The dielectric constants of  $D_2O$  and  $H_2O$  are almost identical, yet the acid dissociation constant of deutacetic acid,  $CH_3COOD$  ( $0.59 \times 10^{-5}$ ) is three times smaller than that of its close analogue, acetic acid,  $CH_3COOH$  ( $1.84 \times 10^{-5}$ ). Since these dissociation constants are expressions of the free energies of hydration of the dissolved proton and the anions, the large difference in dissociation constants indicates the existence of an important difference in the hydration energies. In turn, the difference in hydration energies arises from molecular attributes such as polarizability, the Born repulsion constant (which determines the "size" of the molecule), and dipole moments. Such attributes also underlie the differences between  $K^+$  and  $Na^+$  ions. In a later chapter, the explicit equations relating these molecular attributes to the interaction of protein, ions, and water will be presented in detail. Here we need only mention that since a theory taking into account only the long-range attributes (net charge, dielectric constant, and so on) fails to explain the biological protein-ion-water system in the living state, the correct treatment must consider those properties which determine *short-range interactions*. That is, in the living state, the system of protein, ions, and water must exist in close association. Thus, *association between proteins and interacting particles is the critical difference between the behavior of proteins in dilute salt solutions as treated by the Linderstrøm-Lang theory and the behavior of proteins, salt, and water in biological systems as described by the present theory*.

The Linderstrøm-Lang model of the protein molecule as a sphere with its net charge uniformly smeared over its surface is too simple to account for the specific properties of protein. Therefore, the answer to what attributes do underlie protein specificity must lie in a finer description of protein structure.

Approximately twenty-three amino acids join to form the polypeptide chain (for structural formulas, see Table 0.1). Figure 0.2 demonstrates that a spectrum

of physiological functions as diverse as those subserved by the proteins enumerated can hardly be based on the relatively minor differences in the *total composition* of amino-acid residues as found among the proteins, hormones, allergens, and enzymes listed therein.

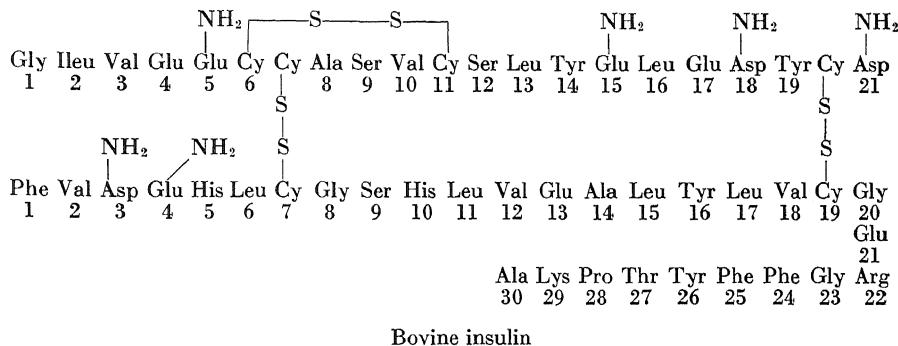
There is an alternative. By permutation, the letters of the alphabet spell out words of great diversity; similarly, the permutations of the amino acids create the diversity among proteins. The painstaking elucidation of amino-acid sequences in work such as that of Sanger (1956) on insulin, Tuppy (1953) and du Vigneaud, *et al.* (1953a,b) on oxytocin, Popenoe and du Vigneaud (1953, 1954) on vasopressin, and Stein and Moore (1961) on ribonuclease illustrate how nature has determined the unique structure of each of these proteins. Some of these structures are depicted in Figures 0.3 through 0.5. More specific examples prove that physiological function, in fact, depends on an exact amino-acid sequence. Thus, a change from the negatively charged glutamic acid to the neutral valine in only one residue of hemoglobin (Table 0.2) produces a change from the normal disc-shaped erythrocyte to one which, under certain conditions, assumes a sickle shape.



**Figure 0.3. STRUCTURAL FORMULAS OF A FEW PROTEIN HORMONES.** For a key to the abbreviations of the names of the amino acids, see Table 0.1. [Data from Tuppy (1953) and du Vigneaud *et al.* (1953a,b): oxytocin; from Popenoe and du Vigneaud (1953, 1954), du Vigneaud *et al.* (1953c), and Acher and Chauvet (1953): porcine and bovine vasopressin; from Harris and Roos (1956): porcine melanocyte-stimulating hormone.]

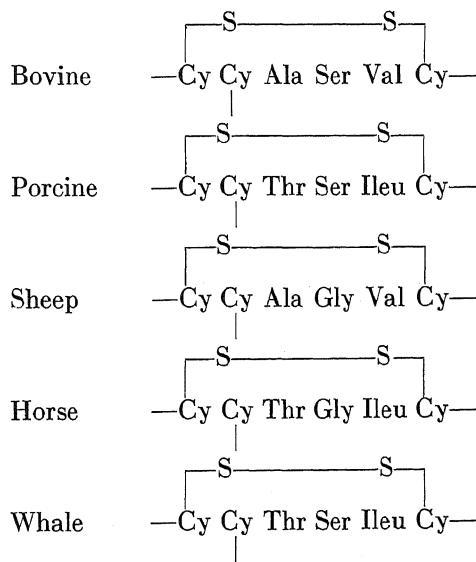
Similar variation of one or two amino-acid residues in otherwise identical polypeptide chains underlies the differences in the normal physiological behavior

of vasopressin and oxytocin (Figure 0.3). The functions of these two hormones are quite different: oxytocin induces contraction of the pregnant uterus and stimulates milk ejection from the lactating breast; vasopressin acts primarily as an antidiuretic. Yet vasopressin differs from oxytocin only in the substitution



Bovine insulin

**Figure 0.4.** THE STRUCTURAL FORMULA OF BOVINE INSULIN. The amino-acid sequence of bovine insulin was worked out by Sanger and his associates (see Ryle *et al.* 1955; J. I. Harris *et al.*, 1956). See Figure 0.3 for the structure of oxytocin elucidated by Tuppy and du Vigneaud, *et al.*



**Figure 0.5.** STRUCTURAL DIFFERENCES BETWEEN BOVINE, PORCINE, SHEEP, HORSE, AND WHALE INSULINS. The sequence of amino-acid residues from the five different species are identical except for the indicated differences in the glyceryl chain. See Figure 0.4 for the complete formula of bovine insulin. (From H. Brown *et al.*, 1955; J. I. Harris *et al.*, 1956.)

of phenylalanine for isoleucine and of arginine for leucine. On the other hand, the difference between porcine and bovine vasopressin consists only in exchanging lysine for arginine, both of which have positively charged groups. This apparently causes no change of function, but does lead to species differences. Similarly, the insulins from beef cattle, pigs, sheep, horses, and whales all share the same potent physiological capacity with respect to functions such as glucose oxidation, glycogen storage, and protein and fat metabolism; the variation between these insulins is produced by the substitution of noncharged groups such as isoleucine for valine, alanine for threonine, and serine for glycine. (See the structural formulas, Figure 0.5.) This leads to minor but significant immunological changes.

Hemoglobin		Structure							
A	S	Val	His	Leu	Thr	Pro	Glu	Glu	Lys
		Val	His	Leu	Thr	Pro	Val	Glu	Lys

**Table 0.2. CHEMICAL DIFFERENCE BETWEEN NORMAL AND SICKLE-CELL HEMOGLOBIN.** Hemoglobin is split into a soluble fraction and an insoluble "core" by digestion with trypsin. The "cores" of hemoglobin A and S are identical (Hunt and Ingram, 1958). Electrophoretic analysis of the products of degradation of the soluble fraction revealed that hemoglobin A (normal) differs from hemoglobin S (sickle-cell) by a single amino-acid residue. (*From* Hunt and Ingram, 1959.)

We conclude that both the nature of the amino-acid components and their sequential order of arrangement give specificity to a protein. The truly specific part of an amino acid lies in its side chain [ $R_1, R_2$  of equation (0-1)]. All protein-forming amino acids contain the same  $\alpha$ -amino and  $\alpha$ -carboxyl groups, which are formed into the uniform and nonspecific polypeptide linkages found everywhere in the protein molecule. This chain of polypeptide linkages, the polypeptide chain, serves as the unique criterion distinguishing all proteins from all nonproteins, and is also instrumental in establishing the specificity of various proteins. Thus, the second major proposition of the present theory is that *the primary function of the highly polarizable resonating chain is to provide a vehicle for the ready transmission of an inductive effect* (see below) *from one functional group to another*. Through this inductive effect the various orders of arrangement of side chains may contribute to the specificity of proteins by adding a dimension beyond the mere summation of the isolated characteristics of all the constituent amino acids. This dimension gives each protein its identity as a distinct functional whole. To understand this, it is necessary to examine the nature of the inductive effect.

From organic chemistry we know that the effective charge carried by a polar group on an organic molecule may be varied by altering other chemical groupings in the molecule. For example, acetic acid,  $\text{CH}_3\text{COOH}$ , is a weak acid, while trichloroacetic acid,  $\text{Cl}_3\text{CCOOH}$ , the well-known protein denaturant, is a very strong acid. Yet both have the same functional COOH group. The substitution of the three hydrogen atoms for the more electronegative (having greater power to draw electrons toward themselves) chlorine atoms effectively diminishes the negative charge of the distant carboxyl group. This redistribution of electrons in the functional groups brings about a decrease in the electrostatic interaction energy between the negatively charged  $\text{COO}^-$  groups and the positively charged  $\text{H}^+$  ion. The result is the conversion of a weak acid, which holds its proton tightly, to a strong acid, which holds its proton loosely. The mechanism that brings about this change is called the inductive effect.

Earlier, we mentioned examples which show that the substitution of charged amino-acid residues for noncharged residues produces important functional changes in proteins; by itself this does not indicate which of these two classes of amino acids critically determines the function. We know that all proteins do not share a high degree of reactivity; silk fibroin, for example, is remarkably inert and "lifeless." Paralleling its inertness is a shortage in its composition of such amino-acid residues as glutamic acid, aspartic acid, lysine, and arginine. On the other hand, proteins which exhibit a considerable degree of functional activity are not only high in trifunctional amino acids, but, as Figure 0.2 demonstrates, each larger functional class appears to possess a characteristic proportion of these residues. Thus the relative reactivity of a protein seems to be prominently related to the possession of the polar groups\* fixed along the "backbone" of the polypeptide chain.

The predominant role played by these polar groups in determining the functional characteristics of protein is understandable if one considers the following: (1) The inductive effect primarily involves the distribution of the negatively charged electrons, and the greatest effect on the distribution of electrons is produced by polar groups bearing net electric charges. Thus, while all the amino-acid residues in a protein play some role in determining the properties of the protein, the polar residues exercise the dominant role. (2) The failure of the long-range interaction theories to explain the behavior of living protoplasm indicates that short-range forces must be operative; ions, water, and protein must be in close association in the living state. It is obvious that charged ions are most likely to associate with proteins at oppositely charged sites; whether a particular ion associates or not varies with the nature of both the free ion and the fixed ion. All these considerations lead to the conclusion that the nature and arrangement of

\* The term "polar groups" will be used throughout this monograph to include monopolar, dipolar, and multipolar groups with or without net charge.

the side chains are expressed in terms of the electron distribution within the various functional groups.

Next let us examine how the changes of electron distributions within the functional groups can determine the properties of proteins. To aid in the mathematical formulation of the differences in electron distributions such as found between the carboxyl groups of acetic acid and trichloroacetic acid, mentioned earlier, we have devised a parameter called the *c-value*, which is expressed in angstrom units. Its full mathematical definition is given in Chapter 4. For the moment, we need only say that a weak acid will have a high *c-value* ( $-2\text{\AA}$ ) and a strong acid will have a low *c-value* ( $-4\text{\AA}$ ). In protein, the *c-value* of a charged group is to a large extent determined by the nature of the neighboring amino-acid residues, in particular the polar ones. As we shall see later, however, this is not the only determinant of the *c-value*.

With the *c-value* defined, it becomes possible to calculate all the energies, short-range as well as long-range, between a counterion ( $\text{H}^+$ ,  $\text{Li}^+$ ,  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{NH}_4^+$ ,  $\text{Rb}^+$ , and  $\text{Cs}^+$ ) and a fixed anion of a specific *c-value*. The results of such calculations show that in an aqueous medium, association between fully hydrated fixed anion-countercation pairs and association between totally dehydrated ion pairs become limiting cases, and all intermediate degrees of hydration are statistically possible. (The history of the development of this formulation is discussed in Chapter 4.) The statistical probability of having a certain number of water molecules between the ion pairs is determined by the nature of the counterions and the *c-value* of the anion. Moreover, given the *c-value* of the anionic group, there will usually be a difference between the adsorption energies of one counterion, say  $\text{K}^+$ , and another, say  $\text{Na}^+$ .

We must also stress the fact that in such associated ion pairs, the interaction energy between ions is very strong. Due to the "freezing" of the water molecules in the electric field thus produced, the dielectric constant of the water surrounding associated ions is not 81 (the value in free solution), but close to unity, giving rise to electrostatic energies sometimes as high as ten times that of the average hydrogen bond (see Table 6.1). When a specific counterion is adsorbed onto a fixed site, an inductive effect is exerted on the *c-value* of the neighboring site. (This inductive effect plus a direct electrostatic effect transmitted through space are collectively called the *F-effect*.) With its *c-value* thus altered, the neighboring site may, in turn, alter its preference for counterions, leading to an exchange at that site. Similarly the next site will be altered, and so on, causing perturbations in a "falling domino" fashion; these ultimately affect the *c-value* and counterions of sites at a distance. Such a process is called an *indirect F-process*. As we shall illustrate later, this process brings about an all-or-none type of response. In the physical world, similar phenomena are found in systems where there is such strong interaction between neighboring individual elements that they no longer act independently, but tend to act in unison. A few examples of

this type of phenomenon, conventionally referred to as cooperative, are found in ferromagnetism, antiferromagnetism, and order-disorder transitions in alloys (Temperley, 1956).

The adsorption energies of alkali-metal ions on fixed anionic sites of varying *c*-values have been calculated; that of the  $\text{NH}_3^+$  ion (in relation to the adsorption energies of hydrogen and the alkali-metal ions) is of paramount importance. This ion is significant because it may serve as a prototype for the amino ( $\text{NH}_3^+—\text{R}$ ) and guanidyl ( $\text{NH}_3^+—\text{C}(\text{NH}_2)—\text{N}—\text{R}$ ) groups which represent the majority of the



fixed cationic groups of all proteins; in addition, it may also serve as a prototype for the amino and the quaternary ammonium groups so frequently present in physiologically active compounds such as drugs and chemical transmitters.

In Chapter 3, evidence will be presented to demonstrate that fixed anions, primarily in the form of  $\text{COO}^-$  groups carried by glutamate and aspartic residues, and the fixed cations in the form of amino and guanidyl groups, are universally present at high concentrations in cellular proteins. Through cooperative interactions, these cellular proteins change reversibly from one metastable state\* to another in an all-or-none fashion. One of the important available states corresponds to that in which an assemblage of fixed anionic groups associates with another assemblage of fixed cationic groups, producing a folded configuration of the protein system. Alternatively, the fixed anions of protein molecules may associate collectively with free counterions such as the  $\text{K}^+$  ion. Thus the protein-water-salt system is fundamentally a labile one, capable of cooperative changes from one metastable state to another. Such cooperative changes may be controlled by "cardinal" sites, sites small in number but of such strategic importance that they can control and modulate, through the indirect *F*-process, the behavior of a large number of fixed sites. It is through these cardinal sites that substances such as ATP, and many hormones and drugs—often present in extremely small quantities—may control biological functions.

This brief summary introduces the main theme of the book, the *association-induction hypothesis*; thus association and induction are the two fundamental mechanisms of the present theory. A brief discussion of the general organization of the monograph may be useful to the reader, especially in the early sections, since the development of this thesis demands that chapters covering widely diverse subjects be juxtaposed.

Since Arrhenius advanced his ionic dissociation theory, and in particular after the development of the limiting law of Debye and Hückel, it has been common knowledge that most alkali-metal ions in free solutions of moderate concentration

\* The use of the term "state" in this context should not be confused with the living state, within the framework of which several varieties of subsidiary metastable states are possible (see Chapter 9).

associate with simple anions (like the halides) only to a small degree. Chapter 2 demonstrates that the lack of association in solutions is not a universal attribute of ions such as  $\text{Na}^+$  and  $\text{K}^+$ . Rather, association is lacking only when these ions exist in solutions as the salts of certain specific anions ( $\text{NaCl}$ , for example). The concepts of nonionic association and of ionic strength can hardly be applied to a solution of charge-bearing groups where these groups are fixed into polyelectrolyte molecules or aggregates (referred to hereafter as *semifixed-charge systems*).<sup>\*</sup> Again, these concepts do not apply when such linear polyelectrolytes are further joined with each other into a three-dimensional latticework (*a fixed-charge system*) in which association between the fixed ions and their counterions is further increased. Here the Debye-Hückel limiting law becomes totally inapplicable (for support of this view, see Debye, 1949). For readers who are not already familiar with the physical basis of this fundamental proposition—the nearly complete ionic association in living protoplasm as a fixed-charge system—I shall include it in Chapter 1, which covers the elementary principles of statistical mechanics. Chapter 2 presents the theoretical reasoning which predicts nearly complete ionic association in living cells; this prediction is confirmed by the definitive experimental evidence presented in Chapters 9 through 11. The applicability of the concept of ionic association to living protoplasm in general is shown in Chapter 3, where the density of fixed ionic sites in cellular and noncellular living matter is comprehensively examined.

In short, Chapters 1 (received March 17, 1961),† 2, and 3 (received April 3, 1961) are primarily concerned with association phenomena and Chapters 4, 5, and 6 (received April 26, 1961) develop the major theme of association and induction. The wide applicability of these concepts is discussed first in Chapter 7 (received May 16, 1961), "The Theory of Proteins," which extensively develops the theme in relation to studies of isolated protein *in vitro*, and then in Chapters 8 to 18 (Chapter 8 received May 16, 1961; Chapters 9 and 10 received May 29, 1961; Chapters 11 through 18 received July 10, 1961), which cover living protoplasm.

## B I B L I O G R A P H Y

### BOOKS AND MONOGRAPHS

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\* A more correct name for a fixed-charge system would be "fixed-polar-group system" because, strictly speaking, the charge-bearing groups are fixed, not the charge itself. However, since such a term is clumsy, we have decided to preserve the term "fixed-charge system."

† Received dates are those on which chapters arrived at Blaisdell Publishing Company.

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# PART I

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## ASSOCIATION



## 1

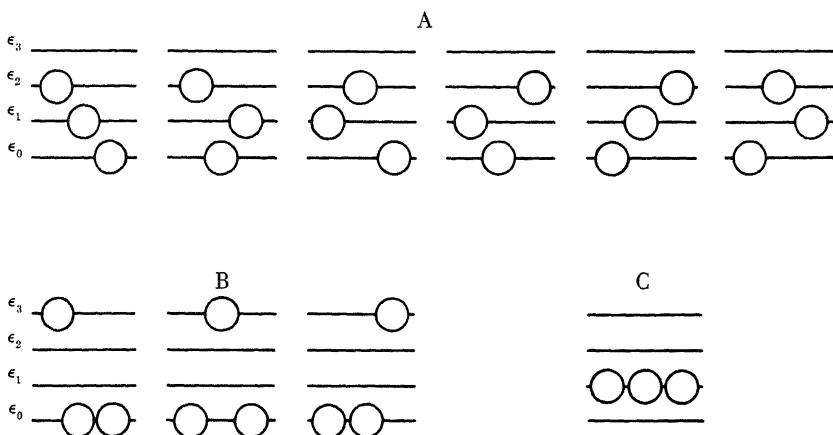
# ENERGY AND ENTROPY IN ASSOCIATION PHENOMENA

In studying the motion of a macroscopic object, we learn that its energy exists in two forms: potential energy, determined by its location in space, and kinetic energy, determined by its velocity. The laws of mechanics state that energy can be neither created *de novo* nor destroyed. Conservation of energy may be demonstrated by an ideal frictionless pendulum; the sum of its kinetic and potential energies is constant during its motion. In reality, a pendulum eventually stops, indicating that the energy is not perfectly conserved. The discovery that this apparent loss of energy is due to its frictional conversion to heat led to the development of the science of thermodynamics which considers heat as a third form of energy, and introduces the concept of entropy.

Studies of the behavior of *microscopic* particles led to the establishment of three separate important conclusions: (1) just as macroscopic bodies possess potential and kinetic mechanical energy, so do atoms, molecules, and similar microscopic systems. (2) The mechanical energies of the atoms and molecules are identifiable with heat (kinetic theory). (3) Classical mechanics may be replaced by quantum mechanics—a more general law that governs the motion of all matter, macroscopic as well as microscopic.

In a discussion of the behavior of ions and molecules, the primary subject of the present volume, one never deals with just a few particles, but always with large collections of them (of the order of  $10^{20}$  to  $10^{23}$ ). The mechanics of the motion of these tremendous populations must be treated statistically much like coin throwing. If one tosses a coin once, he cannot be certain whether it will come up "heads" or "tails." In this difficulty, no known science can help us. On the

other hand, if a coin is tossed an immense number of times, the proportion of the total number of times it will come up "heads" or "tails" becomes predictable. The science of statistics, in fact, states that if we ignore those occasions when the coin lands on its edge, each face will turn up almost exactly  $\frac{1}{2}$  of the time. Dice are another example: if one paints two sides of a die red and the other four sides black, in a very large number of throws one may expect a red side to face upward in almost exactly  $\frac{2}{6}$  of the total number of throws. Thus, we see that



**Figure 1.1. A MODEL FOR THREE LOCALIZED PARTICLES.** The complexions which may be assumed by three identical localized particles when the total energy shared by all particles is three units and energy levels, 0, 1, 2, 3 are available to all of the particles.

the results of "chance" events can be accurately anticipated on the basis of a statistical treatment. The behavior of ions and molecules in any physical system can be treated similarly. This is the subject matter of the science of statistical mechanics.

Consider an isolated ice crystal\* consisting of three water molecules, at lattice sites I, II, and III (Figure 1.1). At a designated temperature, they share a total of three units of energy which may be passed from one molecule to another, but which cannot be increased or decreased. Let us assume that quantum mechanics tells us that there are four allowed energy levels,  $\epsilon_0$ ,  $\epsilon_1$ ,  $\epsilon_2$ , and  $\epsilon_3$ , each higher than the previous level by one energy unit. How many distinguishable ways may the total energy be distributed among the three particles? (1) There are six ways if one molecule is on level two, another at level one, and the last on zero; there are three choices for the  $\epsilon_0$  energy level, two choices for  $\epsilon_1$ , and one

\* In this discussion we are speaking of an idealized ice in which the residual entropy (Pauling, 1948) due to the persistent existence of a degree of waterlike structuring is ignored.

choice for  $\epsilon_2$  (Figure 1.1A). We shall refer to each of these distinguishable configurations as a complexion. Expressing the total number of complexions in an energy distribution as  $t(n_0, n_1, n_2, n_3)$ , where  $n_0, n_1, n_2, n_3$  are the number of particles in the energy levels 0, 1, 2, and 3, respectively, in this distribution we have:

$$t(1,1,1,0) = \frac{N!}{n_0!n_1!n_2!n_3!} = \frac{3!}{1!1!1!0!} = 6 \quad (1-1)$$

where  $N$  is the total number of particles involved. In general,

$$t = \frac{N!}{\prod_i n_i!}. \quad (1-2)$$

(2) There are three complexions if one molecule is on the third level and the other two on zero level (Figure 1.1B):

$$t(2,0,0,1) = \frac{3!}{2!} = 3. \quad (1-3)$$

(3) There is only one complexion if all of the particles occupy the first energy level (Figure 1.1C):

$$t(0,3,0,0) = \frac{3!}{3!} = 1. \quad (1-4)$$

If the total number of *a priori* equally probable complexions is designated by the symbol  $\Omega$ , then

$$\Omega = \sum_i \frac{N!}{\prod_i n_i!} = t(1,1,1,0) + t(2,0,0,1) + t(0,3,0,0) = 10. \quad (1-5)$$

This example, taken from Gurney (1949), illustrates the significance of  $\Omega$  and prepares us for a fundamental equation of statistical mechanics: the entropy  $S$  of an assembly is directly related to  $\Omega$  by

$$S = k \ln \Omega \quad (1-6)$$

where  $k$  is the Boltzmann constant, equal to  $1.38 \times 10^{-16}$  ergs per degree Kelvin. The entropy of the three water molecules in our ice crystal is thus determined by (1) the number of particles, (2) the thermal energy shared among them, and (3) the quantum-mechanically allowed energy levels.

Suppose the molecules can assume an "alternate" state in which the number of allowed energy levels is larger, without a change in total energy of the system. The molecules will then have a larger  $\Omega$ , hence, a larger entropy in the "alternate" state than in the crystalline state. If there is no energy difference between the ground levels (the energy at absolute zero) of the crystalline and the "alternate" states and both are accessible to the water molecules, the probability of finding

the water molecules in the "alternate" state is greater than that of finding them as ice. This is analogous to the greater probability of getting a black side uppermost when tossing a die with four black sides and two red sides. The transformation from ice to the "alternate" state entails an increase in  $\Omega$  and in entropy for this system. Thus, the concept of "entropy," which is somewhat abstruse in thermodynamics, has an explicit explanation in statistical mechanics.

The task of enumerating all the complexions for three water molecules in four energy levels is a relatively simple matter, but when we deal with  $10^{20}$  to  $10^{23}$  molecules, it is virtually impossible. Fortunately, in very large populations, there is only one distribution that makes an effective contribution to the entropy. It is the distribution in which the numbers of particles in the energy levels follow a geometric progression such that each energy level is less populated than the preceding lower level. In this particular  $t$ , the number  $n_1$  of particles occupying the first level and the number  $n_2$  of particles occupying the second level are related by the expression

$$\frac{n_2}{n_1} = \frac{\exp(-\epsilon_2/kT)}{\exp(-\epsilon_1/kT)}. \quad (1-7)$$

Here  $\epsilon_1$  and  $\epsilon_2$  are the quantum-mechanically permissible energies at the two levels;  $kT$ , the product of the Boltzmann constant and the absolute temperature, is a measure of the average kinetic energy per particle and is approximately 0.6 kcal/mole at room temperature. For any  $i$ th or  $j$ th level,

$$\frac{n_i}{n_j} = \frac{\exp(-\epsilon_i/kT)}{\exp(-\epsilon_j/kT)} \quad (1-8)$$

and

$$\begin{aligned} & \frac{n_i}{n_1 + n_2 + \dots + n_i + \dots} \\ &= \frac{\exp(-\epsilon_i/kT)}{\exp(-\epsilon_1/kT) + \exp(-\epsilon_2/kT) + \dots + \exp(-\epsilon_i/kT) + \dots}. \end{aligned} \quad (1-9)$$

If the summation includes all the allowed energy levels then

$$N = \sum_i n_i = n_1 + n_2 + \dots + n_i + \dots \quad (1-10)$$

represents the total number of particles in that particular collection, and

$$\sum_i \exp(-\epsilon_i/kT) = \exp(-\epsilon_1/kT) + \exp(-\epsilon_2/kT) + \dots + \exp(-\epsilon_i/kT) + \dots \quad (1-11)$$

is known as the *partition function* (p.f.) of the collection. It tells how the particles are partitioned or distributed among the allowed energy levels. Equations

(1-9) through (1-11) indicate that

$$n_i = N \frac{\exp (-\epsilon_i/kT)}{\sum_i \exp (-\epsilon_i/kT)}. \quad (1-12)$$

Thus, in the example discussed, if the unit of energy corresponds to 1.0 kcal/mole, the partition function is

$$\text{(p.f.)} = \exp (-0/0.6) + \exp (-1.0/0.6) + \exp (-2.0/0.6) + \exp (-3.0/0.6) \quad (1-13)$$

$$= 1 + 0.19 + 0.04 + 0.01 = 1.24. \quad (1-14)$$

Equation (1-12) shows that 81 per cent of the particles will be found in the 0 level, 15 per cent in the 1 level, 3 per cent in the 2 level, and 1 per cent in the 3 level.

Statistical mechanics cannot indicate what energy levels,  $\epsilon_1, \epsilon_2 \dots \epsilon_i \dots$ , are available; this is the role of quantum mechanics. Once this is known, statistical mechanics provides the important relationship between the partition function and  $\Omega$ :

$$\Omega = (\text{p.f.})^N \exp (E/kT) \quad (1-15)$$

where  $E$  is the total energy of the collection. The Helmholtz free energy  $F$  of the collection is given by

$$F = -NkT \ln (\text{p.f.}). \quad (1-16)$$

Both equations (1-15) and (1-16) are restricted to *localized* systems in which each particle is distinguished by its fixed location in the crystal. Structurally similar particles are distinguishable only by their location in space; thus, in non-localized systems like dilute water vapor where the motion of particles is not restricted, all the particles are indistinguishable from one another. In a system of indistinguishable particles, each of the distributions (0,3,0,0), (2,0,0,1), and (1,1,1,0) has only one possible complexion. For the nonlocalized system, equations (1-5), (1-15), and (1-16) take the forms:

$$\Omega = \sum \frac{1}{\prod_i n_i} \quad (1-17)$$

$$\Omega = \frac{(\text{p.f.})^N \exp (E/kT)}{N!} \quad (1-18)$$

$$F = -kNT \ln (\text{p.f.}) + kT \ln N!. \quad (1-19)$$

Since entropy is  $S = k \ln \Omega$ , it appears to decrease when a system goes from the localized to the nonlocalized state. The opposite usually occurs, as the following considerations demonstrate.

We have represented the partition function as if it were due to a single type

of energy. Actually, the energy of a multiaatomic particle can be resolved into four independent components. A particle moving with translational energy may simultaneously rotate with rotational energy and vibrate with vibrational energy. The complete partition function is written as a product of the several partition functions: translational,  $(p.f.)_{trans}$ ; rotational,  $(p.f.)_{rot}$ ; vibrational,  $(p.f.)_{vib}$ ; and electronic,  $(p.f.)_{electr}$ .

Let us now compare vapor and ice. In the ice state, movement is almost entirely vibrational; each water molecule oscillates about a fixed locus. Freezing completely deprives the water molecules of translational movement. The H-bonds they form with neighboring molecules effectively prevent them from rotating. At room temperature or below, there is insufficient energy to excite the water molecules electronically. Therefore, for most purposes, the total partition function of ice, like that of other crystals, can be represented by its vibrational partition function.

Hypothetically, the partition function could be found by dividing all the quantum-mechanically allowed energy levels by  $kT$ , finding the values of  $\exp(-\epsilon_i/kT)$ , and summing them. This process is usually too laborious to be feasible. But it is possible mathematically to derive equations that give the value of the partition function explicitly. Thus, the vibrational partition function for a crystal of ice is

$$(p.f.)_{vib} = \frac{kT}{h\nu} \quad (1-20)$$

where  $\nu$  is the vibrational frequency of the molecules in the crystal lattice, and  $h$  is the Planck constant, equal to  $6.624 \times 10^{-27}$  erg/sec.

When a water molecule leaves the surface of ice, it gains both translational and rotational freedom. The partition function of the water vapor must consequently consist largely of the product of the translational and rotational partition functions. It can be shown that

$$(p.f.)_{trans} = \frac{(2\pi mkT)^{3/2}}{h^3} V_f \quad (1-21)$$

where  $m$  is the mass of a water molecule,  $k$  and  $T$  have the usual meanings, and  $V_f$  is the "free volume" equal to the total volume of the container in which the vapor is found. Equation (1-21) indicates that the number of allowed energy levels is directly proportional to the free volume.

For a polyatomic nonlinear molecule that has three classical rotational degrees of freedom,

$$(p.f.)_{rot} = \frac{8\pi^2(2\pi kT)^{3/2}(ABC)^{1/2}}{h^3\sigma}. \quad (1-22)$$

Here  $A$ ,  $B$ , and  $C$  are the three principle moments of inertia and  $\sigma$  is a symmetry number equal, for example, to 2 for  $H_2O$  (isosceles triangle) and to 12 for  $CH_4$ .

(regular tetrahedron). Thus we may write the total partition function of ice as

$$(p.f.)_{\text{total}}^{\text{ice}} = (p.f.)_{\text{vib}} = \frac{kT}{h\nu} \quad (1-23)$$

and the total partition function for water vapor\* as

$$(p.f.)_{\text{total}}^{\text{vapor}} = (p.f.)_{\text{trans}}(p.f.)_{\text{rot}} = \frac{64\pi^5(m^{1/2}kT)^3V_f(ABC)^{1/2}}{h^6\sigma}. \quad (1-24)$$

Partition functions are useful for describing the distribution of particles over a set of energy levels; in Figure 1.1 we have labeled these  $\epsilon_0$ ,  $\epsilon_1$ ,  $\epsilon_2$ , and  $\epsilon_3$ , where  $\epsilon_0$  is the ground level for the set. Similarly, we may write  $\epsilon'_0$ ,  $\epsilon'_1$ ,  $\epsilon'_2$ , and  $\epsilon'_3$  for another set of levels representing another state of aggregation. Partition functions offer a means of comparing the relative probability of two such states, but such a comparison is meaningless unless the two sets of levels refer to the same ground level.

In general, a difference exists between the energies of the ground levels of associated and dissociated particles. It is these energy differences to which we refer when speaking of the energies of electrostatic, covalent, hydrogen, or other bonds. This difference in ground-level energy is a potential energy and its exact value is shared by all the particles in an assembly. We need not consider it unless we are comparing assemblies that have different ground-energy levels. If the ground level of an assembly with partition function  $(p.f.)_1$  is higher by  $\Delta E$  than that of another assembly with  $(p.f.)_2$ ,  $(p.f.)_1$  and  $(p.f.)_2$  may not be compared until we have referred the partition functions to a common zero of energy; that is, we must compare  $(p.f.)_1$  with  $(p.f.)_2 \exp(\Delta E/RT)$ , each  $(p.f.)$  expression being referred to the lowest energy level of the system to which it applies.

The sublimation of ice entails both the overcoming of the energy difference between the ground levels of the two states and the performance of work to expand the volume of the system against the pressure  $p$ . The total energy difference between the two ground states is thus  $\Delta E + p\Delta V$ . Thermodynamically, this sum is called  $\Delta H$ , the change in heat content, or enthalpy.

We can now introduce the statistical mechanical equation that describes sublimation:

$$n_v = \frac{(p.f.)_{\text{total}}^{\text{vapor}}}{(p.f.)_{\text{total}}^{\text{ice}}} \exp(\Delta H/kT). \quad (1-25)$$

Here  $n_v$  is the number of molecules in the vapor state. For the sublimation of water,  $-\Delta H$  is 11.3 kcal/mole;† at room temperature  $\exp(\Delta H/kT)$  is thus

\* There is no significant contribution to the entropy by vibrational degrees of freedom in water vapor (Rushbrooke, 1949).

† The standard enthalpy change  $\Delta H$  represents the enthalpy change when both the reactant, water, and product, vapor, are in their respective standard states. For simplicity, we omit the superscript ° from the conventional symbols  $\Delta H^\circ$ ,  $\Delta F^\circ$ , etc.

$5 \times 10^{-9}$ , a figure so small that one wonders how sublimation may occur at all. Of course, sublimation does occur; thus, on a clear subzero wintry day, laundry dries in the open air. From equations (1-25), (1-24), and (1-23),

$$n_v = \frac{64\pi^5(m^{1/2}kT)^3 V_f(ABC)^{1/2}}{h^6\sigma} \frac{h\nu}{kT} \exp(\Delta H/kT). \quad (1-26)$$

It is apparent that  $n_v$  does become a large figure despite the small value of  $\exp(\Delta H/kT)$  because the free volume  $V_f$  is exceedingly large in the open air. Equations (1-19) to (1-22) also enable us to calculate the exact value of the entropy if we use the thermodynamic relationship

$$S = - \left( \frac{\partial F}{\partial T} \right)_{N,V} \quad (1-27)$$

which states that the entropy  $S$  of the assembly is equal to the negative rate of change of free energy with respect to temperature at constant volume and constant number of particles. The rotational entropy ( $25^\circ\text{C}$ ) is found to be 10.48 cal/deg/mole and the translational entropy as high as 34.61 cal/deg/mole; this yields a sum of 45.09 cal/deg/mole for the total entropy of water vapor (Rushbrooke, 1949, p. 146). This great increase in entropy on sublimation arises from the large values of the translational and rotational partition function of water vapor as compared to the vibrational partition function of ice. The difference is large enough to offset the otherwise prohibitively high  $\Delta H$  opposing sublimation.

On the other hand the heat of melting of ice is only 1.43 kcal/mole, and  $\exp(\Delta H_{\text{fusion}}/kT)$  is thus  $9 \times 10^{-1}$  as compared with  $5 \times 10^{-9}$  for sublimation; yet at subzero temperatures ice will sublimate but will not melt. This indicates that the gain of translational and rotational degrees of freedom for water molecules going from the solid to the liquid state must be much smaller than that for molecules going from the solid to the gaseous state. This is demonstrated by the fact that the total entropy of fusion of ice is only 5.25 cal/deg/mole. The following considerations explain this phenomenon:

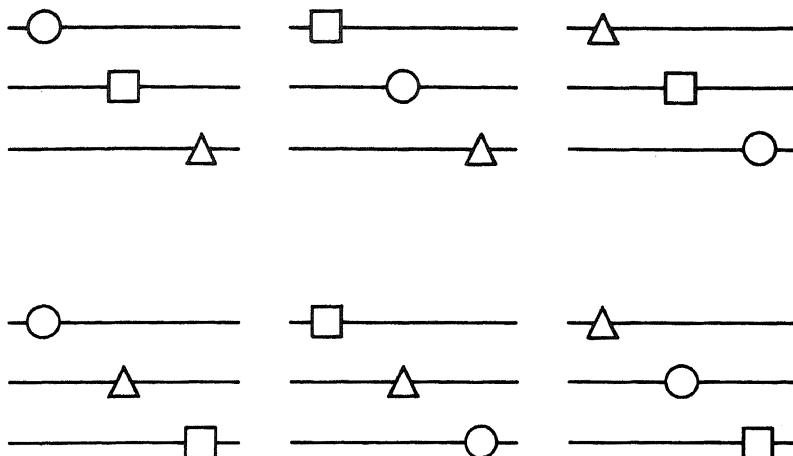
(1) The H-bonds between neighboring water molecules in the liquid greatly hinder rotation and thus diminish the rotational entropy gain on melting.

(2) Since the density of water near its freezing point is greater than that of ice,\* the average volume available to each water molecule is less in water than in ice. Thus the increase of translational entropy on sublimation far exceeds the increase of translational entropy on fusion.

We have discussed four types of entropy: translational, rotational, vibrational, and electronic. For pure substances these include virtually all the complexities distinguishable on the basis of energy levels and spatial localization or

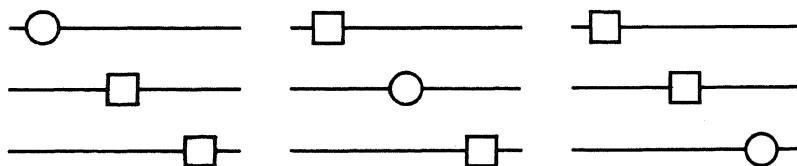
\* Therefore, ice floats.

on the basis of energy levels only. When we deal with a mixed population containing two or more types of molecule, we encounter a fifth form of entropy: configurational entropy. This is because a configuration in which A occupies



**Figure 1.2. THE POSSIBLE ARRANGEMENTS OF THREE PARTICLES OF THREE DIFFERENT SPECIES IN ONE DISTRIBUTION PATTERN.**

a certain site and energy level can be distinguished from a configuration in which B occupies the same site and energy level. Thus there is only one distinguishable complexion, corresponding to Figure 1.1A, if all three particles are of the same species. But if there are three different species, there is a sixfold increase in the



**Figure 1.3. THE POSSIBLE ARRANGEMENTS OF THREE PARTICLES OF TWO DIFFERENT SPECIES IN ONE DISTRIBUTION PATTERN.**

total number of complexions; these are illustrated in Figure 1.2. If there are only two species, the increase is threefold (Figure 1.3).

Let us designate the number of complexions possible when all particles are indistinguishable from each other as  $\Omega_{\text{thermal}}$ , because it varies with the tempera-

ture. The total number of complexions will be indicated as  $\Omega_{\text{total}}$  in which, among the total of  $N$  particles,  $N_A$  of species A and  $N_B$  of species B are found,

$$\Omega_{\text{total}} = \Omega_{\text{thermal}} \frac{N!}{N_A! N_B!} \quad (1-28)$$

$$= \Omega_{\text{thermal}} \frac{N!}{\prod_i N_i!}. \quad (1-29)$$

We then designate  $N!/\prod_i N_i!$  as  $\Omega_{\text{conf}}$  and  $k \ln \Omega_{\text{conf}}$  as configurational entropy.

In Chapter 2, we shall demonstrate that both configurational entropy and thermal entropy play very important roles in determining the degree of association of ions and molecules in various systems.

## B I B L I O G R A P H Y

### BOOKS AND MONOGRAPHS

Gurney, R. W., "Introduction to Statistical Mechanics." McGraw-Hill, New York, 1949.

The first three chapters of the book provide an extraordinarily clear summary of the subject matter of statistical mechanics. The later chapters could perhaps be more profitably read after intensive study of a book like that of Rushbrooke (below). Very little previous knowledge of mathematics or physics is required to read this book.

Rushbrooke, G. S., "Introduction to Statistical Mechanics." Oxford Univ. Press (Clarendon), London and New York, 1949. A remarkably well-written book, particularly for those who are not primarily physicists. The subject, presented in a consistent framework, is at no point abstruse or obscure. The opening chapters of the book by Gurney (above) may be helpful in gaining a general orientation before beginning this more rigorous treatment. The symbols and terminology are the same as those used by Fowler and Guggenheim (below); this book may thus serve as an excellent introduction to the latter important work.

Fowler, R. H., and Guggenheim, E. A., "Statistical Thermodynamics." Cambridge Univ. Press, London and New York, 1939. A more advanced and extremely valuable treatise.

# 2

## SITE FIXATION AND ASSOCIATION PHENOMENA

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The complex systems of protein, water, and ions that we call living matter are invariably found in environments of dilute aqueous salt solution, either as an external environment in the form of fresh or ocean water, or as an internal milieu in the form of tissue fluid. In the introduction, we argued that biological interactions must be determined by specific short-range forces in conjunction with long-range forces, rather than by the latter alone. Yet the most notable achievements in the understanding of dilute aqueous salt solutions (Debye and Hückel, 1923; Bjerrum, 1926) are consistent with, and in fact demand that the interactions between ions in dilute solution be almost entirely due to long-range Coulombic forces. In these theories, ionic association, which makes possible the interplay of short-range forces, is neglected. Thus, there is apparently a conflict between the thesis of short-range interaction and the most important theories of salt solution.

The resolution to this conflict lies in the fact that living protoplasm is not a simple dilute salt solution, but a three-dimensional lattice of protein, water, and salt in which the charge-bearing protein molecules are immobilized into a fixed-charge system. Thus, the conditions that prevail in a dilute aqueous salt solution differ radically from those in protoplasm, and the conclusion that ions are not associated in the former is not applicable to the latter. To clarify this thesis we shall review theories and experiments which support the argument that increasing association follows increasing spatial fixation of one species of ion. Thus, when charge fixation produces a sufficient degree of association, short-range interactions dominate and selectivity of counterions follows.

## 2.1. Association in Mono-Monovalent Strong Electrolyte Solutions

Since the important theories of Debye and Hückel and of Bjerrum were advanced, the association of alkali-metal ions with simple anions in aqueous solution has been regarded as scant. Thus, in a dilute solution of alkali halides, for example, the activity coefficient—an inverse measure of ion-ion interaction—is determined only by the “ionic strength,” which expresses the concentration and net charge of the ions but ignores their specific natures. The Debye-Hückel theory is a limiting law applicable to extremely dilute solutions only. Bjerrum’s theory, however, does consider ion-ion association; defining the fraction of associated ion pairs  $\alpha$ , he derived the equation

$$\alpha = \frac{N_{\pm}}{V} \int_a^q \exp(Z_1 Z_2 \epsilon^2 / D k T r) 4\pi r^2 dr. \quad (2-1)$$

Here  $N_{\pm}$  is the total number of anions and cations present in the volume  $V$ ;  $\epsilon$  is the electronic charge;  $Z_1$  and  $Z_2$  are the valences of the ions forming the pair;  $D$  is the dielectric constant of the medium;  $T$  is the absolute temperature;  $k$  is the Boltzmann constant;  $r$  is the center-to-center distance between the anion and

cation;  $q$  is the critical distance of separation that defines an ion pair; and  $a$  is the distance of closest approach between the interacting ions, equal to the sum of their ionic radii.

At low concentration in aqueous solution, the dissociation of the  $\text{Na}^+$  ion from the  $\text{Cl}^-$  ion is nearly complete. Bjerrum offered the interpretation that the sum of their ionic radii  $a$  is greater than the critical distance of separation  $q$ . Similarly, experimental observations on other 1-1 strong electrolytes such as  $\text{KCl}$  and alkylammonium chlorides agree with this theory (see Harned and Owen, 1958).

But, when we investigate the alkylammonium fluorides, even in fairly dilute solution, we detect ion-pair formation. Furthermore, even in the case of sodium chloride, Bjerrum's theory predicts that increasing ionic association  $\alpha$  follows increasing ion concentration  $N_{\pm}$ . Fuoss and Kraus (1935) presented theoretical arguments along Bjerrum's line of reasoning and experimental observations to show that triple and quadruple associated ionic clusters form at high ionic concentrations. The state of complete association for salt ions is clearly reached in the ionic crystal; theoretically, we can crystallize all strong electrolytes from their aqueous solutions by lowering the temperature and increasing the concentration. Thus, complete dissociation of alkali-metal-ion salts occurs only under specific conditions determined by the nature of variables such as the anion, ionic concentration, and the dielectric constant of the medium; *no ion pair may be said never to associate*.

## 2.2. Association in Mono-Multivalent Strong Electrolyte Solutions

The change of the value of  $Z_2$  in equation (2-1) from 1, for  $\text{NaCl}$ , to 2, for multivalent salts such as  $\text{Li}_2\text{SO}_4$  and  $\text{Na}_2\text{SO}_4$ , indicates a higher degree of ionic association at low ionic concentrations. Both conductance, as in Table 2.1, and isopiestic measurements demonstrate this (Righellato and Davies, 1930; also Robinson *et al.*, 1941). Contrasting these results with the smaller degree of association in the 1-1 strong electrolytes, one sees the simplest example of the effects of fixation; the fixation of two negative charges together to form a divalent anion increases the degree of association. In this case, the two charges happen to be in very close proximity.

Now let us consider multivalent anions whose ionic groups are farther apart than those of the sulfates; oxalic acid ( $\text{HOOC}-\text{COOH}$ ), for instance. Although the two carboxyl groups on this divalent acid are physically indistinguishable, they have very different dissociation constants. Bjerrum (1923b)\* established two reasons for this: (1) For the first dissociation, there are two choices; therefore, the

\* See also Westheimer and Shookhoff, 1939.

Salt	Molarity	$\mu$	$\log K$	
$\text{Li}_2\text{SO}_4$ $(\Sigma B = 1.60)$	0.005	0.01475	-0.642	
	0.01	0.02916	-0.645	
	0.025	0.07130	-0.647	
	0.05	0.1390	-0.641	
	0.1	0.2674	-0.632	
	0.25	0.6255	-0.462	
$\text{Na}_2\text{SO}_4$ $(\Sigma B = 1.30)$	0.005	0.01458	-0.880	
	0.025	0.07092	-0.709	
	0.05	0.1383	-0.711	
	0.1	0.2688	-0.688	
$\text{K}_2\text{SO}_4$ $(\Sigma B = 1.05)$	0.005	0.01462	-0.833	
	0.01	0.02878	-0.834	
	0.025	0.07005	-0.820	
	0.05	0.1368	-0.810	
	0.1	0.2651	-0.815	
	0.25	0.6415	-0.722	
$\text{Ag}_2\text{SO}_4$ $(\Sigma B = ?)$	0.0025	0.0073	-1.154	
	0.005	0.01409	-1.259	
	$\text{Tl}_2\text{SO}_4$ $(\Sigma B = 1.99)$	0.001	0.002924	-1.405
	0.0025	0.007160	-1.336	
	0.005	0.01391	-1.323	
	0.01	0.02658	-1.325	
$\text{CaSO}_4$ $(\Sigma B = 1.00)$	0.025	0.06110	-1.324	
	0.05	0.1122	-1.321	

**Table 2.1.** THE EXTENT OF DISSOCIATION OF UNIBIVALENT SALTS ( $\text{C}_2\text{A}$ ) IN WATER. For the reaction  $\text{C}^+ + \text{A}^- \rightleftharpoons \text{CA}^-$ , the dissociation constant  $K$  is defined as

$$K = \frac{(\text{A}^-)(\text{C}^+)}{(\text{CA}^-)} \frac{f_{\text{A}} f_{\text{C}}}{f_{\text{CA}}},$$

where  $(\text{A}^-)$  is the concentration of  $\text{A}^-$  anion,  $f_{\text{C}}$  is the activity coefficient of the  $\text{C}^+$  cation, and  $\mu$  is the ionic strength. These data, calculated from the conductivities, indicate a considerable degree of ionic association at low ionic strength. (Data from Righellato and Davies, 1930.)

dissociation of the first proton is favored by a factor of  $\ln 2$ . For the first association, there are also two choices, thus favoring the association of the second proton. Combined, these factors make the first dissociation constant ( $pK$ ) differ from the second by  $\ln (2 \times 2) = \ln 4$ ; this is an entropy factor (Chapter 1). (2) After the first proton has dissociated, the anionic charge acts electrostatically on the second proton and increases the energy needed to effect a dissociation.

Finally, let us consider dicarboxylic tetradecanedioic acid (Peek and Hill,

1951). Measured in 20 per cent methanol in water, the *pK* values of the two equivalent carboxyl groups are, respectively, 5.91 and 5.10. After correction for the entropy factor, an energy difference of some 0.29 kcal/mole remains, even though the two acid groups are separated by 14 carbon atoms.

We may conclude that the fixation of two charged groups together makes dissociation from the second group more difficult than it would be if the groups were not fixed. In other words, an increase in the average association follows this most simple type of ionic-site fixation. The effect in question occurs also when many charged groups are fixed together either by covalent bonds or through micellar aggregation resulting from the action of short-range van der Waals' forces.

### 2.3. Association Phenomena in Semifixed-Charge Systems

#### A. MICELLAR AGGREGATES

In an aqueous solution, organic electrolytes such as sodium acetate and ethylamine hydrochloride behave as normal 1-1 strong electrolytes; they show complete dissociation up to fairly high concentrations. In compounds with longer alkyl chains, however, the nearly ideal behavior,\* as shown by their equivalent conductances, breaks down abruptly at a critical concentration. A very sharp drop in equivalent conductance is associated with further increase of concentration. This phenomenon has been explained as a result of micelle formation (J. W. McBain, 1913; G. S. Hartley, 1936). The equivalent conductance is the sum of the conductances due to the anion and the cation. The fractions of current carried by the cation and the anion are called the transference numbers  $t_+$  and  $t_-$ ; these are determined by the mobilities  $u_+$  and  $u_-$  and the ionic concentrations  $C_+$  and  $C_-$ :

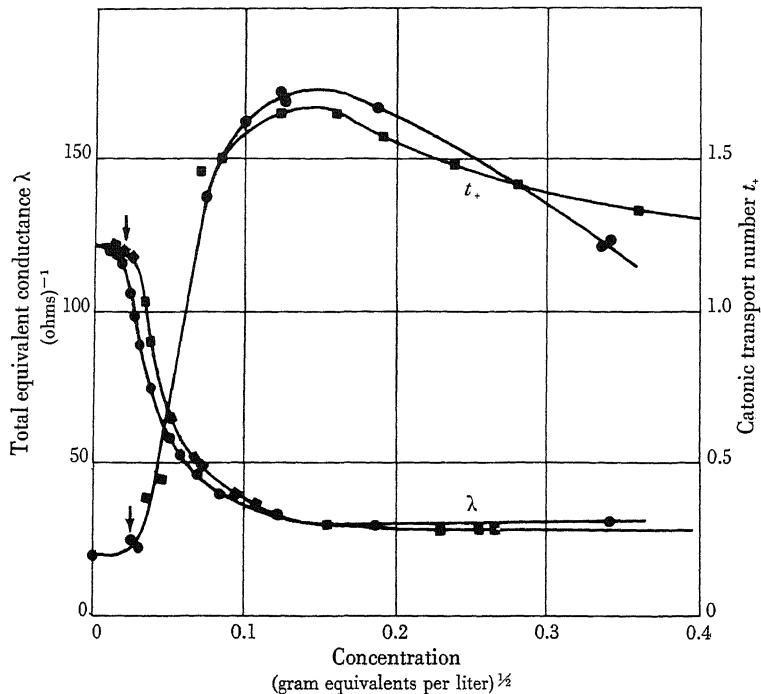
$$t_+ = \frac{C_+u_+}{C_+u_+ + C_-u_-} \quad (2-2)$$

$$t_- = \frac{C_-u_-}{C_+u_+ + C_-u_-}. \quad (2-3)$$

McBain reasoned that the mobility of the long-chain electrolyte increases with micellar aggregation. The experimental results shown in Figure 2.1, taken from G. S. Hartley *et al.* (1936) fully support this deduction. The equivalent conductance  $\lambda$  drops abruptly and the cationic transference numbers  $t_+$  of cetylpyridinium and cetyltrimethylammonium bromide simultaneously rise steeply to such an extent that they exceed unity. Hartley's explanation is most convincing: In this condition, "more bromine is being carried toward the cathode than is migrating

\* Strictly speaking, the behavior is not entirely ideal even in the low concentration range, but departure of the conductivity from the Onsager theory is not great (see M. E. L. McBain, 1955).

in the free state to the anode, and therefore the bromide transport numbers are negative." Considering that the equivalent conductance has fallen from 120 to less than 30, the conclusion is obvious. The long-chain cations aggregate into a semifixed-charge system while the bromide ion shifts from an essentially dissociated condition to a high degree of association; see Figure 2.2. The strong



**Figure 2.1. THE EFFECT OF MICELLE FORMATION ON CONDUCTANCE.** The curves show the total equivalent conductance  $\lambda$  and the transport number of the paraffin chain cations  $t_+$  of cetylpyridinium • and cetyltrimethylammonium ■ bromides at 35°C. The inflection ↓ marks the critical point for micelle formation with its associated charge fixation. Cationic transfer numbers were determined by the moving boundary method. (Figure after G. S. Hartley *et al.*, 1936.)

dependence of the critical concentration on the nature of the mobile counterion shown in Grieger's data (1949), Figure 2.3, agrees perfectly with this view. Similar dependence of the critical concentration of dodecyl sulfate on the nature of the alkali-metal countercation,  $K^+$ ,  $Na^+$ , and so on, has also been reported by Meguro *et al.* (1956).

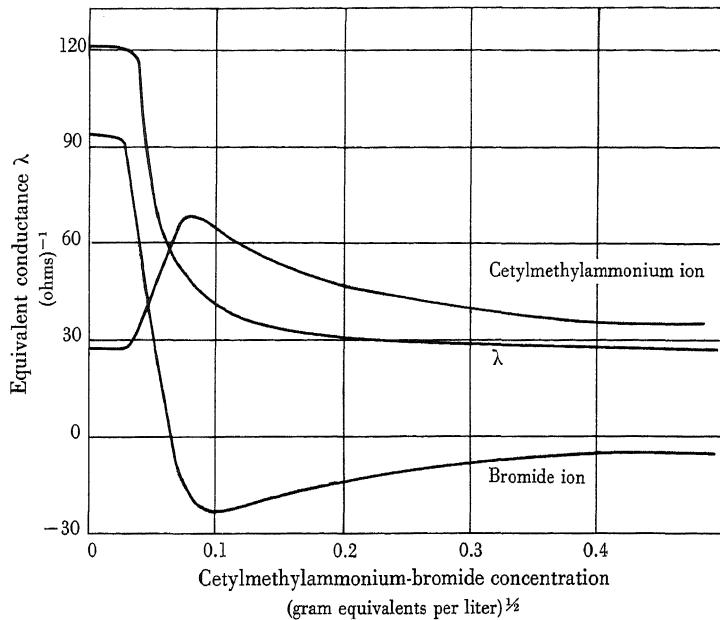
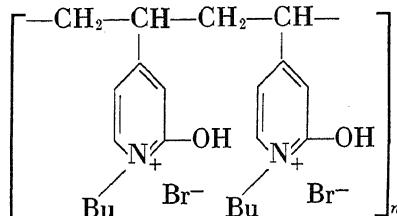


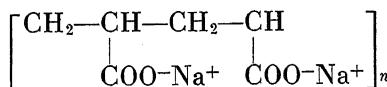
Figure 2.2. THE EQUIVALENT CONDUCTANCE OF THE COMPONENT IONS AND THE TOTAL EQUIVALENT CONDUCTANCE  $\lambda$  OF CETYLTRIMETHYLMONIUM BROMIDE AT 35°C. (Figure after G. S. Hartley *et al.*, 1936.)

## B. LINEAR POLYELECTROLYTES

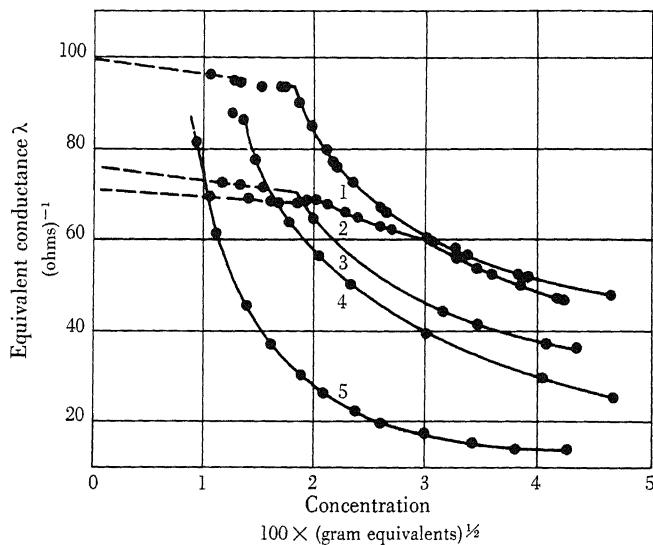
Figure 2.4 presents data of Edelson and Fuoss (1950) on conductances of poly-*p*-vinyl-*N*-*n*-butylpyridonium bromide (PVBB)



and of sodium polyacrylate (SPA)



These data show both that the individual conductances of these polyelectrolyte solutions are much lower than those of equivalent solutions of NaBr and that the sum of the conductances of the Na-SPA and the Br-PVBB solution is lower than the conductance of NaBr.\* No reasonable correction for the viscosity of the system explains this difference. The simplest explanation for these observations is



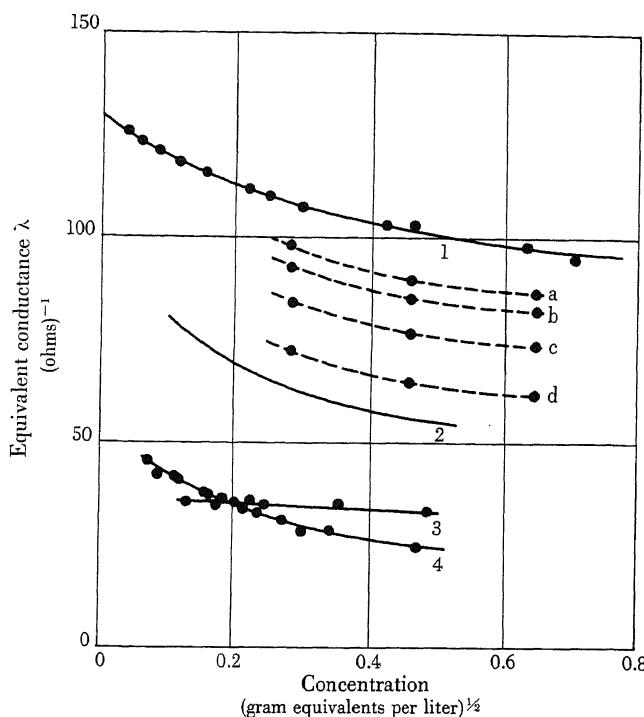
**Figure 2.3.** EQUIVALENT CONDUCTANCE OF THE OCTADECYLTRIMETHYLLAMMONIUM SALTS OF VARYING ANIONS. (1) chloride, (2) formate, (3) bromate, (4) nitrate, (5) oxalate. The curves demonstrate the significance of the specific nature of counterions in determining micellar aggregation. (Figure after Grieger, 1949.)

that a higher degree of association exists between the free counterions and the ionic groups on the respective macromolecules than between  $\text{Na}^+$  and  $\text{Br}^-$  ions in solutions of NaBr at equivalent concentrations. The behavior of linear polyelectrolytes is thus similar to that of micellar aggregates.

The experimental facts show that, as more and more charged groups are fixed together, the over-all degree of association between ions of opposite charge increases. Two points are worth mentioning: (1) In dicarboxylic acids, the dissociation of the first cation affects the dissociation of only one other cation. In polyelectrolytes, the dissociation of the first cation influences more than one subsequent dissociation. The average association thus increases with the total

\* Edelson and Fuoss conclude from these data that "polyelectrolyte *gegenions* behave much like the same ions in simple electrolytes."

number of charges fixed. When the fixed-charge system assumes macroscopic dimensions, very few ions can diffuse away; this is usually understood in terms of the maintenance of macroscopic electroneutrality. (2) As the total number of charges increases, the net charge that arises from their dissociation begins to



**Figure 2.4. THE CONDUCTANCE OF POLYELECTROLYTE SOLUTIONS.** The curves represent (1) NaBr, (2) the sum of (3) and (4), (3) sodium polyacrylate (SPA), and (4) poly-*p*-vinyl-*N*-*n*-butylpyridonium bromide (PVBB). The conductance of NaBr in various concentrations of polyvinyl alcohol (dotted lines a-d) shows that no reasonable correction for viscosity can cause line 1 to coincide with line 2. Furthermore, extrapolation of lines 2, 3, or 4 to a concentration of zero, at which neither viscosity nor "chain interference" would surpass that of NaBr, cannot reasonably be expected to alter the conclusion that conductance is being reduced through ionic site fixation. (Figure after Edelson and Fuoss, 1950.)

create a long-range electrostatic field so that it becomes more and more likely that the dissociated ions will be found in a diffuse ion cloud surrounding the polyelectrolyte. Each particle of polyelectrolyte acts as a droplet of concentrated ionic solution (see Florey, 1945); it is influenced relatively little by the total ionic strength of the medium and is capable of preserving a constant high degree of ionic

association (see equation 2-1). Thus, if 10 charges are fixed together, dilution of the system with a very large volume of pure water will not change it much. But if these 10 charges are entirely separate, such dilution leads to the dispersion and dissociation of all the ions.

These factors probably play a predominant role in producing a high degree of ionic association in linear polyelectrolytes or micellar aggregates to which we refer collectively as *semifixed-charge systems*. In the next section, we shall discuss how other statistical-mechanical considerations suggest other mechanisms, which are less important in semifixed-charge systems but become highly significant in true fixed-charge systems. As the degree of ionic association is enhanced, short-range forces begin to play an important role in determining the dissociation energy between members of an ion pair. Thus we can expect differences between the dissociation energies of different ions to emerge. This, in turn, leads to ionic specificity. An examination of experimental evidence confirms this expectation.

#### 2.4. Association of Counterions in True Fixed-Charge Systems

If a fixed-charge system is either purely or effectively anionic or cationic, dissociation of a counterion leaves a net charge that discourages the dissociation of a second otherwise equivalent counterion. The principle in question is similar to that illustrated by the difference in the free energies of dissociation of the first and second  $H^+$  ions of a dicarboxylic acid. However, ionic dissociation in a three-dimensional macroscopic anionic or cationic fixed-charge system always has two components: (1) dissociation from the surface layer into the free solution, similar to dissociation from a linear polymer, and (2) dissociation in the bulk phase in which macroscopic electroneutrality is conserved and the great majority of counterions are confined within the fixed-charge system. The dissociation of counterions inside such a macroscopic fixed-charge system is thus not dissociation away from the fixed-charge system as a whole; rather it is a matter of how a population of counterions will distribute itself statistically among the possible positions *within* the system.

If such a fixed-charge system is a rigid three-dimensional lattice, charges borne on it will, like those in crystals, occupy specific positions in space. The system may then be represented as consisting of minute microcells, each enclosing one fixed ionic site, Figure 3.1. In this ideal case, the most probable distribution of ions is one in which they are distributed one to a microcell. Any other configuration entails multiple occupancies which are energetically unfavorable and therefore improbable. To aid in the visualization of this model, one may imagine that each volume containing 10 or 20 microcells is a large polyelectrolyte molecule. The detachment of one counterion will make it less probable that a second counterion will dissociate. Furthermore, even the first counterion will have

difficulty in dissociating because the dissociated counterion in a true fixed-charge system cannot move to an "external" location, but must remain within the system to maintain macroscopic electroneutrality. Thus, a counterion must either remain in its "own" microcell or occupy an energetically unfavorable position. Ionic association and dissociation in a true fixed-charge system thus becomes a question of the probabilities of either association or dissociation within the unit microcell.

#### A. THE EFFECTS OF ENERGY AND ENTROPY ON DISSOCIATION PHENOMENA

The development of a satisfactory theory of the liquid state is a difficult problem and has not been solved. The earliest models of liquids were built on the assumption that the molecules were distributed randomly.\* Since x-ray analytical work such as that of Stewart (1927), there has been little doubt that, near their freezing points (for example, water at room temperature), liquids retain the type of short-range order found in crystals. We have chosen a statistical-mechanical model for the treatment of association phenomena; it treats aqueous solutions and fixed-charge systems as if they were crystals with a very large number of sites, each of which may be occupied by a solvent molecule or a solute ion.

Let us consider an aqueous salt solution; in such a solution, association phenomena are understood in terms of chemical equilibrium. If we represent the free anion as A and its counterion as B, then  $A + B \rightleftharpoons AB$ . Since the process occurs in aqueous solution, the equilibrium constant  $K$  is derived from the equation:

$$K = \frac{X_A X_B}{X_{AB}} = \frac{(p.f.)_A (p.f.)_B}{(p.f.)_{AB}} \exp(\Delta E / RT). \quad (2-4)$$

Here  $X_A$ ,  $X_B$ , and  $X_{AB}$  are the mole fractions of A, B, and the associated ion pair AB, respectively;  $\Delta E$  is the energy of association;  $R$  is the gas constant; and  $T$  is the absolute temperature. If  $N_A$ ,  $N_B$ , and  $N_{AB}$  are the number of A, B, and AB species in the assembly, then

$$\frac{N_A N_B}{N_{AB}} = N' \frac{(p.f.)_A (p.f.)_B}{(p.f.)_{AB}} \exp(\Delta E / RT) \quad (2-5)$$

where  $N'$  is the total number of solute and solvent molecules. Let

$$\alpha = \frac{N_{AB}}{N_A + N_{AB}} = \frac{N_{AB}}{N_A^\circ} \quad (2-6)$$

the fraction of component A which is associated;  $N_A^\circ$  is the total number of A

\* In our earlier consideration of the entropy of dissociation of an ion, we adopted a similar model in which the aqueous phase was regarded as a continuous medium; such a view is more consistent with a random-distribution model of liquid (Ling, 1960).

present. Since A and B are present in equal numbers, we have also

$$\alpha = \frac{N_{AB}}{N_B + N_{AB}} = \frac{N_{AB}}{N_B^\circ} \quad (2-7)$$

and

$$N_A = N_B = (1 - \alpha)N_A^\circ. \quad (2-8)$$

Equation (2-5) can now be written

$$\frac{(1 - \alpha)^2}{\alpha} = \frac{N'}{N_A^\circ} \frac{(p.f.)_A(p.f.)_B}{(p.f.)_{AB}} \exp(\Delta E/RT). \quad (2-9)$$

If we let

$$\frac{N'}{N_A^\circ} \frac{(p.f.)_A(p.f.)_B}{(p.f.)_{AB}} \exp(\Delta E/RT) = s \quad (2-10)$$

then

$$\alpha = \frac{2 + s \pm \sqrt{s^2 + 4s}}{2}. \quad (2-11)$$

The partition-function ratio  $(p.f.)_A(p.f.)_B/(p.f.)_{AB}$  for the dissociation of NaCl into its ions is estimated by Fowler and Guggenheim (1939) to have a value of about 100 if both  $\text{Na}^+$  and  $\text{Cl}^-$  ions are assumed to be rigid hard spheres, with rotational partition functions equal to unity.

A typical value for  $\Delta E$  is  $-12$  kcal/mole. Substituting into equations (2-6) and (2-7), we obtain a value of  $\alpha$  equal to unity; there should be no dissociation of NaCl in water at all. Yet it is completely dissociated. Fowler and Guggenheim have shown that this error arises from ignoring the effect of hydration. Neither  $\text{Na}^+$  nor  $\text{Cl}^-$  ions exist as single rigid spheres; they possess a shell of rigidly attached water molecules. Thus, these hydrated ions are effectively polyatomic particles. A rigorous evaluation of the rotational partition function of such aggregates is difficult; but its important effect on ionic association is demonstrated by the following example. The dissociation of trimethylammonium nitrate, both ions of which are polyatomic, may be represented as



Fowler and Guggenheim have estimated that the ratio of the partition functions for this dissociation is close to  $10^8$ . They obtained this large value, which is due to the rotational entropies of the polyatomic particles, by assuming that the ions dissociate in a continuous dielectric with no hindrance to free rotation; this condition may be realized only in a vacuum. Nevertheless, the rotational movements of hydrated ions in a uniformly fluid and mobile population of water molecules can depart but little from the ideal case; thus, to make a rough estimate, we shall say that the partition function ratio is  $10^7$  rather than  $10^8$ . If  $N_A^\circ$  has a value of 0.1 moles per liter,  $N'/N_A^\circ$  is equal to  $55.6/0.1 = 556$ , where 55.6 is the molar concentration of water in a dilute salt solution. Substituting these values into

equations (2-10) and (2-11), one obtains

$$\mathfrak{S} = 556 \times 10^7 \times \frac{1}{\exp(12/0.6)} = 11.5$$

and  $\alpha$  approaches 0. Here the use of such estimated values leads to the conclusion that ions are dissociated in dilute aqueous solutions, a conclusion consistent with observations of real solutions.

Assuming that there is no difference between the rotational-entropy gain on dissociation in a free solution and that in a fixed-charge system, we shall investigate the effects of configurational entropy on dissociation in a fixed-charge system. Then we shall incorporate the rotational entropy effect. Finally we shall discuss association energy within the fixed-charge system.

### (1) The configurational entropy of dissociation in a fixed-charge system

Using the crystal-lattice model we derive the equation:<sup>\*</sup>

$$\frac{N_{\text{dissoc}}}{N_{\text{assoc}}} = \frac{\sigma(\text{p.f.})_{\text{dissoc}}}{\rho(\text{p.f.})_{\text{assoc}}} \exp(\Delta E/RT). \quad (2-13)$$

Here  $N_{\text{dissoc}}$  is the number and  $(\text{p.f.})_{\text{dissoc}}$ , the partition function of the counterions in the dissociated state;  $N_{\text{assoc}}$  is the number and  $(\text{p.f.})_{\text{assoc}}$ , the partition function of the counterions in the associated state;  $\Delta E$  is the average energy difference per mole between the associated and dissociated states and is a negative value;  $\sigma$  and  $\rho$  are the total number of sites that a counterion can occupy within a microcell. Let  $\rho$  be the number of sites at which a counterion may be said to be associated, and  $\sigma$ , the number of sites at which a counterion may be said to be dissociated. Since the number of sites in a unit volume is constant, the ratio  $\sigma/\rho$  is equal to the ratio of the volume that can be occupied by associated ions to the volume that can be occupied by dissociated ions.

Let  $\alpha$  represent the fraction of ions that are associated;  $(1 - \alpha)$ , the fraction of ions that are dissociated; and define the relation,<sup>†</sup>

$$\mathcal{R} \equiv \frac{\sigma(\text{p.f.})_{\text{dissoc}}}{\rho(\text{p.f.})_{\text{assoc}}} \exp(\Delta E/RT);$$

then

$$\frac{N_{\text{dissoc}}}{N_{\text{assoc}}} = \frac{(1 - \alpha)}{\alpha} = \mathcal{R} \quad (2-14)$$

and

$$\alpha = \frac{1}{1 + \mathcal{R}} \quad (1 - \alpha) = \frac{\mathcal{R}}{1 + \mathcal{R}}. \quad (2-15)$$

\* A full derivation appears in Appendices A and B.

† For simplicity,  $\sigma(\text{p.f.})_{\text{dissoc}}$  and  $\rho(\text{p.f.})_{\text{assoc}}$  will be represented by  $(\text{p.f.})_{\text{dissoc}}$  and  $(\text{p.f.})_{\text{assoc}}$ , respectively. Equation (2-13) then becomes

$$\frac{N_{\text{dissoc}}}{N_{\text{assoc}}} = \frac{(\text{p.f.})_{\text{dissoc}}}{(\text{p.f.})_{\text{assoc}}} \exp(\Delta E/RT).$$

To evaluate  $\alpha$ , we assume that, for thermal entropy, the conditions, (a) and (b), hold.

(a) The partition function of the free anion (*p.f.*)<sub>A</sub> is equal to the partition function of the associated ion pair (*p.f.*)<sub>AB</sub>. (b) The partition function for the associated ion with the fixed-charge system has a value of unity and the partition function for the dissociated ion in the fixed-charge system is equal to the partition function for the same dissociated ion in free solution,

$$(\text{p.f.})_{\text{dissoc}} = (\text{p.f.})_B.$$

Then

$$\left[ \frac{(\text{p.f.})_{\text{dissoc}}}{(\text{p.f.})_{\text{assoc}}} \right]_{\text{fix}} = \frac{(\text{p.f.})_A (\text{p.f.})_B}{(\text{p.f.})_{AB}}. \quad (2-16)$$

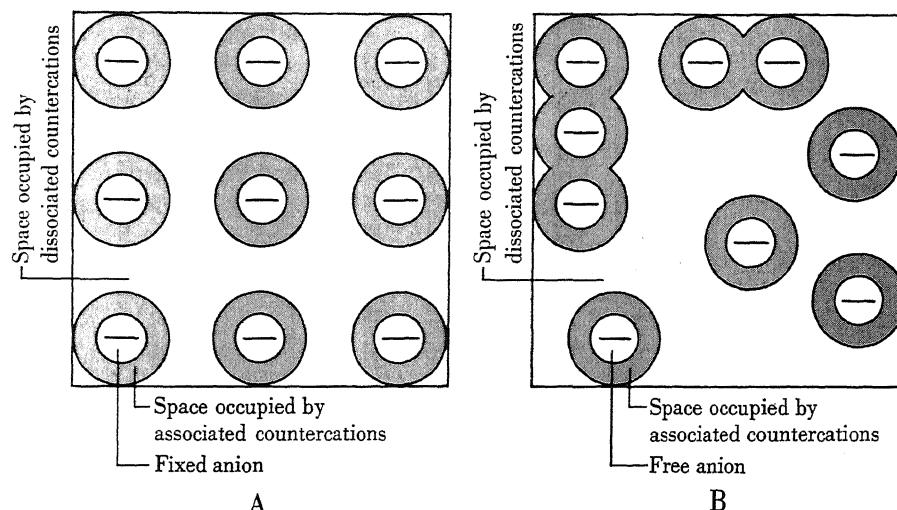
To estimate dissociation within a fixed-charge system, we specify an anionic fixed-charge system with a microcell radius  $r_3$ . A counterion bearing a unit positive charge that occupies a position in a spherical shell with an inner radius of  $r_1$  and an outer radius of  $r_2$ , both measured from the center of the fixed anion, is defined as associated. If the counterion occupies a position in an outer shell with radii  $r_2$  and  $r_3$ , respectively, it is considered dissociated. The value of  $r_2$  is determined by the limit of complete dielectric saturation. Assume that it has a value of 7 Å. If  $r_1$  is 1 Å,  $r_3$ , corresponding to a microcell of a diameter of 20 Å, is 10 Å; and we assume that  $\Delta E$  is -12 kcal/mole. Equations (2-14) and (2-15) yield:

$$\begin{aligned} \mathcal{R} &= \frac{\frac{4}{3}\pi r_3^3 - \frac{4}{3}\pi r_2^3}{\frac{4}{3}\pi r_2^3 - \frac{4}{3}\pi r_1^3} \times \frac{(\text{p.f.})_{\text{dissoc}}}{(\text{p.f.})_{\text{assoc}}} \exp(-12/0.59) \\ &= \frac{1000 - 343}{343 - 1} \times 10^7 \times \frac{1}{6.294 \times 10^8} = 3.05 \times 10^{-2} \\ (1 - \alpha) &= \frac{3.05 \times 10^{-2}}{1 + 3.05 \times 10^{-2}} = 3\% \quad \alpha = 97\%. \end{aligned}$$

This estimation suggests that a relatively high degree of ionic association may be expected on the basis of the theoretical model. The value of  $r_2$  is crucial in this estimation. If  $r_2$  is not 7 Å, but 2 Å,  $\alpha$  will be only 31.7%. To understand this difference, we must consider the mechanism by which ionic association is increased due to the configurational entropy.

In the fixed-charge system, the fixed sites are quite evenly distributed and have a unique configuration. In free solution, the anions may have a very large number of configurations; one of these is shown in Figure 2.5B. Comparing this with Figure 2.5A, one sees that the ratio  $\sigma/\rho$  is maximized in the fixed-charge system. In the configuration shown in B, the total number of associated sites is considerably diminished due to overlap and the total number of dissociated sites is correspondingly increased. Since the average configuration in free solution is more like B than A, a higher degree of dissociation is predicted on the basis of configurational entropy alone. The lower configurational entropy gain on dis-

sociation in a fixed-charge system may also be represented by a difference in "free volume" if the aqueous medium is considered not as a modified crystalline system, but as a continuous dielectric (Ling, 1960). Dr. C. N. Yang at the Institute for Advanced Studies, Princeton, suggested the mechanism by which the configurational entropy of dissociation is decreased in a fixed-charge system. The



**Figure 2.5. THE EFFECT OF CONFIGURATIONAL ENTROPY ON ION DISSOCIATION IN A FIXED-CHARGE SYSTEM AND IN FREE SOLUTION.** Part A shows diagrammatically the relatively uniform distribution of fixed ionic sites in a fixed-charge system. This creates a maximal ratio of the total associated sites (shaded) to the dissociated sites (unshaded), leading to a higher degree of ion association. Part B typifies a more probable configuration of the ion in a free solution. Here a smaller ratio results from the overlapping of associated sites and the consequent increase of dissociated sites. This leads to a lower degree of ion association.

criteria for the operation of this mechanism are two: (1) The volume of the associated sites ( $\rho$ ) is fairly large. (2) The edge of the boundary between associated and dissociated sites is sharp. The large value of  $\rho$  is due to the existence of more than one configuration corresponding to the associated state (see Chapter 4) and the sharp edge is due to the steep drop of dielectric constant as one approaches an ion (see Figure 6, Ling, 1952).

## (2) The rotational entropy of dissociation in a fixed-charge system

When a symmetrical molecular substance changes its state from solid to gaseous, the latent heat of vaporization is relatively small: it is 0.22 kcal/mole for hydrogen, 2.08 for oxygen, 1.69 for nitrogen (Slater, 1939, p. 259). For ice and for solid ethyl alcohol, the latent heats of vaporization are 11.3 and 10.4 kcal/mole,

respectively. The hydrogen bonds in ice and in solid alcohol determine this difference; vaporization is possible only when enough energy is absorbed to break these bonds (Pauling, 1948).

In melting, molecules are not completely dispersed. It takes 0.028 kcal/mole to melt solid hydrogen, 0.096 to melt solid oxygen, and 0.218 to melt solid nitrogen, but it takes 1.43 kcal/mole to melt ice and 1.10 kcal/mole to melt solid ethyl alcohol. Thus, there is a reduction of the number of hydrogen bonds in the melting of ice.

The entropy of ice is largely vibrational.\* The increase of translational entropy on sublimation is due to the great increase of free volume. The increase in rotational entropy also follows naturally: since  $\text{H}_2\text{O}$  is asymmetrical, its full rotational entropy [see equation (1-22)] is attained only in the vapor state. Although water has the same molecular asymmetry in the ice state, the formation of hydrogen bonds effectively inhibits free rotation so that there is relatively little rotational entropy in ice.

Let us now examine the entropies of melting of various substances: that of hydrogen is 2.0 cal/deg/mole; oxygen, 1.78; and nitrogen, 3.46; the entropies of melting of water and ethyl alcohol are 5.25 and 7.10 cal/deg/mole, respectively. Most theories of the entropy of melting are built on the premise that a volume expansion accompanies melting. This leads to an increase of free volume and a consequent increase of translational (communal) and vibrational entropy (see Slater, 1939, p. 260; Eyring and Hirschfelder, 1937; Rice, 1939). In the case of water, melting is accompanied by a decrease of volume, not an increase. Yet the entropy of melting is considerably higher than the entropies of melting for many substances that occupy larger volumes in the liquid state than in the solid state. Since this extra entropy cannot be translational, vibrational, or electronic,† we can conclude that melting of ice is accompanied by an increase of rotational entropy. The ice-liquid water transformation involves the breaking of permanent energy barriers; this allows the molecules to rotate. The fixed hydrogen bonds in the solid state thus restrict free rotation more than do the fluid energy barriers of the liquid state.

A fixed-charge system bears a relation to free solution similar to the relation of ice to liquid water. The proteinaceous fixed-charge system possesses a high density of permanently fixed hydrogen-bonding sites; these can inhibit the free rotation of dipolar molecules like water, and also of ions and polar molecules with their hydration shells of water. Let us assume that this restriction of rotation in the dissociated state, within a fixed-charge system, reduces the partition function ratio  $(\text{p.f.})_{\text{dissoc}}/(\text{p.f.})_{\text{assoc}}$ , equation (2-13), to  $10^2$ . Under these conditions, dissociation will be only 29 per cent, even if  $\Delta E$  is as low as -5 kcal/mole.

\* To a small degree, other entropies (residual entropy) seem to exist due to lattice imperfections (Pauling, 1948, p. 301).

† At 0°C or thereabout, the temperature is too low for electronic excitation.

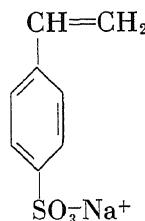
(3) The effect of the adsorption energy on ionic association

The exponential relation between association energy and ion association is such that if  $\Delta E$  is very high, say  $-30$  kcal/mole, association will be virtually complete in a fixed-charge system and even in a free, fairly dilute solution. Chapters 4 and 9 present evidence that the association energy of  $H^+$  ions with a carboxylic group often exceeds this value and that adsorption is independent of the fixation of the anionic groups. For the association of ions such as  $K^+$ ,  $Na^+$ , and  $NH_4^+$ , the association energy is usually about  $-10$  kcal/mole. We can now understand why the crucial importance of the  $H^+$  ion is retained in protein interaction whether the protein exists as a fixed-charge system in the living cell or is dispersed in a dilute aqueous solution. But alkali-metal ions like  $Na^+$  and  $K^+$ , in many cases, seem to have no action on protein in dilute aqueous solutions other than their contribution to the nonspecific ionic strength; they demonstrate their full specificity only when interacting with the living protoplasm in the natural state as a fixed-charge system.

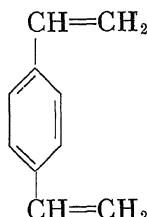
## B. NONLIVING THREE-DIMENSIONAL FIXED-CHARGE SYSTEMS

(1) Exchange resins

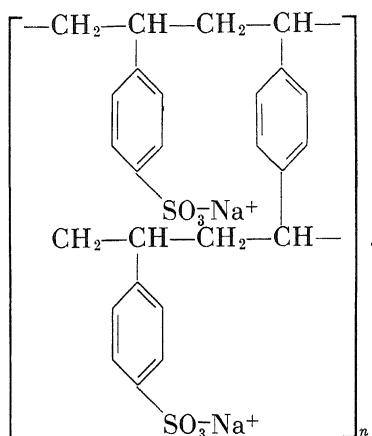
At low concentrations long-chain electrolytes are completely dissociated, but above a critical concentration when these molecules suddenly aggregate into micelles, they show a high degree of ionic association. Similarly, the polymerization of simple monomeric ions into linear polymers leads to an increased association of their counterions. It follows that we should expect an increase in the degree of association between  $Na^+$  and sulfonate ions following the polymerization of styrene sulfonate, into polystyrene sulfonate. If a cross-linking agent such as



divinylbenzene, is added during this polymerization process, instead of a linear

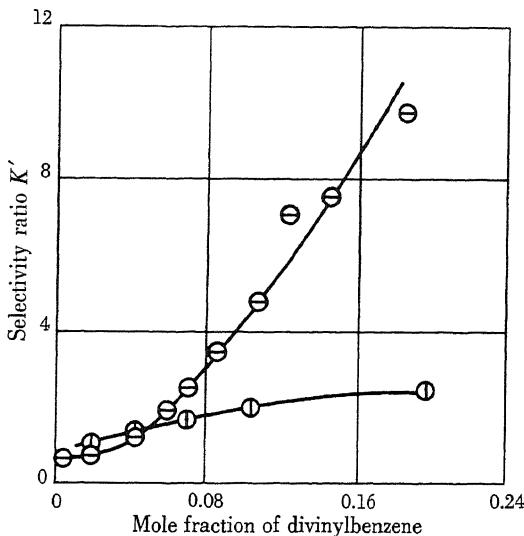


polymer we obtain a three-dimensional network of charged sulfonate groups, that is, a three-dimensional fixed-charge system. This is an example of an ion exchange resin:



To understand the high degree of association between fixed ions and their counterions and the attendant selective ionic accumulation, we must examine the decrease in entropy of dissociation within such systems. Configurational entropy decreases have been discussed in Section 2.4A(1); we must now elaborate on the problem of rotational entropy. Under normal conditions Amberlite exchange resins contain 50 per cent or less water by weight (see Rohm and Haas, 1949). Thus, in one liter of resin 500 grams of carbon chains form a network consisting of basic C—C units. Given a C—C bond length of 1.54 Å (Pauling, 1948, Chapter 5), suppose that each residue is a —CH<sub>2</sub>— with an average weight of 14; if all the residues were joined into a single chain, it would be  $\frac{50}{14} \times 6.023 \times 10^{23} \times 1.54 \times 10^{-8} = 3.3 \times 10^{17}$  cm in length. If it is divided into 10-centimeter segments, there should be  $(3.3 \times 10^{17})/10 = 3.3 \times 10^{16}$  such segments per 100 square centimeters, or  $1.82 \times 10^8$  segments per 10 centimeters. The distance between adjoining segments is thus  $10/(1.82 \times 10^8) = 5.49 \times 10^{-8}$  centimeters. Since a water molecule is about 2.7 Å in diameter (Pauling, 1948, p. 304), a hydrated ion with a shell of water molecules cannot be smaller than 5 Å in diameter. Rotation of a 5 Å molecule in a lattice with average interstices of 5 Å must be greatly restricted. Thus the restriction of both configurational and rotational entropy gain on dissociation depends largely on the spatial fixation of the lattice.

Since the degree of fixation in ion exchange resins varies directly with the percentage of cross-linking agent, one would expect an increasing degree of association and, therefore, of ionic selectivity to be exhibited sharply with an increasing percentage of cross-linking agent. This concept is consistent with the findings of Gregor (1951) and Bonner (1954): In the synthesis of an exchange resin, the ionic selectivity coefficient increases as the percentage of cross-linking agents in the polymer increases. See Figure 2.6 and Table 2.2.



**Figure 2.6.** THE EFFECT OF THE DEGREE OF CROSS LINKING ON THE SELECTIVITY OF A SYNTHETIC SULFONIC EXCHANGE RESIN. The degree of cross linking varies with the percentage of divinylbenzene present. For the exchange  $p_{fr}^+ + q_{ads}^+ \rightleftharpoons p_{ads}^+ + q_{fr}^+$ , the selectivity coefficient  $K'$  is equal to  $\frac{[p^+]_{fr}[q^+]_{ads}}{[p^+]_{ads}[q^+]_{fr}}$ , where  $[p^+]_{fr}$ ,  $[q^+]_{ads}$  refer to the concentrations of the  $p$ th free and  $q$ th adsorbed ion, respectively. ⊖ represents potassium-tetramethylammonium exchange, and ⊕, potassium-sodium exchange. (Figure after Gregor, 1951.)

	4% DVB	8% DVB	16% DVB
$\text{Li}^+$	1.00	1.00	1.00
$\text{H}^+$	1.30	1.26	1.45
$\text{Na}^+$	1.49	1.88	2.23
$\text{NH}_4^+$	1.75	2.22	3.07
$\text{K}^+$	2.09	2.63	4.15
$\text{Ag}^+$	4.00	7.36	19.4

**Table 2.2.** THE EFFECT OF VARYING THE PERCENTAGE OF CROSS-LINKING AGENT ON THE SELECTIVITY COEFFICIENTS OF SULFONIC EXCHANGE RESINS. The selectivity ratios refer to the  $\text{Li}^+$  ion as having a selectivity of unity and are calculated from the equilibrium constants  $K$ . The constant  $K$ , in turn, is related to the selectivity coefficient  $K'$  by the relation  $\log K = \int_0^1 \log K' dX_p^+$ , where  $X_p^+$  refers to the mole fraction of the resin sites associated with the exchanging ion  $p^+$ . The selectivity coefficient  $K'$  is defined in Figure 2.6. DVB stands for divinylbenzene. (Data from Bonner, 1954.)

## (2) Ion-selectivity properties of other nonliving fixed-charge systems

Long-chain electrolytes and synthetic exchange resins demonstrate the sequence: fixation of charges to increased counterion association to increased ionic selectivity based on differences of ionic properties manifested in short-range interactions. Given the conditions of substantial charge fixation, ionic selectivity always follows.

Fixed-charge system	Selectivity coefficient	Source
Glass, glass electrode	$\frac{K}{Na} = 6.0$	Eisenman <i>et al.</i> , 1957 (see also Urban and Steiner, 1931)
Permutit, synthetic aluminum silicates (artificial zeolites)	$\frac{K}{Na} = \frac{45.07}{29.46} = 1.53$	Jenny, 1932
Clays		
Putman clay ( $NH_4$ -form)	$\frac{K}{Na} = \frac{63.7}{32.8} = 1.95$	Jenny, 1932
Bentonite clay ( $NH_4$ -H-form)	$\frac{K}{Na} = \frac{53.0}{50.6} = 1.05$	Jenny, 1932
Zeo-Karb; sulfonated coal	$\frac{K}{Na} = 2^b$	Walton, 1943
Dowex 50, sulfonated polystyrene resin with 16% DVB	$\frac{K}{Na} = \frac{4.15}{2.23} = 1.85$	Bonner, 1954

**Table 2.3.<sup>a</sup> THE  $K^+$ / $Na^+$  SELECTIVITY COEFFICIENTS OF SOME NONLIVING FIXED-CHARGE SYSTEMS.**

<sup>a</sup> See also Hechter and Lester (1960) for their work on the silicate mineral, orthoclase feldspar, and on Wyoming Bentonite clay.

<sup>b</sup> Selectivity coefficient at 50–50 per cent saturation.

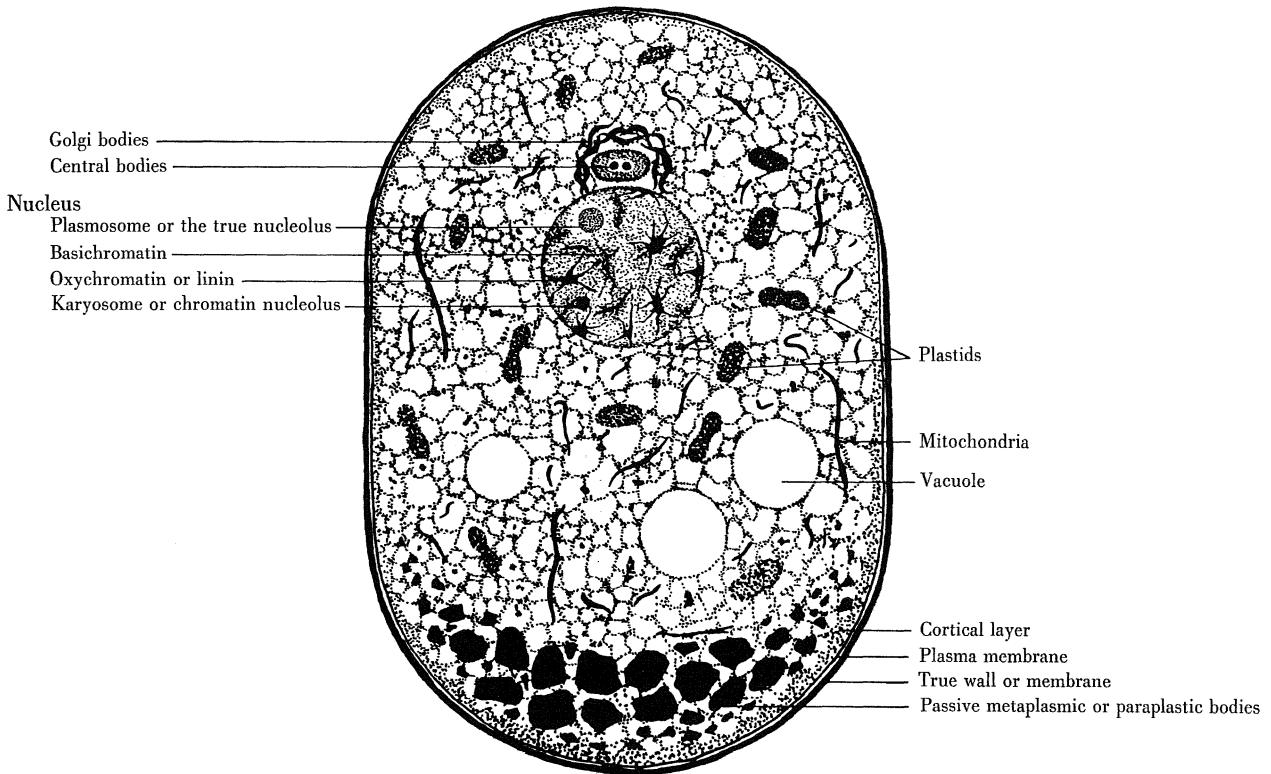
Typical examples are quoted in Table 2.3. Whether the system is crystalline as in minerals and zeolites, vitreous as in glass, or a dense aggregation of polymeric molecules as in dried oxidized collodion, each is a fixed-charge system.

These examples show that ionic selectivity (but not a particular sequential order of ion selectivity) is independent of the nature of the fixed ionic groups. The only common features shown by all these systems are the possession of charged groups and the fixation of these in space.

### C. THE LIVING CELL AS A TRUE FIXED-CHARGE SYSTEM

#### (1) Components of cells as fixed-charge systems

According to our hypothesis, living protoplasm consists of various types of fixed-charge systems, the universal constituents of which are proteins, water, and salt ions. To clarify this we distinguish three classes of cellular components (see Figure 2.7).



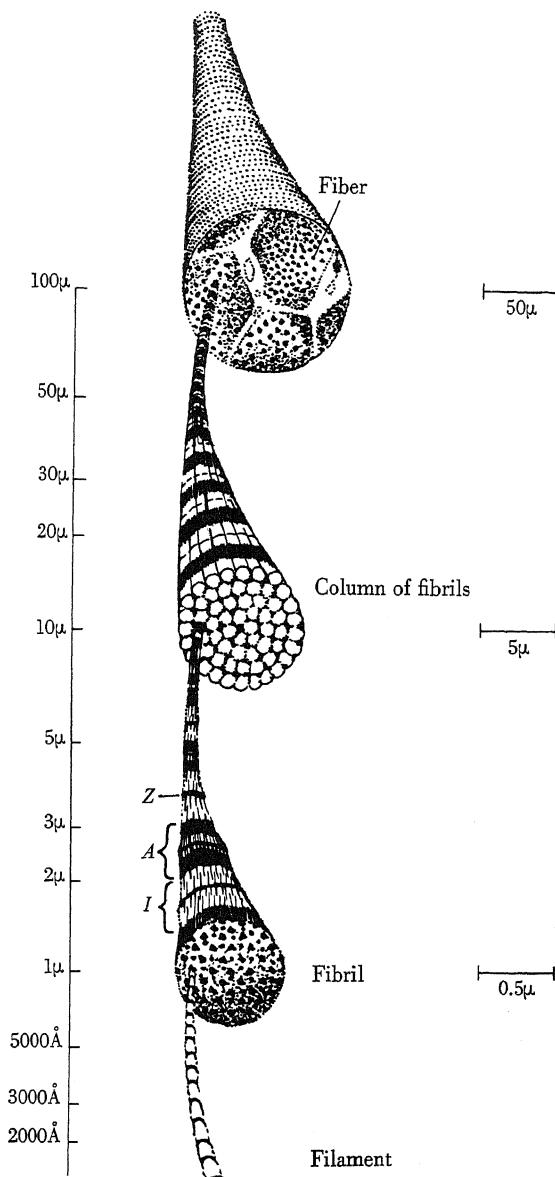
**Figure 2.7. DIAGRAM OF A TYPICAL CELL.** The cytoplasm is shown as a granular network in which various cytological particles are suspended. (Data from Wilson, 1928.)

(a) *Cytological structures.* All cytological structures truly demonstrable as membranes,\* mitochondria, nuclei, and so on, are usually seen in preparations that have undergone extensive treatment with aqueous and organic solvents such as xylol and benzene. Thus, if the constituent material of these structures were not fixed, these solvents would extract and remove it. Since such cellular elements persist for microscopic observation they must be structurally rigid. These rigid structures of protoplasm must be protein; thus the essential components, whatever their chemical composition, must be fixed onto a protein lattice. As the proteins of living cells, essential lipids, etc., are all charged, they constitute a fixed-charge system when fixed in space. Different structures and their diverse substructures must have fixed ionic groups of varied nature and density.

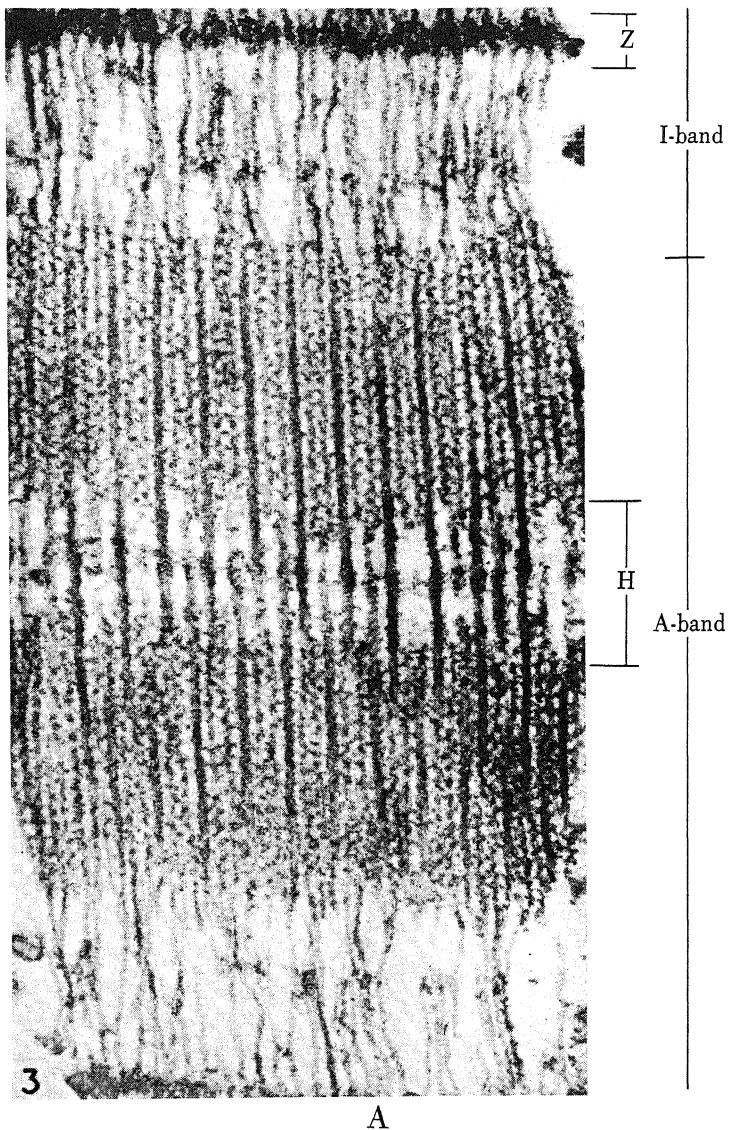
(b) *Vacuoles and similar cytological inclusions.* These subcellular compartments are obviously not fixed-charge systems. Not conspicuous in most animal cells, they are found in plant cells to be generally larger and to contain saps that have the physical consistency and chemical composition of true dilute salt solutions, and must be so regarded. The fundamental concepts developed in this thesis provide both an explanation for the ionic behavior of cytoplasm and an explanation for the ionic behavior of cells which contain a conspicuous vacuolar system.

(c) *Cytoplasm.* Figures 2.8 and 2.9 depict the fine structures of the bulk matter of the cytoplasmic content of a muscle cell; they show a highly regular long-range order in the range of  $10^8\text{\AA}$ . Another degree of order in the  $10\text{\AA}$  range is revealed by wide-angle x-ray diffraction pattern studies (Astbury, 1947) shown in Figures 2.10 and 2.11. The possession of both long-range and short-range order establishes that muscular cytoplasm is a fixed-charge system. Spatial fixation, of course, may also occur without the regularity required for the production of either regular x-ray diffraction patterns or recognizable homogeneous cytological patterns. In any case, charge fixation resides in a (sometimes labile) gel structure observable in all living cells (Seifriz, 1944). The concept that protoplasm is not a dilute water solution but a gel was advanced by Dujardin (1835). He described protoplasm as "living jelly" and von Mohl (1846) characterized it as a "glutinous substance insoluble in water." Although the cytoplasmic gel usually appears microscopically homogeneous in the living state (for squid axon axoplasm, see Hodgkin and Keynes, 1956), this normal labile condition is not generally preserved by the denaturation and dehydration processes usually employed in the preparation of microscopic specimens. The experimental demonstrations of artifacts in the form of vacuoles and filaments shown by earlier cytologists are most instructive. With these clarifications, we can discuss the living cell as a fixed-charge system.

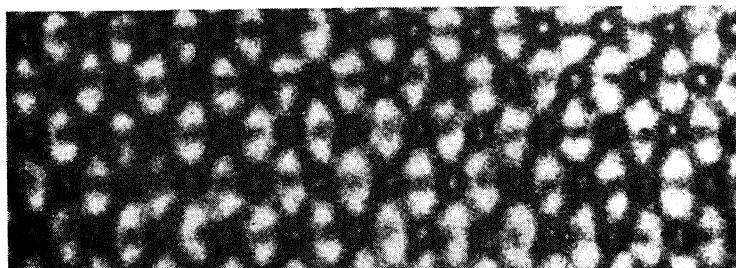
\* For a further discussion on cell membranes, see Chapter 10.



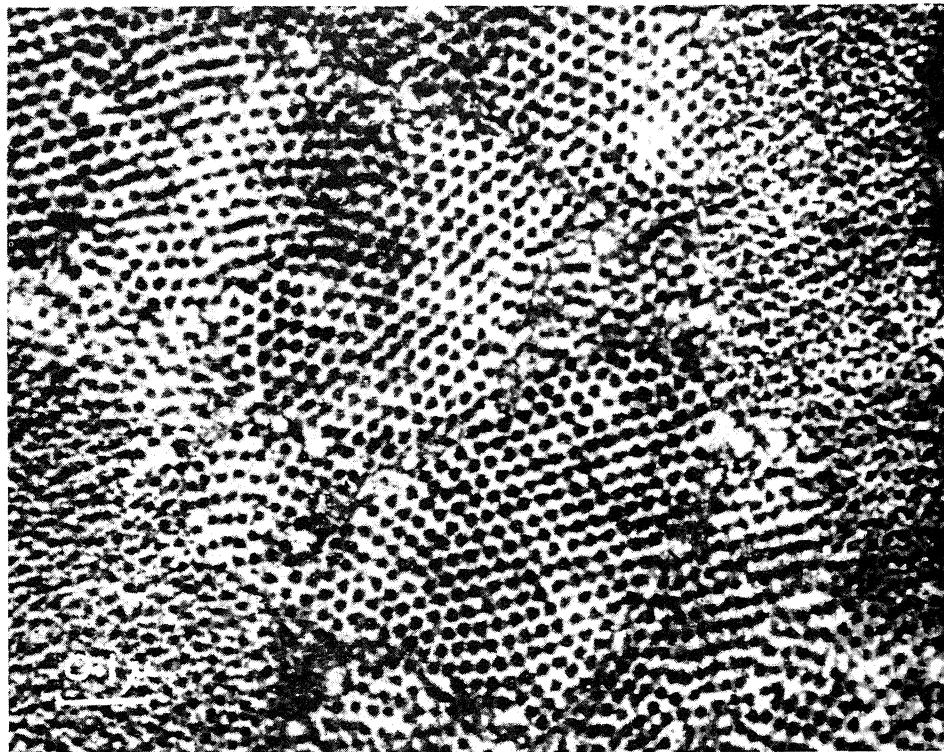
**Figure 2.8. THE ORDERING OF MUSCLE COMPONENTS.** A high degree of regularity is indicative of the substantial spatial fixation that is enjoyed by the muscle components as shown by this schematic view of the microscopic and submicroscopic structure of voluntary muscle. The positions of the *Z*, *A* and *I* zones of the myofibril are indicated. (Figure after Buchthal *et al.*, 1951.)



A

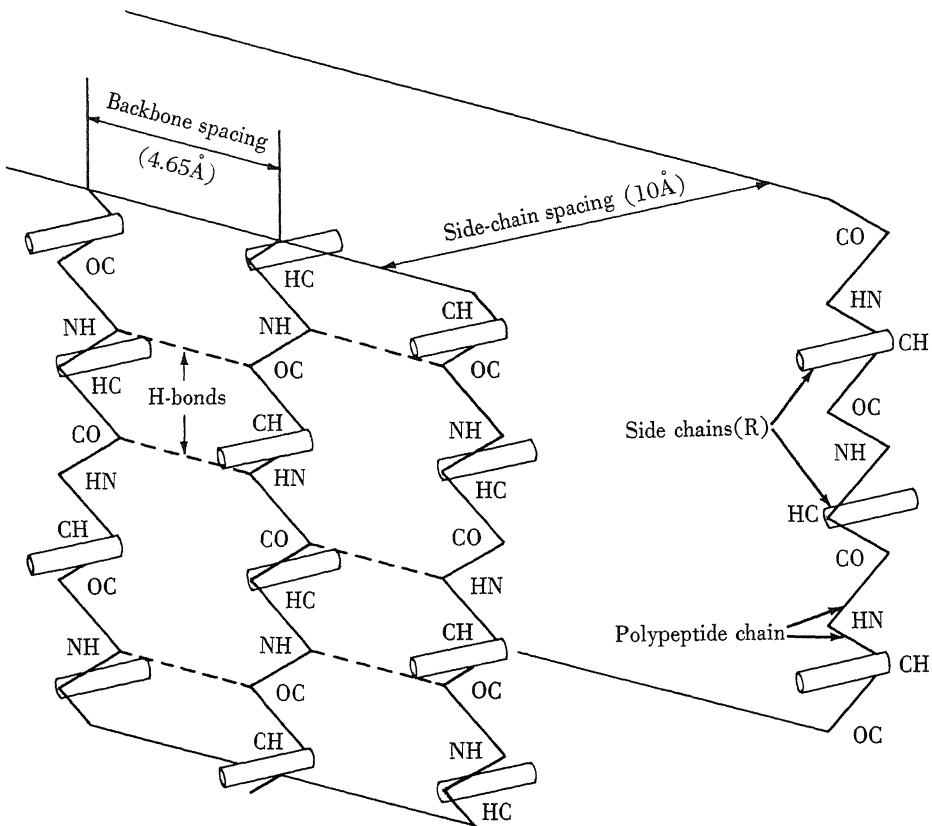


C



B

**Figure 2.9. THE ORDERING OF MUSCLE COMPONENTS.** A, an electron micrograph of a longitudinal section of myofibril of rabbit *psoas* muscle fixed and stained in osmium tetroxide. The *I*, *A*, *H*, and *Z* bands are indicated. The high degree of order seen at this level is evident. (Figure from H. E. Huxley, 1957.) B, an electron micrograph of a transverse section of frog *sartorius* muscle. Both the simple hexagonal array of the *H* band in the center of the picture and the more complex array of filaments in portions of *A* band to either side show a considerable regularity. (Figure from H. E. Huxley, 1953.) C, a transverse section through the *A* band of a blow-fly flight muscle. Note the regular hexagonal array of compound filaments. (Figure from Hodge, 1956.) The distance between these myofilaments is 200–300 Å (see Buchthal and Rosenfalck, 1957). An additional level of order of approximately 10 Å is disclosed by x-ray diffraction studies (see Astbury, 1947).

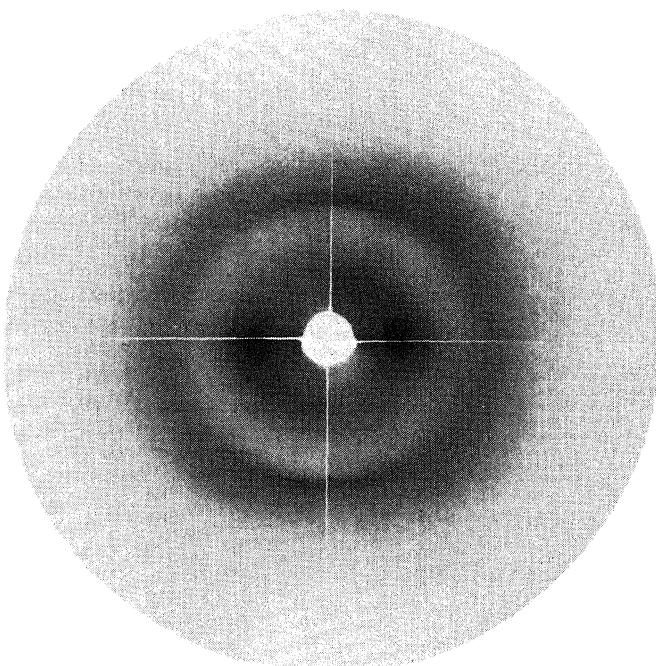


**Figure 2.10. DIAGRAMMATIC SCHEME FOR THE STRUCTURE OF  $\beta$ -KERATIN.** [Redrawn from the figures of Low (1953) and Astbury and Woods (1933).] Diagram shows both “backbone spacing” ( $4.65\text{\AA}$ ) and side-chain spacing ( $10\text{\AA}$ ). It should be pointed out that another scheme, where the protein molecules are arranged in parallel-chain pleated sheets, appears in the light of recent knowledge to be more correct. (For this, the reader should consult Pauling and Corey, 1951, 1953.) Nonetheless, to illustrate the general three-dimensional order in fibrous proteins this rougher diagram is sufficient. The close relationship between the protein structures of keratin and myosin, the major muscle proteins, is shown in Figure 2.11.

## (2) The entropy of dissociation within the living cell

Two criteria for a high degree of ionic association within a fixed-charge system stand out: the rigid fixation and the relatively even distribution in space of the fixed ionic sites, and the restriction in free rotation of the dissociated ion and its shell of water molecules. Together, these factors increase greatly the degree of ionic association within a fixed-charge system.

Practically all living cells contain from 15 to 25 per cent protein. Since proteins are basically long chains, there is enough protein in any cell to form



**Figure 2.11. THE X-RAY DIFFRACTION PATTERNS OF  $\alpha$ -KERATIN AND MYOSIN.** While stretched keratin assumes the extended  $\beta$ -keratin configuration shown in Figure 2.10, unstretched keratin assumes the  $\alpha$ -configuration, probably helical in nature. [See Edsall and Wyman (1958), Chapter 3, for an excellent review.] The present x-ray diffraction diagram shows a quadrant photograph comparing an  $\alpha$ -myosin (upper right and lower left quadrants) and an  $\alpha$ -keratin (horn, lower right and upper left quadrants) on the same film. These two proteins show not only spacing identity, but a complete and general likeness of spot shape, size, and over-all intensity distribution. Thus we are justified in assuming that the short-range order seen in  $\beta$ -keratin is probably also present in myosin. (Figure from Astbury and Dickinson, 1940.)

a uniform three-dimensional lattice with chain-to-chain spacing of 20 $\text{\AA}$ . In exchange resins, the fixation of the chains is effected through the introduction of cross-linking agents such as divinylbenzene. In commercial preparations, the percentage of cross-linking agents is usually about 8 per cent. Thus a large portion of the chain is not fixed at the microscopic level. In cellular protoplasm, however, a very large proportion of the atoms on both the polypeptide backbone and the side chains serve as sites of anchorage. The total number of amino-acid residues in cells averages 1.68 moles per liter. Each amino-acid residue provides two hydrogen bonding sites (NH, CO). At least 30 per cent of these amino acids possess side chains which have polar groups such as the carboxyl and amino groups, hydrogen bonding groups such as phenolic groups, or disulfide linkages.

Thus, there are, on the average,  $1.68 \times 2.3 = 3.86$  moles of potential anchoring sites per liter of cells; these potential anchoring sites are uniformly distributed along the whole framework of the 20 $\text{\AA}$  lattice. This profusion of anchoring sites must be the basis of the high degree of fixation observed in living cells, for a large number of these sites certainly pair off to form bridges between neighboring chains.

The NH and CO groups of the backbone are also able to form strong bonds with water molecules. The living-cell fixed-charge system thus allows the accumulation of a large number of water molecules as well as hydrated ions. The configurational and rotational entropy of the water molecules entering the system is decreased in much the same way as the ice crystal lattice decreases the entropy of the individual water molecules within it.\*

## 2.5. Summary

Complete ionic dissociation is limited to special conditions of monomeric, mono-monovalent salts at low concentrations. Charge fixation increases the average degree of ion association through its effect on both the energy and the entropy of dissociation. The ultimate state of charge fixation is visualized as a three-dimensional lattice-bearing fixed-charge system. In this true fixed-charge system, the entropy of dissociation differs from the entropy of dissociation in a free solution of similar ionic strength because of restrictions on both the configurational and the rotational entropy gain on dissociation. This provides an increase in the degree of ionic association within the fixed-charge system.

# B I B L I O G R A P H Y

## BOOKS AND MONOGRAPHS

- Falkenhagen, H., "Electrolytes" (transl. by R. P. Bell). Oxford Univ. Press (Clarendon), London and New York, 1934. Chapter 3 contains a valuable section (8) on electrolytic dissociation; Chapter 11 contains a valuable section on the experimental determination of the degree of ionic dissociation.
- Fowler, R. H., and Guggenheim, E. A., "Statistical Thermodynamics." Cambridge Univ. Press, London and New York, 1939. Chapter 9 of this book, which deals with solutions of electrolytes, contains reviews of the theories of Debye and Bjerrum of electrolyte solution

\* Dr. George Karreman recently informed me of the experimental demonstration of restricted rotation of water molecules in tissues by Berendsen (1960) from his studies of nuclear magnetic resonance of partially dried collagen.

and an important semiquantitative discussion on the statistical mechanics of ionic dissociation in aqueous media.

Glasstone, S., "Textbook of Physical Chemistry," 2nd ed. Van Nostrand, Princeton, New Jersey, 1946. Chapter 12 includes an excellent account of electrolyte conduction phenomena as well as the Debye-Hückel theory of dilute ionic solutions.

Robinson, R. A., and Stokes, R. H., "Electrolyte Solutions," 2nd ed. Academic Press, New York, 1959. Chapter 14 presents a rather extensive discussion of ionic association in solutions of "monomeric" electrolytes.

Wilson, E. B., "The Cell in Development and Heredity," 3rd ed. Macmillan, New York, 1928. The comprehensive review of the morphological structure of a typical cell given in the first chapter of this treatise may be of help to the nonbiologist reader; further, the many pitfalls of deducing subtle structural details from cytological specimens which have been subject to the destructive action of fixations are pointed out.



# 3

## THE CALCULATION OF THE DENSITY OF FIXED IONIC SITES IN CELLULAR AND NONCELLULAR BIOLOGICAL MATERIAL

An adequate concentration of fixed sites is the first requirement for ionic association in living cells (Ling, 1952). This chapter will demonstrate that a high density of fixed ionic sites is characteristic of muscle cells (as shown in the 1951-1952 version of the hypothesis) and a wide sampling of living material.

In Table 3.1, the percentage of amino-acid residues that bear residual positively and negatively charged groups is given for representative specimens chosen from diverse phyla. These data show that living organisms possess a high percentage of trifunctional amino-acid residues in their protein composition. Table 3.2 lists the average densities of ionic sites (positive and negative) of proteins from a large number of mammalian organs. Again, the high density of ionic sites is universally apparent. Table 3.3 presents the densities of acidic and basic amino groups of a few familiar proteins. We must make a critical evaluation of these data before using them in our argument.

(1) We do not include the histidine residues among the cationic groups in Tables 3.1, 3.2, and 3.3, as is usually done (Haurowitz, 1950) because the  $pK$  value of the imidazole group of histidine has not been determined exactly.\*

\* Cohn and Edsall (1943), Chapter 4, Table 5D.

(2) The amide nitrogen was subtracted from the sum of the glutamic- and aspartic-acid residues to obtain the total free anionic groups listed in Table 3.3. However, the amide nitrogen value probably includes not only true amide nitrogen, but also nitrogen from amino acids that comprise easily hydrolyzable peptide linkages which are decomposed during acid hydrolysis.\* This leads to spuriously high values of amide nitrogen and spuriously low values of "free"

Class	Species	Total basic group	Total acidic group	Source
Virus	Tobacco mosaic virus	8.3	21.7	Knight, 1947
Bacteriophages	T <sub>3</sub>	13.5	22.4	Fraser and Jerrell, 1953
Bacteria	<i>Escherichia coli</i>	16.5	19.2	Polson and Wyckoff, 1948
	<i>Staphylococcus aureus</i>	4.2	19.6	Mondolfo and Hounie, 1951
Fungus	Brewers' yeast	8.4	21.3	Lindan and Work, 1951
Algae	<i>Chlorella vulgaris</i>	12.1	19.3	Fowden, 1952
Spermatophytes	Average of many	7.9	15.4	Lugg, 1949
Protozoa	<i>Entamoeba histolytica</i>	12.6	12.7	Becker and Geiman, 1954
Crustacea	Shrimp	11.3	16.7	Dunn <i>et al.</i> , 1949
Fish	Tuna	9.2	14.2	Dunn <i>et al.</i> , 1949
Bird	Chicken	7.7	12.6	Dunn <i>et al.</i> , 1949
Mammal	Rat	7.7	13.8	Dunn <i>et al.</i> , 1949

**Table 3.1. POLAR AMINO-ACID CONTENTS OF VARIOUS PROTEINS.** The basic and acidic trifunctional amino-acid residues given as a percentage of the total amino-acid residues of the protein of representative living organisms.

anionic groups; the examples of myosin illustrate this. If all the easily hydrolyzable nitrogen in this protein (Table 3.3) were derived from the amide groups of asparagine and glutamine residues, the free anionic groups would comprise 10.2 per cent of the total residues. At pH 5.4, there would then be a total negative charge of, at most, 10.2 per cent.

A total positive charge of at least 16.0 per cent is present in the form of ionized lysine, arginine, and histidine residues. The concentration of cationic groups borne on these trifunctional amino-acid residues is the same as the total molar concentration of these residues and is not complicated by such factors as the amide groups of the anionic residues. Thus, at pH 5.4, one would expect a large surplus of positive charges. Actually pH 5.4 is the isoelectric point of

\* See Kominz *et al.* (1954), Tristram (1949), also Moore and Stein (1951), for discussion on loss of trifunctional amino acids and, in particular, lysine, arginine, and histidine in the basic column.

Tissue	Protein, %	Species	Total basic group	Total acidic group	Source
Brain	—	Swine	13.8	14.1	Camien <i>et al.</i> , 1949
Lens	—	Human	11.4	18.2	Schaeffer and Shankman, 1950
Heart	16 <sup>a</sup>	Human	10.9	12.5	Müting and Wortman, 1954
Muscle	—	Average	13.3	19.4	Block and Bolling, 1951
Muscle	21 <sup>a</sup>	Rat	9.0	11.5	Müting and Wortman, 1954
Muscle	19–25 <sup>a</sup>	Rabbit	6.3	12.5	Müting and Wortman, 1954
Muscle	18.5 <sup>a</sup>	Human	6.3–10.7	10.2–14.2	Müting and Wortman, 1954
Kidney	18 <sup>a</sup>	Human	9.1	12.4	Müting and Wortman, 1954
Lung	14.9–16.7 <sup>a</sup>	Human	8.0	12.1	Müting and Wortman, 1954
Pancreas	—	Human	11.4	13.3	Müting and Wortman, 1954
Spleen	17.5 <sup>a</sup>	Human	7.3	12.1	Müting and Wortman, 1954
Thyroid	—	Human	6.4	10.9	Müting and Wortman, 1954
Liver	16–22 <sup>a</sup>	Rat	8.9	11.3	Block and Bolling, 1951
Adrenal	—	Human	6.8	11.7	Müting and Wortman, 1954
Bone <sup>b</sup>	—	Ox	5.9	24.7	Eastoe and Eastoe, 1954
Pituitary	—	Human	6.9	12.8	Müting and Wortman, 1954
Ovary	—	Swine	8.2	12.9	Camien <i>et al.</i> , 1949
Erythrocyte <sup>c</sup>	—	Horse	8.3	12.8	Granick, 1949
Egg	—	Sea urchin (unfertilized)	11.4	15.2	Kavanau, 1954
Egg	—	Chicken	8.1	15.4	Rutgers Univ., 1950

Table 3.2. THE BASIC AND ACIDIC TRIFUNCTIONAL AMINO-ACID RESIDUES AS A PERCENTAGE OF THE TOTAL AMINO-ACID RESIDUES OF THE PROTEINS OF VARIOUS MAMMALIAN ORGANS AND TISSUES.

<sup>a</sup> Spector, 1956 (Table 55).

<sup>b</sup> Mucopolysaccharide protein complex.

<sup>c</sup> Data from analysis of hemoglobin which makes up about 95 per cent of total erythrocyte dry weight.

myosin (Szent-Györgyi, 1951). The true number of free carboxyl groups must, therefore, lie between the sum of aspartic- and glutamic-acid residues—an upper limit—and this sum minus the easily hydrolyzable nitrogen—the lower limit—as in Table 3.3. The studies of lysozyme provide another illustration of a

Protein	Total muscle protein, %	Arg + Lys + His, %	Arg + Lys, %	Glu + Asp, %	Glu + Asp - NH <sub>2</sub> , %	Source
<b>Muscle Proteins</b>						
Tropomyosin	2.6 <sup>a</sup>	18.5	17.9	35.3	27.7	Kominz <i>et al.</i> , 1954
Actin	15 <sup>b</sup>	12.6	10.4	21.2	13.6	Kominz <i>et al.</i> , 1954
Myosin	39-57 <sup>c</sup>	16.7	14.9	28.4	10.2	Kominz <i>et al.</i> , 1954
<b>Nerve Proteins</b>						
Fraction A (lobster)	—	—	9.7	26.1	13.8	Koechlin and Parish, 1953
Hair	—	—	8.3	14.9	—	Block and Bolling, 1951
Horn	—	—	9.4	14.6	—	Block and Bolling, 1951
Wool	—	—	8.5	14.1	—	Block and Bolling, 1951
Bovine serum albumin	17.4	15.5	21.8	14.6	Stein and Moore, 1949	
Ovalbumin	10.9	9.0	21.7	14.2	Tristram, 1949	
$\beta$ -Lactoglobulin	14.3	12.9	25.9	16.3	Stein and Moore, 1949	
Horse hemoglobin	16.2	9.5	16.3	8.4	Tristram, 1949	

**Table 3.3. THE BASIC AND ACIDIC TRIFUNCTIONAL AMINO-ACID RESIDUES OF SOME ISOLATED PROTEINS GIVEN AS A PERCENTAGE OF THE TOTAL AMINO-ACID RESIDUES.** Abbreviations are those given in Table 0.1. NH<sub>2</sub> is amide nitrogen.

<sup>a</sup> Bailey, 1955.

<sup>b</sup> Hasselbach and Schneider, 1951.

<sup>c</sup> Weber and Meyer, 1933; E. C. Bate Smith, 1937.

spuriously high amide N-value. Fromageot and deGarinhe (1950) found 1.3 free  $\beta$ - and  $\gamma$ -carboxyl groups per lysozyme molecule when they computed this value as the sum of aspartic and glutamic residues minus measured amide nitrogen; J. C. Lewis *et al.*, (1950) found a value of 6.5 by the same method. Careful titration by Tanford and Wagner (1954), however, revealed as many as 10.5 freely titratable carboxyl groups. This number presumably included one free terminal carboxyl group.

(3) In order to present a comprehensive survey of the density of fixed ionic.

sites in the cells of different animals and organs of animals, many of the data tabulated in Tables 3.1 through 3.3 were taken from Müting and Wortman (1954) and from Dunn and co-workers (1949). There is reason to suspect that values for both cationic and anionic residues obtained by these investigators are too low.

All values of the amino-acid composition of the voluntary muscle of the rat, rabbit, and human that were reported by Müting and Wortman (1954) fall below the average for mammals tabulated by Block and Bolling (1951). In Table 3.3, we have quoted the analyses of tropomyosin, actin, and myosin (Kominz *et al.*, 1954); these agree with the analyses by Bailey (1948). The fraction of each of these proteins in the total protein of rabbit muscle is known; myosin makes up at least 39 per cent; actin, 15 per cent; and tropomyosin, 2.6 per cent (Table 3.3); 43 per cent is not accounted for. If we know the total concentration of trifunctional amino acids in muscle, subtraction of the values for tropomyosin, actin, and myosin should yield the trifunctional amino-acid content of the rest of the muscle proteins. If we use Müting and Wortman's values for the concentration of the dicarboxylic trifunctional amino-acid residues in total rabbit muscle proteins, we find that the unaccounted for 43 per cent of muscle proteins must have a concentration of glutamic and aspartic residues equal to minus 2.7 per cent and a concentration of arginine and lysine residues equal to minus 1.5 per cent. In fact, a large part of this 43 per cent must be enzymes such as aldolase (myogen a), lactic dehydrogenase, and phosphorylase; these contain a high percentage of dicarboxylic, lysine, and arginine residues (Tristram, 1953). We conclude that the experimental methods used by Müting and Wortman have underestimated the lysine, arginine, glutamic-acid, and aspartic-acid residues in voluntary muscle. Since they used the same techniques for their determinations of these amino-acid residues in other tissues, we anticipate a similar underestimation of these values.

The data of Dunn and co-workers (1949) reveal a like discrepancy. About 50 per cent of the weight of mammals and birds is due to muscle. The high percentages of basic and acidic groups in muscle protein as well as in other important organs have already been shown in Table 3.2; this sets a lower limit on the total trifunctional amino-acid content of whole carcasses of chickens. This lower limit calculated from muscle is higher than Dunn's values for whole chicken and rat carcasses; that is, there appear to be fewer trifunctional amino-acid residues in the whole carcasses than in the muscle tissue alone. One must conclude that these figures suffered underestimations similar to those of Müting and Wortman.

If we consider these facts, we can safely conclude that tissue proteins contain among their total amino-acid residues not less and probably more than 8 per cent free cationic groups (lysine + arginine + ionized histidine) and not less than 10 per cent free anionic groups (glutamic + aspartic — true amide nitrogen). If this 18 per cent of the amino-acid residues is distributed evenly along the

polypeptide chains and each peptide linkage measures  $3.5\text{\AA}$  in length,\* there will be, on the average, one ionizable group every 5.55 peptide linkages, or every  $5.55 \times 3.5 = 19.4\text{\AA}$ , let us say  $20\text{\AA}$ , along the fully extended chain.

The next problem is to determine the average distance between each of the fully extended polypeptide chains. Table 3.2 presents data on the percentage of protein in various organs (Spector, 1956). If we assumed that 16 per cent is the average concentration of nonfat protein in fresh tissue, we would be conservative. Fresh tissue weight, however, includes at least 10 per cent extracellular space; this contains a relatively insignificant amount of protein (7 per cent).† The percentage of protein in cells should thus be further multiplied by a factor of no less than  $100/90$ ; this yields  $16 \times 100/90 = 17.8$  per cent. From Block and Bolling's (1951) data we can calculate the average molecular weight of amino-acid residues to be 128.9 for voluntary muscle and 126.6 for liver; from Koechlin and Parish's (1953) data, 132.3 for fraction A of lobster nerve proteins. If we assume that these are representative proteins and subtract the weight of the one water molecule lost by each amino acid in the formation of the peptide chain, we obtain 112 for the average molecular weight of the amino-acid residues in cell proteins. Since cells contain about 17.8 per cent protein, we find an average concentration of  $1.58M$  amino-acid residues per kilogram of cells. Tsai and Lin (1939) found that the specific gravity of nervous tissue is 1.04 to 1.05. Morales *et al.* (1945) estimated the specific gravity of guinea-pig muscle to be 1.066 and that of skin, 1.06. The specific gravity of human red blood cells ranges from 1.05 to 1.06 (Best and Taylor, 1955). Assuming an average value of 1.05, we arrive at 1.66 moles per liter of fresh cells as the total amino-acid concentration in the average living cell. The total length of amino-acid peptide linkage would thus be  $1.66 \times 6.06 \times 10^{23} \times 3.5 \times 10^{-8} = 3.52 \times 10^{15}$  cm. This would yield  $3.52 \times 10^{15}$  filaments measuring 10 cm in length. Uniformly arranged in a square area  $10 \text{ cm} \times 10 \text{ cm}$ , there would be  $(35.2 \times 10^{14})^{1/2} = 5.93 \times 10^7$  such filaments on each side, separated from each other by  $10/(5.93 \times 10^7) = 16.9 \times 10^{-8}$  cm. To be liberal we assume that the filaments are  $20\text{\AA}$  apart.

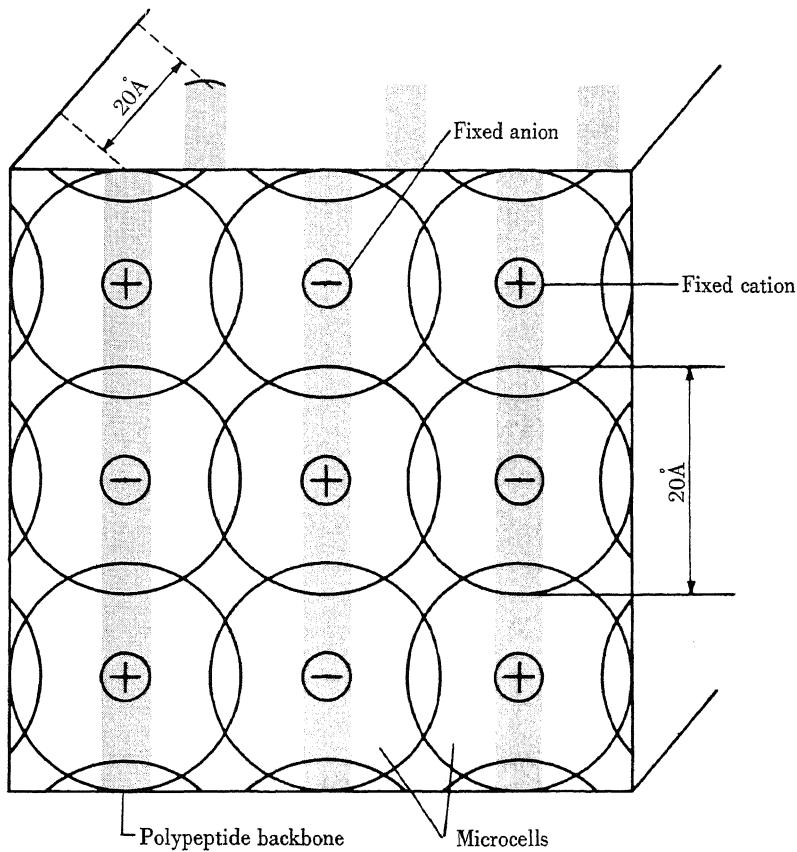
Since the chain-to-chain distance and the charge-to-charge distance on the same chain are each at most  $20\text{\AA}$ , we can draw a diagram representing a microscopic portion of the inside of a typical cell (Figure 3.1). Such a cell would consist of numerous identical or nearly identical microscopic units, each of these units or microcells containing one fixed ionic group, either positive or negative, at its center. The average distance from the center of one fixed ion to the center of an immediately neighboring fixed ion would be  $20\text{\AA}$ .

We have demonstrated marked resemblances between a fixed-charge system

\* Corey (1948), Astbury (1933), Kratky and Kuriyama (1931), Trogus and Hess (1933).

† Höber (1945), Boyle *et al.* (1941), Hajdu (1953), Horvath (1954).

and a typical ionic crystal. However, the fixed-charge system differs from the ionic crystal in two important respects: (1) In crystals, both species of ion are "fixed" in position. In the fixed-charge system, only one species of ion is so markedly restricted in motion; the counterion retains a considerable amount of freedom. (2) Although the fixed ion and its counterion are close to each other



**Figure 3.1.** A MODEL OF THE LIVING CELL FIXED-CHARGE SYSTEM. The average microcell radius is 10 Å.

in fixed-charge systems such pairs are in general farther apart than in crystals, where spacing is uniform. Thus, in a first approximation, we may neglect the interaction of neighboring sites. We can thus estimate the average free counterion distribution in a living cell by investigating the distribution of a counterion in a single microcell.

## B I B L I O G R A P H Y

### BOOKS AND MONOGRAPHS

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# PART II

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## INDUCTION



# 4

## THE MODEL OF THE BIOLOGICAL FIXED-CHARGE SYSTEM

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#### 4.1. Introduction—The 1951–52 Model

In 1951 and 1952, the author presented a version of the present hypothesis. According to this early version, selectivity of one alkali-metal ion over another is achieved by a difference in association energy and by an enhanced degree of ionic association through fixation of one species of ion; the fixed ions were called “fixed charges.”\*

In the early model, the author followed Bjerrum’s theory of ion-pair formation (1926). The treatment differed from that of Bjerrum in that it included the effect of dielectric saturation, a phenomenon of particular importance to the present case. If  $dn$  is the average number of counterions within a spherical shell of thickness  $dr$  and at a distance  $r$  from the center of a fixed ionic group, then

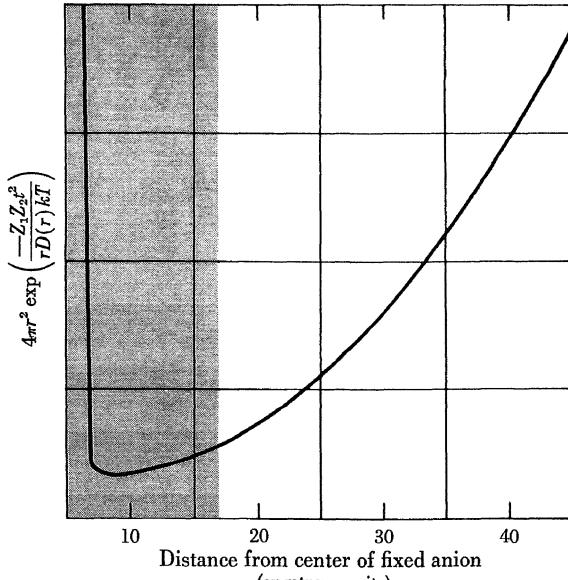
$$dn = \frac{N_{\pm}}{V} 4\pi r^2 \exp\left(\frac{-Z_1 Z_2 \epsilon^2}{D(r) k T r}\right) dr. \quad (4-1)$$

Here  $N_{\pm}$  is the number of counterions in the volume  $V$ ;  $Z_1$  and  $Z_2$  are the valences of the charges;  $\epsilon$  is the electronic charge;  $k$  is the Boltzmann constant;  $T$  is the absolute temperature; and  $D(r)$  is the effective dielectric value, which varies with  $r$  as a result of the freezing in (dielectric saturation) of the water molecules in the intense field immediately surrounding an ion (Sack, 1926, 1927; Debye, 1929; Webb, 1926; Hasted *et al.*, 1948; Grahame, 1950). We considered  $pdr$ ,

$$pdr = \frac{dn}{N_{\pm}/V} \quad (4-2)$$

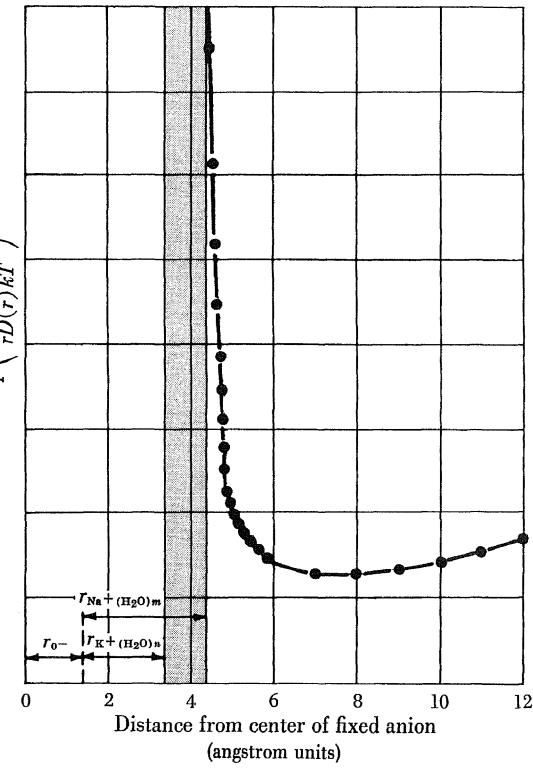
as the probability of finding a counterion within the shell. Using the values of 2.0 Å for the radius of the hydrated  $K^+$  ion and 2.8 Å for the hydrated  $Na^+$  ion and a microcell radius of 20 Å, we estimated a selectivity ratio  $K^+/Na^+$  of 7. The details of this calculation are given in Appendix C; Figure 4.1 presents the results. We have reproduced Bjerrum’s curve showing the probability of finding a cation in a shell  $r$  Å (abscissa) away from the anion as part B of this figure. The shaded area corresponds to the volume within the microcell of 12 Å radius illustrated in part A.

\* For the historical background of the term “fixed charge,” see Chapter 9.



A

**Figure 4.1. THE PROBABILITY OF FINDING A COUNTERCATION AT VARIOUS DISTANCES FROM THE CENTER OF A MICROCELL.** The shaded area in A represents the volume available to the smaller hydrated  $K^+$  ion (radius =  $r_{K^+(H_2O)_n}$ ) but not to the larger hydrated  $Na^+$  ion



B

(radius =  $r_{Na^+(H_2O)_m}$ );  $r_0$  stands for the radius of the singly charged oxygen. In B, taken from Bjerrum (1926), the shaded area corresponds to the volume of a microcell of the size shown in A.

This earlier model served many useful purposes, but the theory, as such, had serious limitations. First, despite its general acceptance (Fowler and Guggenheim, 1939; Harned and Owen, 1958; Kortüm and Bockris, 1951), the concept of hydrated ionic radii lacks clear physical significance. The hydrated ionic radius is usually only a few tenths of an angstrom unit larger than the crystal radius although a single water molecule is 2.7 Å in diameter (see Appendix C; interpretation according to the present model is given in Section 4.4D). The use of the hydrated ion concept thus prevented quantitative improvement of the model. Second, the rigidity imposed upon the theory by the acceptance of a set of hydrated diameters of constant magnitude, and hence a fixed order of preference for different ions, is at variance with an increasing amount of evidence demonstrating the variability of ionic preference.

It was not until some years after 1952 that I became aware of any system, living or nonliving, that shows an unequivocal selective ionic accumulation of alkali-metal ions in any order other than  $K^+ > Na^+$ . Thus my early model seemed adequate. In the years that followed, however, it gradually became clear that, in a number of other systems in which ionic selectivity is a matter of greater simplicity, different and opposite orders of preference exist. Investigators in these fields had, in fact, already offered qualitative explanations to account for the diverse orders of preference observed for the monovalent cations.

Following the work of Wiegner and Jenny (1927), Jenny (1932) concluded from studies of clays and permutites that "the observed irregularities in the lyotropic series of natural aluminum silicates may be interpreted as various stages in the reversal of the normal hydration order of the exchanging cations. From the viewpoint of hydration of ions, it is logical to connect the reversal of the lyotropic series with a dehydration of ions." Bungenberg de Jong (1949) ascribed the difference in the order of selectivity for different ions among systems possessing unlike functional groups to the different polarizabilities of these groups (see also Bregman and Murata, 1952; Bregman, 1953). After Eisenman *et al.* (1957),\* as quoted by Isard (1959), postulated that the variation in the lyotropic series was due to a change in the electrostatic field strength of the fixed anionic sites, a convincing system could be erected to explain the selectivity orders observed in nature. These investigators hypothesized that with the increase of the field strength the interacting cations would lose their hydration shells in an orderly fashion, the least hydrated cations losing their hydration shells first.†

Eisenman and co-workers postulated eleven orders out of the 120 possible

\* The theories of Jenny and Bungenberg de Jong, as well as that of Eisenman, and co-workers, up to 1957 all dealt with the effects of hydration and dehydration in a qualitative manner. In deriving a quantitative theory one must take into account the effects of dielectric saturation as well as the hydrated diameters; only in this way can a significant selectivity between  $K^+$  ion and  $Na^+$  ion be theoretically derived.

† This order of dehydration is the reverse of that envisioned by Jenny (1932); he stated that the most hydrated ion will be the first affected by the dehydration process.

permutations for the five alkali-metal ions. They then showed that these eleven orders could account for a large number of the "irregular" lyotropic orders encountered in various ionic studies.\* This hypothesis led to refinements in the calculation of the association energies of the various ions mentioned in the introduction (for an abstract, see Ling, 1957).

## 4.2. The Present Model

### A. THE DEFINITION OF $c$ - AND $c'$ -VALUES AND THEIR ANALOGUES

Acids bearing the same carboxylic groups may have widely varying acid-dissociation constants: the  $pK$  value of acetic acid is 4.76, but the  $pK$  value of trichloroacetic acid is less than 1.0; the  $pK$  value of the carboxyl groups of uncharged glycine  $\text{NH}_2\text{CH}_2\text{COO}^-$  is 4.30 (Edsall and Blanchard, 1933), that for the charged glycine  $\text{NH}_3^+\text{CH}_2\text{COO}^-$  is 2.31 (Zief and Edsall, 1937). Thus, although the three substituent chlorine atoms of trichloroacetic acid and the amino group of glycine are spatially separated from the dissociating carboxyl group, they markedly weaken the attractive force between the carboxyl group and its proton. This phenomenon is called the inductive effect.

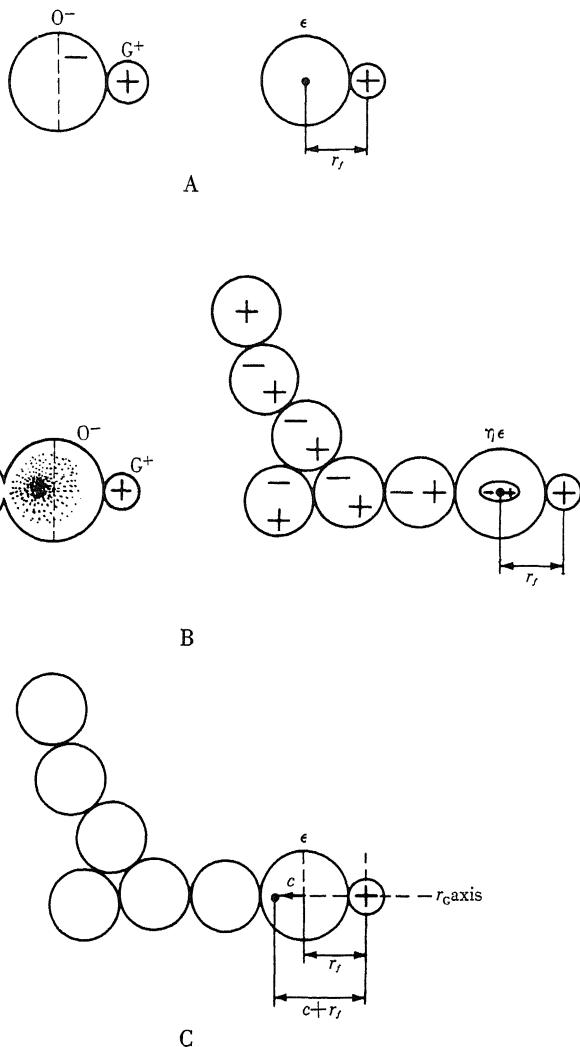
G. N. Lewis in 1916 and 1923 proposed that the inductive effect is a result of an electrical dissymmetry caused by the unequal sharing of electrons between unlike atoms. Pairs of shared electrons are thus displaced without being dissociated from their original atomic octets; this produces a similar displacement in the next link and the effect is propagated along a multiatomic molecule. The electronegativity of an atom or group in a molecule is the measurement of the tendency of this atom or group to draw electrons toward itself (Pauling, 1948). Substitution of hydrogen atoms by the more electronegative chlorine atoms, as in trichloroacetic acid, leads to withdrawal of electrons from an attached residue and hence a reduction of electron density in it. This is a negative inductive effect,  $-I$ -effect. The  $\text{NH}_3^+$  group which is electronegative also exerts a  $-I$ -effect. Substitution of hydrogen by less electronegative groups releases electrons and increases the electron density of a connected atom creating a  $+I$ -effect (Ingold, 1953).

The inductive effect is an electrostatic effect mediated through intervening atoms and intimately dependent upon their number, polarizability, and other characteristics. Another electrostatic effect emanates from substituent groups and is transmitted through space along the axis of shortest separation of the interacting atoms. This is called a direct effect,  $D$ -effect. The difference between the acid dissociation constants of acetic acid and of glycine may be accounted for by the combined  $D$ -effect and  $I$ -effect, together termed the

\* Eisenman (1962) has summarized the views of these authors.

*F*-effect. This nomenclature, used mostly by European authors (see Hermans, 1954), has been adopted in this monograph for its simplicity.

Let us begin with a singly charged and isolated oxygen ion  $O^-$  and assume that it is associated with a monovalent cation  $G^+$  (Figure 4.2A). Let us also



**Figure 4.2.** THE DEFINITION OF THE  $c$ -VALUE.

assume that the equilibrium distance between the “center of gravity” of the extra electron of this singly charged oxygen and the geometrical center of the cation is  $r_f$ . We now build up a complex molecule such as that shown in Figure 4.2B, bearing one or more chains of varying dipolar groups as well as single

charges. These groups interact with the cation  $G^+$ ; their interaction energy may be analyzed and resolved into three terms.

(1) Direct electrostatic effects produced by charge-bearing monopolar or dipolar groups and transmitted through the shortest spatial distance. Resolving the dipoles into single charges one may represent this class of effects by

$$\sum_{i=1}^n \frac{Z_G Z_i \epsilon^2}{D_i r_i}.$$

Here  $Z_G$  and  $Z_i$  are the valences;  $\epsilon$  is the electronic charge;  $r_i$  is the shortest distance in space between the charge-bearing group and the center of the cation  $G^+$ ; and  $D_i$  is the effective dielectric constant for that particular interaction.

(2) The  $I$ -effect, which produces induced dipoles along the whole molecule. The induced dipoles may again be resolved into single charges and absorbed in the first term.

(3) The  $I$ -effect and the  $M$ -effect (mesomeric or resonance effect), which create a change of electron density and a displacement of the center of gravity of the electron cloud of the oxygen atom on the functional group. This displacement of the center of gravity of the functional group is analogous to other induced dipoles mentioned under (2); it too will be absorbed in the first term. The varying electron density may be represented by a varying charge  $\eta\epsilon$ , where  $\eta$  is a positive number and  $\epsilon$  is the electronic charge located at a distance  $r_f$  from the center of the cation  $G^+$ .  $D_f$  is the effective dielectric constant within this distance. The net effect corresponds to the sum of (1) the  $D$ -effect, and (2) and (3), the  $I$ -effects:

$$\frac{Z_G \eta \epsilon^2}{D_f r_f} + \sum_{i=1}^n \frac{Z_G Z_i \epsilon^2}{D_i r_i}.$$

Although the detailed meaning of this expression may be quite complex, its net effect is an increase, a decrease, or no change in the energy of interaction between the anion and the cation  $G^+$ . Exactly the same increase or decrease may be simulated by assuming a constant unit excess charge (valence electron) on the anion and by moving this excess charge along the  $r_G$  axis toward or away from  $G^+$ . This change in the separation of the unit excess electron from  $G^+$  may be represented in angstrom units; it will be called  $c$ . The distance  $c$  is positive if the excess electron is displaced toward  $G^+$ , negative if it is displaced away from  $G^+$ . Thus, the total separation, as shown in Figure 4.2, will be  $r_f - c$ . We may then set  $Z_G \epsilon^2 / (r_f - c)$  equal to the combined actions of the three terms above. Cancelling  $Z_G \epsilon^2$  and rearranging, we have the definition of the  $c$ -value:

$$c = r_f - \frac{1}{\frac{\eta}{D_f r_f} + \sum_{i=1}^n \frac{Z_i}{D_i r_i}}. \quad (4-3)$$

In this treatment, the *c*-value is defined to be independent of the nature of the cation  $G^+$  and its exact location. That part of the interaction which does depend on the nature and location of  $G^+$  is absorbed in another variable, the group polarizability  $\alpha$  (see Section 4.3A).

A fall of the *c*-value (a change from 0 to  $-1\text{\AA}$ ) parallels a decrease in the electron density of the functional group; for example, acetic acid (a weak acid)  $\rightarrow$  trichloroacetic acid (a strong acid). A rise of *c*-value parallels an increase of electron density; for example, trichloroacetic acid  $\rightarrow$  acetic acid. A *c'*-value may be formulated for a change in the density of the excess positive charge on an amino group; we use a hypothetical  $\text{NH}_3^+$  group. Here, a rise of *c'*-value parallels a decrease of electron density and a fall of *c'*-value parallels an increase of electron density.

We can broaden the concepts of the *c*- and *c'*-values so that their application will include proton-accepting and proton-donating groups such as alcoholic, amide, ester, and ether groups as well as charged or dipolar groups other than  $\text{O}^-$  and  $\text{NH}_3^+$ . In these cases, the particular group is matched by a hypothetical singly charged  $\text{O}^-$  or  $\text{NH}_3^+$  group with the equivalent *c*- and *c'*-values. These values are then called the *c*- and *c'*-analogues of these different groups.

## B. THE LINEAR MODEL AND THE FOUR CONFIGURATIONS

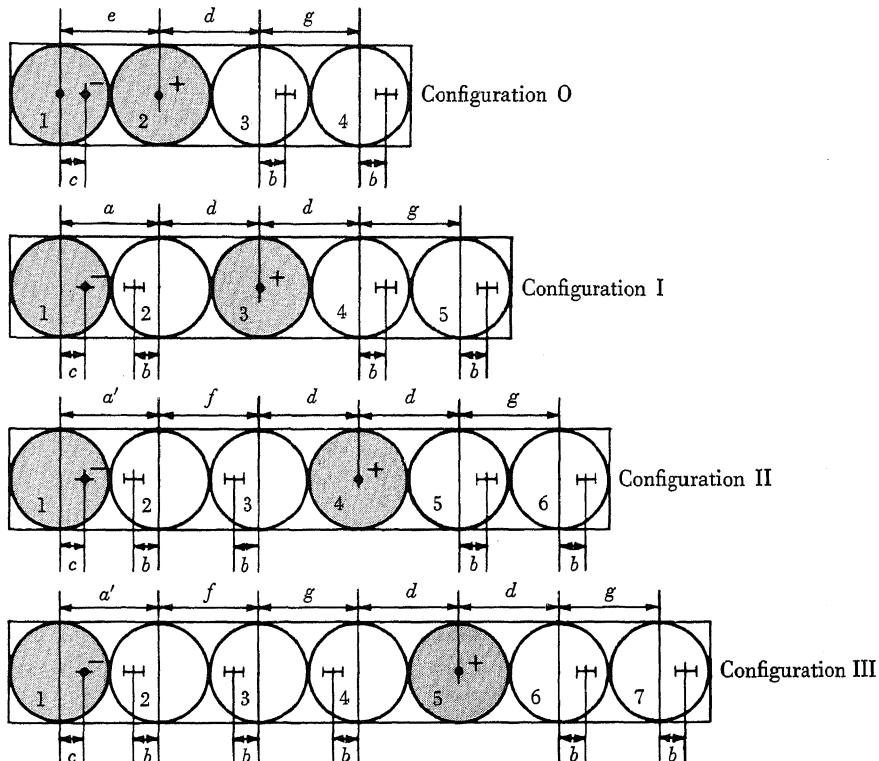
Having defined the *c*-value, the *c'*-value, and their analogues, let us insert a hypothetical cylindrical cavity in a microcell of a fixed-charge system. One end of the cavity encloses a negatively charged oxyacid group, represented as a negatively charged oxygen atom (Figure 4.3). Adjacent to the oxygen atom and near the middle of the cavity, we place a cation; farther away, we place two water molecules in a linear array. This configuration is called configuration 0. We then insert, between the fixed anion and its countercation, one, two, and three water molecules successively and call these configurations, respectively, configurations I, II, and III.\* We assume that all the water molecules within this cavity are completely frozen in (dielectrically saturated) and that variation of polarization outside the cavity from one configuration to another may be neglected.

## C. THE VALUE OF THE DIELECTRIC COEFFICIENT *D*

Dielectric saturation in the immediate vicinity of an ion has long been recognized (Sack, 1926, 1927). According to the Debye theory of dielectric saturation

\* There is not enough room to accommodate the two water molecules distal to the anion within the microcell of  $20\text{\AA}$  radius. However, the linear arrangement is a model of the three-dimensional system in which water molecules are joined, not end-to-end, but in a zigzag. In that case, the problem disappears. Calculations were also made with the two distal water molecules removed from the model; the results showed no significant change except that the crossover points, to be discussed later, occurred at lower *c*-values.

(1929), the radial dielectric coefficient has a constant value of 3 in the space extending from the center of a monovalent ion radially to a distance of about  $3\text{\AA}$ , where it begins to rise gradually to the macroscopic value of 81, at about  $16\text{\AA}$ . The more recent calculations of Hasted *et al.* (1948) and of Grahame (1950)



**Figure 4.3. THE LINEAR MODEL.** The interaction energies were calculated for each of the monovalent cations in each of the four configurations of fixed anions and water.

show a more abrupt rise of dielectric coefficient immediately beyond the first layer of water. Nevertheless, the consensus of opinion is that the first layer of water is almost completely saturated dielectrically.

Hasted assumed that the first layer of water around an anion is much less saturated than the first layer around a cation. This opinion is quite widely held (Hasted *et al.*, 1948; Grahame, 1950). I adopted this assumption in my treatment of the 1951-52 model; however, little experimental evidence exists to support this view. Lorenz and Posen (1916) presented measurements of the mobilities of a large number of polyatomic organic anions. Applying Stokes'

law, they calculated the effective volume of the ions and then compared these effective volumes with the molecular volume and concluded that no room could be left for an additional layer of water. Judging from the uncertain applicability of Stokes' law and the large ionic size, one sees no conclusive evidence for a general lack of hydration around anions, although the particular contention of these authors may prove correct. On the contrary, anions are known to possess both greater heats of hydration and greater entropies of hydration than cations of equal size; for example,  $K^+$  (crystal radius = 1.33 Å) has a  $\Delta H$  of -75 kcal/mole and a  $\Delta S$  of -11 cal/deg/mole; the corresponding values for  $F^-$  (crystal radius = 1.36 Å) are -121.5 kcal/mole and -26 cal/deg/mole, respectively (Ketelaar, 1953, Table 13; Verwey, 1942). Water molecules oriented in the first hydration shell of an anion should thus be more rigidly frozen and have less freedom than water molecules around a cation of equal size. We may conclude that the first layer of water in immediate contact with either anions or cations is dielectrically saturated. Therefore, the water molecules between the anionic oxyacid group and a cation in configurations I and II should be completely saturated.

The third water molecule in configuration III, found between the two water molecules immediately adjacent to the carboxyl group and the cation, should also be completely saturated dielectrically. The following facts suggest that this middle water molecule is more polarized than any second-nearest neighboring water molecule around a single isolated cation or anion: (1) The interaction between the permanent dipole moment of a water molecule and the cation and that between the permanent dipole moment of the water molecule and the anion are additive. (2) The interaction between induced dipole and permanent dipole and that between induced dipole and induced dipole are enhanced through reinforcement of cationic and anionic induction. (3) These effects combine to shorten the equilibrium distances between the interacting molecules and ions, and thereby secondarily further intensify the cohesive forces of all these as well as those of the London dispersion energies.

#### 4.3. Calculation of the Association Energies and Distribution Ratios

The difference in thermodynamic internal energy between the appropriately defined associated and dissociated states ( $\Delta E = E_{\text{assoc}} - E_{\text{dissoc}}$ ) is represented by  $\Delta E$ , which is usually a negative quantity. The terms "association energy" and "dissociation energy" are both used in this monograph. These energies are equal in absolute magnitude but differ in sign. We have chosen to represent the association energy by  $\Delta E$ ; the dissociation energy, then, is given by  $-\Delta E$ . We use the terms "adsorption energy" and "association energy" as synonyms. Having presented the basic assumptions of the linear model, we can

calculate the association energy  $\Delta E$  for each cation at varying  $c$ -values. Two steps are involved in this process; first, given a cation, a particular  $c$ -value of the fixed anion, and an unlimited number of water molecules, we want to find the statistical probability of having no water molecule, one water molecule, two, or three water molecules between the cation and the fixed anion pair; that is, we want to determine the probability of finding each of the configurations 0, I, II, III. To evaluate this, we must find the total of all energies between cation and anion, between ions and water, between water and water on an absolute basis for each configuration. By comparing the total energies of the various configurations, we can calculate their relative abundance at a given temperature. Having found this, we can calculate the association energy  $\Delta E$  for each particular cation in its particular statistical distribution (for example, 95 per cent in configuration III, 4 per cent in configuration II, and 1 per cent in configuration I). The association energy  $\Delta E$  is the difference between the energy of the cation in the fixed-charge system as a counterion of a fixed anion of a given  $c$ -value and the energy of the same cation in an infinitely dilute solution at an infinite distance. In a truly accurate three-dimensional model,  $\Delta E$  could be obtained by comparing the energy calculated from the first step, properly weighted statistically, with the experimentally measured energy of the hydrated cation extrapolated to infinite dilution. Since we have only a linear model, this comparison cannot be made; instead we use the charging method of Born (1920). Since the major energy difference between the associated ion pair and the dissociated ion pair is the electrostatic interaction energy between the cation and the fixed anion, we can determine the approximate  $\Delta E$  by evaluating the work done in bringing the cation from infinity to the particular equilibrium location estimated in the first part of our calculation. Once the association energies have been evaluated we can find the macroscopic equilibrium distribution ratios between various ion pairs.

#### A. CALCULATION—PART I. THE EVALUATION OF THE STATISTICAL WEIGHTS OF THE VARIOUS CONFIGURATIONS AT EQUILIBRIUM

We shall follow the treatment of ionic hydration of Moelwyn-Hughes (1949). We represent a water molecule as one that has a permanent dipole located  $b\text{\AA}$  away from the geometrical center of an otherwise spherically symmetrical molecule. The repulsion constant between the oxygen end of the water molecule and a cation is then assumed to be similar to that between the same cation and a fluoride ion (which resembles oxygen, Moelwyn-Hughes); the repulsive field is represented as inversely proportional to the ninth power of the distance between the centers of the two interacting atoms. The whole array is first assumed to be at  $0^\circ\text{K}$ . The total potential energy  $U$  of a particular configuration can be calculated from the equation:

$$\begin{aligned}
 u &= \sum_i \sum_{j>i} \frac{Z_i Z_j \epsilon^2}{|r_{ij}|} - \sum_i \sum_{j \neq i} \frac{Z_i \epsilon \mu_j (r_{ij} + b_j)}{|r_{ij} + b_j|^3} \\
 &\quad (1) \qquad \qquad \qquad (2) \\
 &\quad - \sum_i \sum_{j \neq i} \sum_{k \neq i} \frac{\alpha_i Z_j Z_k \epsilon^2 r_{ji} r_{ki}}{2|r_{ji}|^3 |r_{ki}|^3} - \sum_i \sum_{j>i} \frac{2\mu_i \mu_j}{|r_{ij} - (b_i - b_j)|^3} \\
 &\quad (3) \qquad \qquad \qquad (4) \\
 &\quad - \sum_i \sum_{j \neq i} \sum_{k \neq i} \frac{2\alpha_i Z_j \epsilon r_{ji} \mu_k}{|r_{ji}|^3 |r_{ki} - b_k|^3} \\
 &\quad (5) \\
 &\quad - \sum_i \sum_{j>i} \frac{2}{|r_{ij}|^3} \left( \sum_{k \neq i} \frac{\alpha_i Z_k \epsilon r_{ki}}{|r_{ki}|^3} \right) \left( \sum_{l \neq j} \frac{\alpha_j Z_l \epsilon r_{lj}}{|r_{lj}|^3} \right) \\
 &\quad (6) \\
 &\quad - \sum_i \sum_{j>i} \frac{9}{4} \frac{\alpha_i \alpha_j}{|r_{ij}|^6} \frac{U_i U_j}{U_i + U_j} + \sum_i \sum_{j>i} \frac{A_{ij}}{|r_{ij}|^9}. \\
 &\quad (7) \qquad \qquad \qquad (8)
 \end{aligned} \tag{4-4}$$

In succession, the terms on the right-hand side of the equation represent (1) the classical energy of interaction between charge and charge, (2) the energy of interaction between charge and permanent dipole, (3) the energy of interaction between charge and induced dipole, (4) the energy of interaction between permanent dipole and permanent dipole, (5) the energy of interaction between permanent dipole and induced dipole, (6) the energy of interaction between induced dipole and induced dipole, (7) the London dispersion energy, and (8) the Born repulsion energy.

We number the ions and molecules consecutively from the fixed site to the end of the linear array, and define the positive direction as that of increasing numbers. The valency (0, +1, or -1) of the  $i$ th entity is represented by  $Z_i$ ;  $\epsilon$  is the electronic charge;  $r_{ij}$  is a scalar quantity representing the distance from the  $i$ th to the  $j$ th entity and is positive if  $i < j$ ;  $\mu_j$  is the scalar permanent dipole moment of the  $j$ th entity and is positive or negative as the dipole lies parallel or antiparallel to the positive direction;  $b_j$  is equal to  $\pm b$ , the sign being determined by the direction of displacement of the permanent dipole from the geometrical center of the species in a particular configuration. Thus the quantity

$$\frac{b_j}{\pm b} = \frac{\mu_j}{|\mu_j|} \tag{4-5}$$

depends only upon the orientation of the dipole within the species. We determine the equilibrium orientation of the dipoles with respect to the linear system by minimizing the total energy as a function of the possible orientations. The polarizability of the  $j$ th entity is represented by  $\alpha$ ;  $A_{ij}$  is the specific energy constant

of the repulsive field between the  $i$ th and  $j$ th entities in immediate contact;  $U_j$  is the zero-point energy of the  $j$ th entity. The zero-point energy of a cation is taken as the second ionization potential; that of an anion is taken as the electron affinity (see J. E. Mayer, 1933). The zero-point energy for water molecules is calculated from the value of refractive indices of Tilton and Taylor (1938) to be  $20.92 \times 10^{-12}$  ergs per molecule.\* This agrees closely with the

Constant	H	Li	Na	K	NH <sub>4</sub>	Rb	Cs	H <sub>2</sub> O	Carboxyl oxygen
$d \times 10^8$ cm	1.61	2.21	2.50	2.90	3.06	3.09	3.38		
$g \times 10^8$ cm	2.42	2.50	2.53	2.55	2.55	2.56	2.58		
$\alpha \times 10^{24}$ cm	0 <sup>c</sup>	0.075 <sup>a</sup>	0.21 <sup>a</sup>	0.87 <sup>a</sup>	1.65 <sup>b</sup>	1.81 <sup>b</sup>	2.79 <sup>a</sup>	1.444 <sup>a</sup>	0.876 <sup>c</sup>
$U \times 10^{12}$ erg/mole	0 <sup>e</sup>	120.6 <sup>d</sup>	75.4 <sup>d</sup>	50.7 <sup>d</sup>	50.7 <sup>e</sup>	43.8 <sup>d</sup>	37.5 <sup>d</sup>	20.9 <sup>e</sup>	3.4 <sup>f</sup>
$\mu \times 10^{18}$ e.s.u.								1.834 <sup>a</sup>	
$A \times 10^{32}$ erg/cm <sup>9</sup>									
(against oxygen end of water molecule)	0.208 <sup>i</sup>	3.38 <sup>i</sup>	8.5 <sup>h</sup>	26.5 <sup>h</sup>	35.01 <sup>i</sup>	42.5 <sup>h</sup>	82.5 <sup>h</sup>	14.14 <sup>j</sup>	14.14 <sup>j</sup>
(against fluoride ion)			(6.29) <sup>k</sup>	(28.4) <sup>k</sup>		(49.4) <sup>k</sup>	(90.3) <sup>k</sup>		

Table 4.1. PHYSICAL CONSTANTS USED IN THE COMPUTATION OF INTERACTION ENERGIES.

<sup>a</sup> B. E. Conway, 1952 (Table II 3).

<sup>b</sup> Ketelaar, 1953.

<sup>c</sup> See text.

<sup>d</sup> Handbook of Chemistry and Physics, 1956-57 (p. 2347, II).

<sup>e</sup> Calculated from refractive indices of Tilton and Taylor, 1938.

<sup>f</sup> Latimer, 1952 (p. 18).

<sup>g</sup> Moelwyn-Hughes, 1949.

<sup>h</sup> Calculated from experimental heat of hydration, Moelwyn-Hughes, 1949.

<sup>i</sup> Calculated.

<sup>j</sup> Calculated from Searcy, 1949.

<sup>k</sup> Calculated from Lennard-Jones, 1936 (Table 32, p. 327).

ionization potential of water,  $21.1 \times 10^{-12}$  ergs per molecule (International Critical Tables).

In calculating the dispersion energy, we introduce the additional factor of  $3/2$  into the London expression,

$$-\frac{3}{2} \frac{\alpha_i \alpha_j}{|r_{ij}|^6} \frac{U_i U_j}{U_i + U_j}$$

following Born and Mayer (1932) and Bernal and Fowler (1933). The various other constants adopted and their sources are listed in Table 4.1.

\* Sellmeier's equation of the form

$$n_i = 1 + \frac{a}{\nu_0^2 - \nu_i^2}$$

was used. Here  $n_i$  is the refractive index at wave length  $\lambda_i$ ;  $\nu_i$  is the corresponding frequency;  $\nu_0$  is assumed to be the only natural frequency; and  $a$  is a constant. The wave lengths  $\lambda_i$  chosen correspond to the following: 7065.2 Å (He), 5892.6 Å (Na), 4861.3 Å (H), 4046.6 Å (Hg), and  $\nu_i = c_{\text{light}}/\lambda_i$  ( $c_{\text{light}}$  is the velocity of light). The  $\nu_0$  values calculated for each pair of frequencies were averaged, giving  $20.92 \times 10^{-12}$  ergs per molecule.

### (1) The polarizability, zero-point energy, and other constants

The value  $\alpha$ , assigned to the oxyacid group, represents the polarizability of the negatively charged oxygen and includes contributions from nearby atoms and other secondary effects (see the top of page 60). Consequently, we choose two values representing a reasonable range, rather than a single value. The lower value is arbitrarily taken as  $0.876 \times 10^{-24} \text{ cm}^3$ . This is not an unreasonable lower limit since the carboxyl oxygen has a polarizability of  $0.84 \times 10^{-24} \text{ cm}^3$ ; the fluoride ion has a polarizability of  $0.81 \times 10^{-24} \text{ cm}^3$ ; the hydroxyl oxygen,  $0.59 \times 10^{-24} \text{ cm}^3$ ; and the ether oxygen,  $0.64 \times 10^{-24} \text{ cm}^3$  (Ketelaar, 1953, Table 9). For the upper limit, we choose the value  $2.0 \times 10^{-24} \text{ cm}^3$  (the  $\text{OH}^-$  group has a polarizability of  $1.89 \times 10^{-24} \text{ cm}^3$ , Ketelaar, 1953).

The  $\text{H}^+$  ion is given zero values for both its polarizability and its zero-point energy. The zero-point energy for the ammonium ion  $\text{NH}_4^+$  is not available. Mulliken (1933) pointed out the chemical similarity between the ammonium ion and the potassium ion, and showed that the ionization potential of the  $\text{NH}_4^+$  ion is very close to that of the potassium ion. We have assumed that the  $\text{NH}_4^+$  ion has the same value for its second ionization potential as the potassium ion has. The best approximation for oxyacid oxygen is that of the electron affinity of a singly charged oxygen atom (Latimer, 1952).

The Born repulsion constants for the  $\text{Na}^+$  ion, the  $\text{K}^+$  ion, the  $\text{Rb}^+$  ion, and the  $\text{Cs}^+$  ion against either the oxyacid oxygen or the oxygen end of a water molecule were calculated from experimentally determined heats of hydration using Moelwyn-Hughes' equation 3 (1949, p. 479). For comparison, the repulsion constants between alkali-metal ions and the fluoride ion (which resembles oxygen) are also listed (Lennard-Jones, 1936, p. 327). We calculate the repulsion constant between water molecules from the data of Searcy (1949), who equated the repulsion term to  $C/r^{12}$ , while we use the approximation  $A/r^9$ . The value of  $C$  a constant, is obtained from Searcy. The equilibrium distance  $r$  between water molecules in ice is  $2.76\text{\AA}$  (Pauling, 1948). Equating  $C/r^{12}$  to  $A/r^9$ , we obtain  $A = 14.14 \times 10^{-82} \text{ erg cm}^9$ . We assume the same value for the repulsion between the hydrogen end of a water molecule and an oxyacid oxygen atom.

### (2) Calculation of equilibrium distances

Figure 4.3 represents a scheme for the linear arrangement in the cylindrical cavity mentioned earlier and the designation of the distances:  $a$ ,  $a'$ ,  $b$ ,  $d$ ,  $e$ ,  $f$ ,  $g$ . The next step is to select or calculate the equilibrium values of these distances. The value of  $b$ , the distance between the center of a water molecule and the center of its permanent dipole moment, is  $0.274\text{\AA}$ ;  $d$ -values (roughly the sum of the radii of the cation and a water molecule) for the  $\text{Na}^+$  ion, the  $\text{K}^+$  ion, the  $\text{Rb}^+$  ion, and the  $\text{Cs}^+$  ion are, respectively,  $1.55\text{\AA}$ ,  $1.57\text{\AA}$ ,  $1.61\text{\AA}$ , and  $1.69\text{\AA}$ .

greater than their respective Pauling crystal radii. Adding the average value of 1.61 $\text{\AA}$  to the crystal radius of zero for proton and of 1.45 $\text{\AA}$  for the ammonium ion (Ketelaar, 1953, Table 3E), we obtain an equilibrium  $d$ -value equal to 1.61 $\text{\AA}$  for the  $\text{H}^+$  ion and to 3.06 $\text{\AA}$  for the ammonium ion. Substituting these into  $a_0$  of equation 3 of Moelwyn-Hughes, we obtain the repulsion constant  $A$  with a value of  $0.208 \times 10^{-82}$  erg cm $^9$  for the  $\text{H}^+$  ion and  $35.0 \times 10^{-82}$  erg cm $^9$  for the  $\text{NH}_4^+$  ion. It now remains only to find the values of  $a$ ,  $a'$ ,  $e$ ,  $f$ , and  $g$ .

(a) *The distance g.* The  $g$ -value represents the center-to-center distance between the two water molecules distal to the anion (Figure 4.3). Since this distance is far removed from the anionic charge we assume that it varies only with the nature of the cation and that it does not depend on the anion. Neglecting the less important terms, we solve for the equilibrium distance  $g$  by assuming  $d$  constant for each cation and equating the repulsion force to the combined cohesive forces\* due to the dispersion energy and to the energy of interaction between ion and dipole. We obtain

$$\frac{9A}{g^{10}} = \frac{2\epsilon\mu_w}{(d + g + b)^3} + \frac{6\alpha_w\mu_w\epsilon}{(g + b)^4 d^2} + \frac{6\mu_w^2}{g^4} + \frac{54}{8} \frac{\alpha_w^2 U_w}{g^7} \quad (4-6)$$

where  $A$  represents the repulsion constant, equal to  $14.14 \times 10^{-82}$  erg cm $^9$  between a pair of water molecules; and the subscript  $w$  refers to the water molecule. The equation is then solved for the different cations with the results given in Table 4.1. These and following equations are solved by the method of successive approximation.

(b) *The distance e.* After similar simplification by neglecting less important terms, one obtains at equilibrium

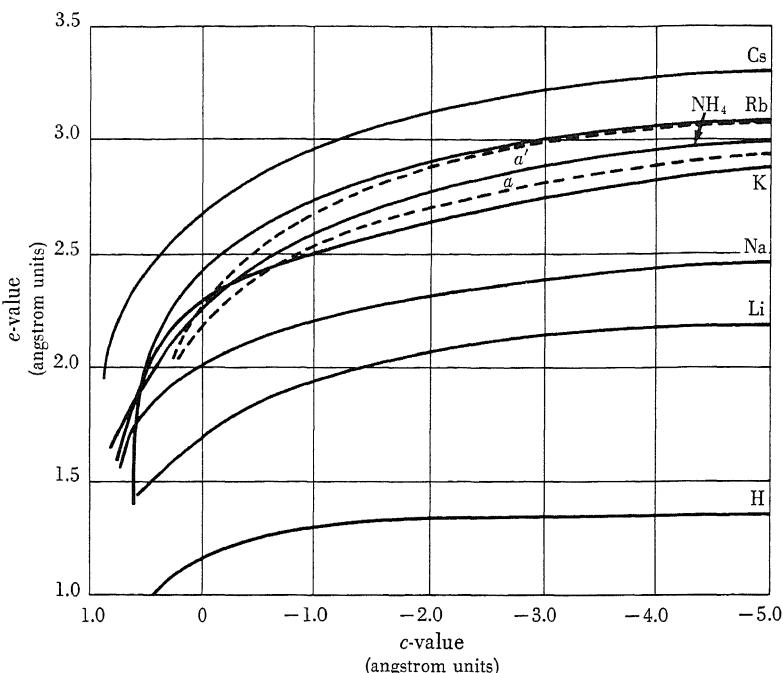
$$\frac{9A'}{e^{10}} = \frac{\epsilon^2}{(e - c)^2} + \frac{2\alpha_- \epsilon^2}{e^5} + \frac{2\alpha_+ \epsilon^2}{(e - c)^5} \quad (4-7)$$

where  $A'$  is the repulsion constant between a cation and the oxygen end of a water molecule; and the subscripts + and - refer to the cationic and anionic groups, respectively. Defining a variable  $k$  by the equation  $e - c = ke$ , we substitute  $ke$  for  $(e - c)$ . Rearranging the above equation, we obtain

$$e^8 + \left( 2\alpha_- k^2 + \frac{2\alpha_+}{k^3} \right) e^5 - \frac{9A' k^2}{\epsilon^2} = 0 \quad (4-8)$$

which we then solve for different cations and different  $k$ -values. From the relation  $e - c = ke$ , we find the value of  $c$  that corresponds to each  $k$ -value; a plot of  $e$  versus  $c$  for each alkali-metal ion is shown in Figure 4.4.

\* Both the repulsion force and the combined cohesive forces are derived from equation (4-4) for the various energies and from the relation that the force between two interacting particles is equal to the derivative of the energy between them with respect to  $r$ , the distance of separation.



**Figure 4.4.** THE RELATION BETWEEN THE  $c$ -VALUE AND THE COMPUTED VALUE OF  $e$ ,  $a$ , AND  $a'$ . The equilibrium distance  $e$  between the centers of the fixed anion and the counter-cation in the 0-configuration is represented by the solid line. The dotted lines show the distances  $a$  and  $a'$  as functions of the  $c$ -value. Here  $a$  is the distance between the center of the fixed anion and the first water molecule in configuration I and  $a'$  is the analogous distance in configurations II and III.

(c) *The distances  $a$  and  $a'$ .* Letting  $a - c = k'a$ , we find that

$$\frac{9A}{a^{10}} = \frac{2\alpha_w\epsilon^2}{(k')^5 a^5} + \frac{2\mu_w\epsilon}{(k')^3 \left(a - \frac{b}{k}\right)^3} + \frac{27}{2} \frac{\alpha_w U_- U_w}{U_- + U_w} \frac{1}{(k')^7 a^7} + \frac{\epsilon^2}{(k')^2 \left(a + \frac{d}{k'}\right)^2}. \quad (4-9)$$

Calculations of  $a$ -values using the above equation, first with the smallest  $d$ -value for the  $H^+$  ion and then with the largest  $d$ -value for the  $Cs^+$  ion, show that these differences of  $d$ -value affect the  $a$ -value very little. We decided to use the average  $d$ -value of  $2.68\text{\AA}$  for all cation calculations and  $2.68 + 2.7\text{\AA}$  ( $2.7\text{\AA}$  is the diameter of a water molecule) =  $5.38\text{\AA}$  for the calculation of all  $a'$ -values. The result is also given in Figure 4.4.

(d) *The distance  $f$ .* Following the same reasoning as above, and again neglecting the lesser terms, we derive

$$\frac{9A}{f^{10}} = \frac{2\mu_w\epsilon}{(d + f)^3} + \frac{\epsilon^2}{(d + f + a - c)^2} + \frac{6\mu_w^2}{f^4} \quad (4-10)$$

for configuration II. The same  $f$ -values were used for configuration III. The results are plotted in Figure 4.5.

(3) The calculation of the total potential energies of the various configurations

Having determined all the equilibrium distances, we calculate the total potential energies of the various configurations. In summing the individual terms, we neglect a particular term in equation (4-4) only after computing that term for each cation and finding that the term for the ion with the highest value is less than 0.15 kcal/mole. The total energy for each ion at each  $c$ -value is then plotted against the  $c$ -value after subtracting the value  $n \times 4.98$  kcal/mole; here  $n$  represents the number of water molecules inserted between the cation and the anion for that configuration and 4.98 kcal/mole corresponds to the energy needed to

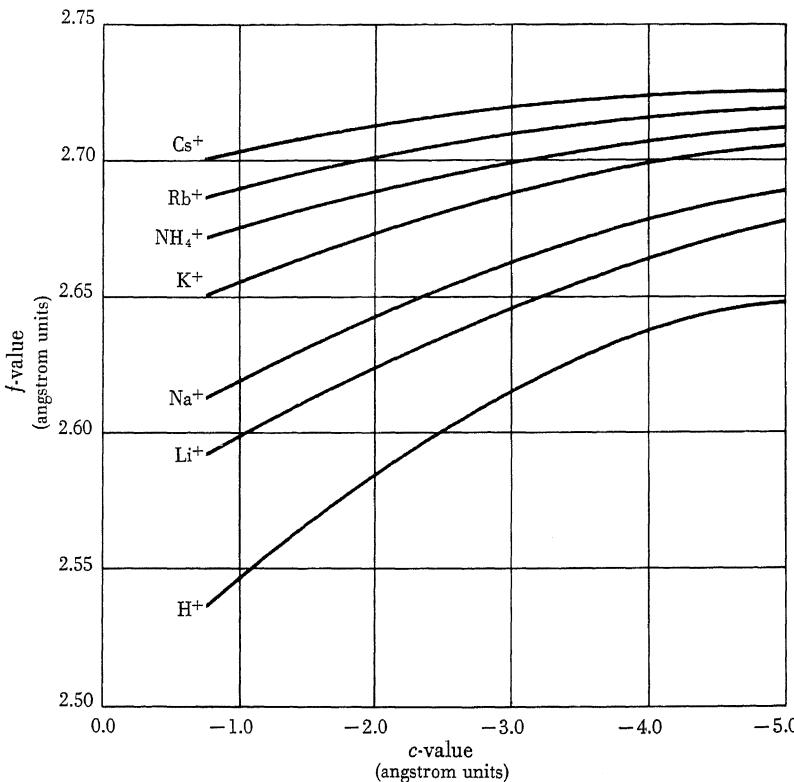


Figure 4.5. THE RELATION BETWEEN THE  $c$ -VALUE AND THE COMPUTED VALUE OF  $f$ . The distance between the first and second water molecules in configurations II and III is represented by  $f$ .

bring a water molecule from pure liquid water at an infinite distance into the linear array.\*

Figures 4.6, 4.7, and 4.8 plot the computed energies of the various configurations of the different ions against the *c*-value. In Figure 4.6, the polarizability of the oxyacid group is assumed to be  $0.876 \times 10^{-24} \text{ cm}^3$ ; in Figure 4.7,  $1.25 \times 10^{-24} \text{ cm}^3$ ; and in Figure 4.8,  $2.0 \times 10^{-24} \text{ cm}^3$ .

#### (4) The calculation of the contributions of different configurations

For each ion at a particular *c*-value, one configuration represents a state of lowest energy. In general, the relationship for each ion is such that at lower *c*-values the higher configuration with a greater number of water molecules intervening between the fixed anion and the counterion is preferred. As the *c*-value increases, the preference is shifted to lower and lower configurations until eventually, at the highest *c*-value, the 0-configuration is invariably the preferred one. This trend is followed by each ion, although the *c*-value at which the preference changes from one configuration to another varies from ion to ion.

From Appendix B, we derive for the species  $p_i$

$$\frac{n_i^s}{\sum_{s=0,I,II,III} n_i^s} = \frac{(\text{p.f.})_i^s \exp(-\mathfrak{U}_i^s/RT)}{\sum_{s=0,I,II,III} (\text{p.f.})_i^s \exp(-\mathfrak{U}_i^s/RT)} \quad (4-11)$$

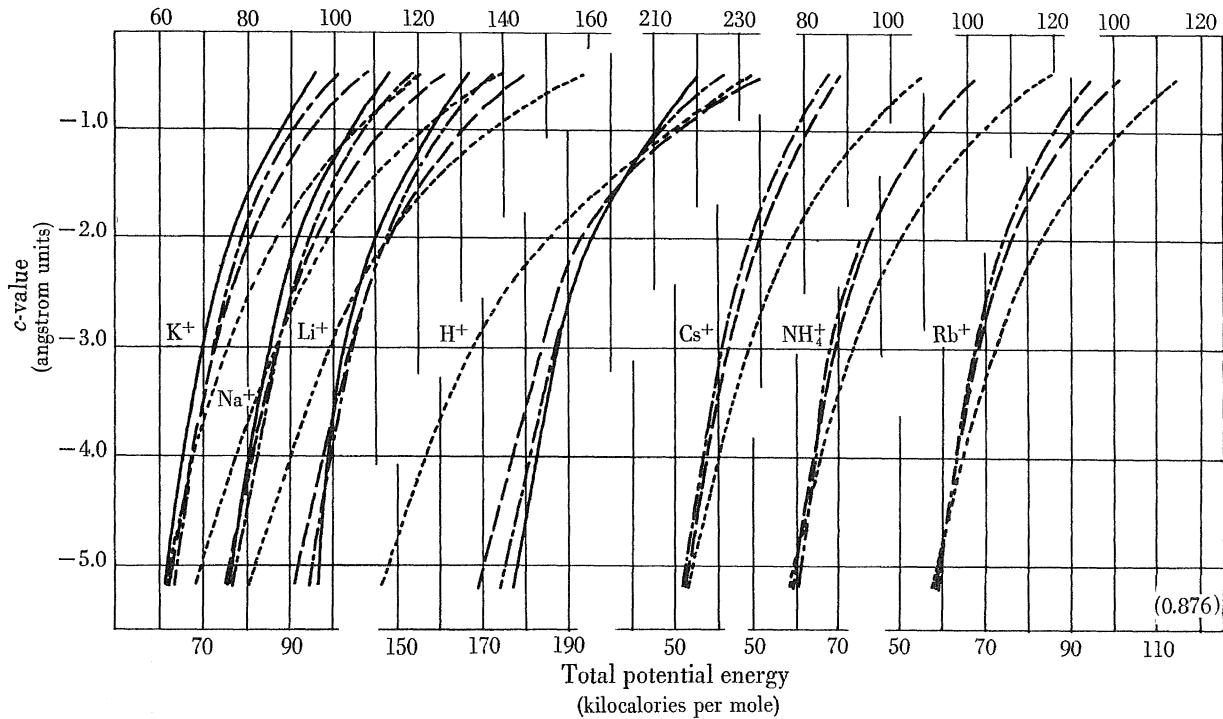
where  $n_i^s$  is the number of  $p_i$  ions in a particular configuration *s*;  $(\text{p.f.})_i^s$  is the partition function for that ion in that configuration; and  $\mathfrak{U}_i^s$  is the total energy of the *i*th ion assembly in configuration *s*.

If we assume that only the configurational partition function varies significantly among the associated ions (see Section 2.4), the relative distribution of the same ion  $p_i$  in the different configurations would be

$$\frac{n_i^s}{\sum_{s=0,I,II,III} n_i^s} = \frac{\rho_i^s \exp(-\mathfrak{U}_i^s/RT)}{\sum_{s=0,I,II,III} \rho_i^s \exp(-\mathfrak{U}_i^s/RT)}. \quad (4-12)$$

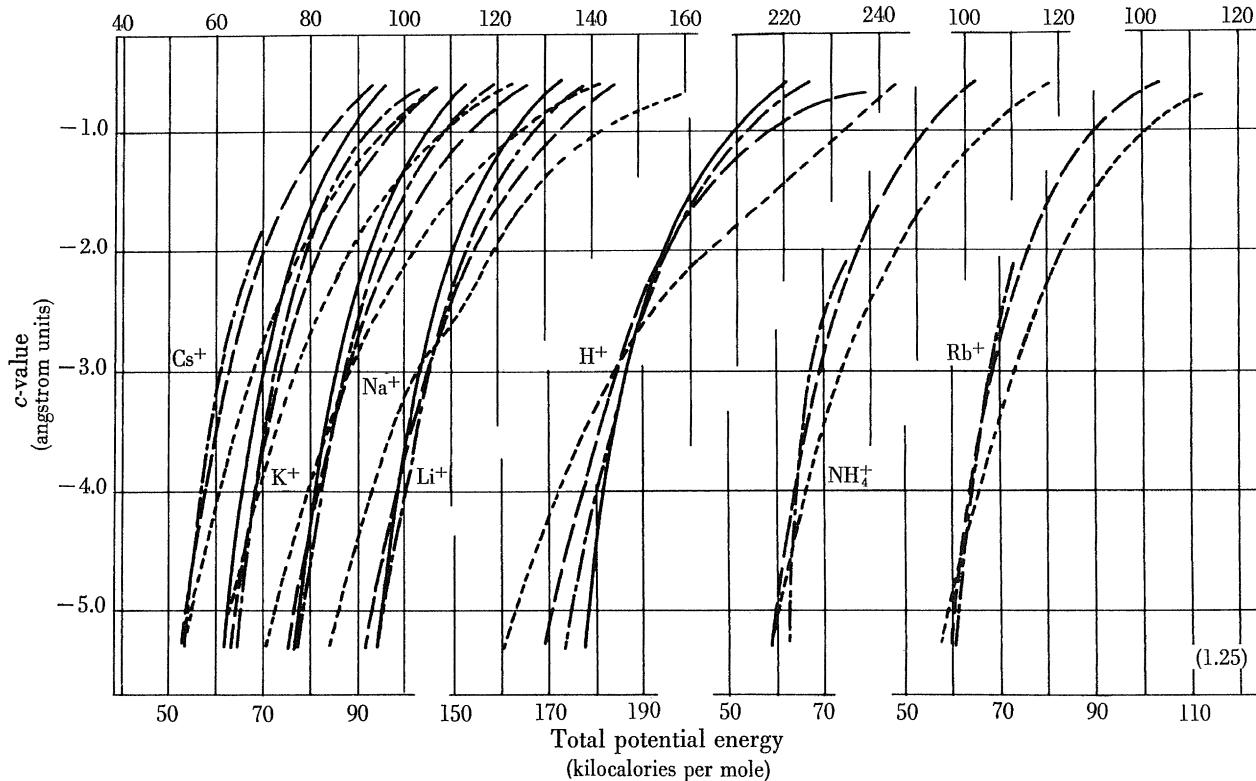
where  $\rho_i^s$  is the number of sites (see Section 2.4A) available to  $p_i$  in configuration *s*, a function of the species of the ion, its configuration, and the *c*-value as shown in Figures 4.6 to 4.8. If  $r_i^s$  is the equilibrium distance from the center of  $p_i$  in configuration *s* to the center of gravity of the negative charges ( $r_f - c$ ), on first approximation the ion may be assumed to be constrained to move within a spherical shell of radius  $r_i^s$  and thickness *h*. We make the further assumption

\* Each oriented water molecule possesses a total energy equal to the latent heat of vaporization (9.955 kcal/mole) minus  $RT$  (0.582 kcal/mole at 20°C). Each water molecule has four "half bonds" (coordination number = 4, each bond counted twice) of 2.489 kcal/mole each. We assume that two of these half bonds are altered in transplanting one water molecule to the linear array.



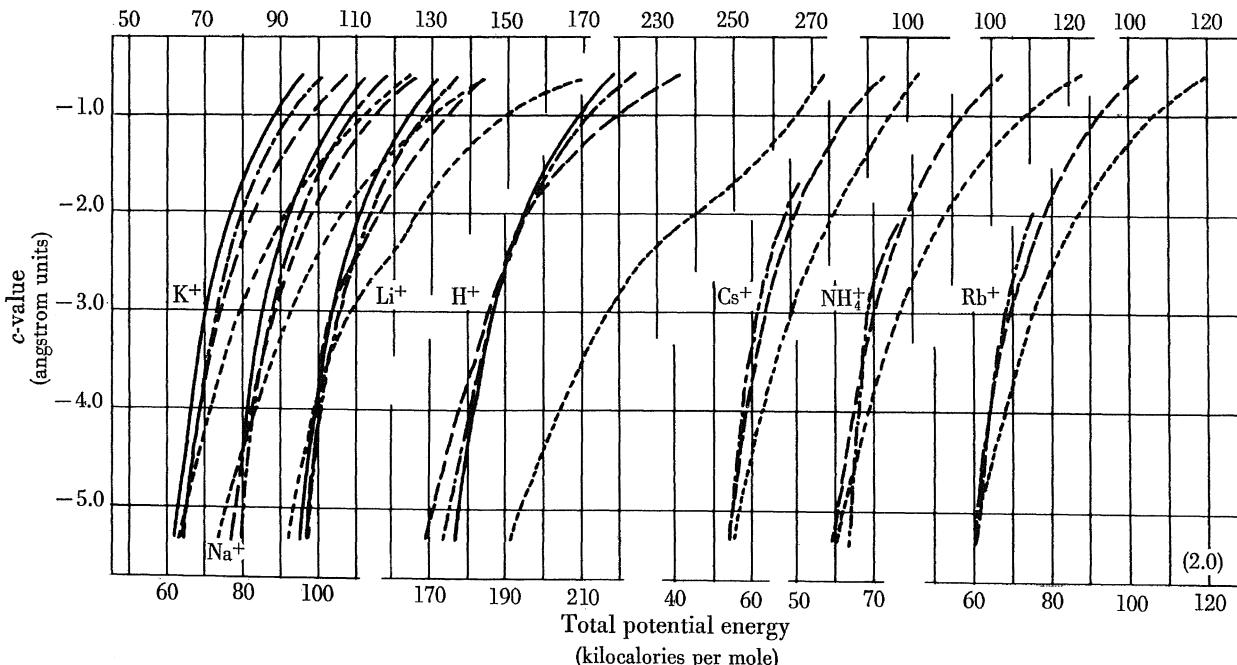
**Figure 4.6. THE VARIATION OF THE TOTAL POTENTIAL ENERGY  $U$  OF THE FIXED ANION-COUNTERCATION-WATER SYSTEMS AS A FUNCTION OF THE  $c$ -VALUE AT  $0^{\circ}\text{K}$ . In this figure, the fixed anion has**

a polarizability of  $0.876 \times 10^{-24} \text{ cm}^3$ . Values are plotted for each cation in each of the configurations 0 ——, I ——, II ——, III ——.



**Figure 4.7.** THE VARIATION OF THE TOTAL POTENTIAL ENERGY  $U$  OF THE FIXED ANION-COUNTERCATION-WATER SYSTEM AS A FUNCTION OF THE  $c$ -VALUE AT  $0^\circ\text{K}$ . In this figure, the fixed anion has a

polarizability of  $1.25 \times 10^{-24} \text{ cm}^3$ . Values are plotted for each cation in each of the configurations 0 ----, I - - -, II - - - - , III —.



**Figure 4.8.** THE VARIATION OF THE TOTAL POTENTIAL ENERGY  $U$  OF THE FIXED ANION-COUNTERCATION-WATER SYSTEM AS A FUNCTION OF THE  $c$ -VALUE AT 0°K. In this figure, the fixed anion has a

polarizability of  $2.0 \times 10^{-24} \text{ cm}^3$ . Values are plotted for each cation in each of the configurations 0 ----, I —, II ——, III ——.

that the empirical equation

$$h = \left( \frac{RT}{\mathfrak{U}} \right)^{\frac{1}{2}} \text{Å}$$

which holds for almost all diatomic molecules, also applies to the fixed anion-counterion pair (see Mayer and Mayer, 1940). In this equation,  $\mathfrak{U}$  stands for the absolute magnitude of the energy of the bond and one may write for ion  $p_i$  in configuration  $s$

$$\rho_i^s = 4\pi \left( \frac{RT}{\mathfrak{U}_i^s} \right)^{\frac{1}{2}} (r_i^s)^2.$$

Substitution into equation (4-12) yields

$$\frac{n_i^s}{\sum_{s=0, I, II, III} n_i^s} = \frac{[(r_i^s)^2 / (\mathfrak{U}_i^s)^{\frac{1}{2}}] \exp(-\mathfrak{U}_i^s/RT)}{\sum_{s=0, I, II, III} [(r_i^s)^2 / (\mathfrak{U}_i^s)^{\frac{1}{2}}] \exp(-\mathfrak{U}_i^s/RT)}. \quad (4-13)$$

In the present case, since only those configurations with the greatest (negative) total energies count, the ratio of  $(\mathfrak{U}_i^s)^{\frac{1}{2}}$  for two such configurations is always close to unity; hence

$$\frac{n_i^s}{\sum_{s=0, I, II, III} n_i^s} = \frac{(r_i^s)^2 \exp(-\mathfrak{U}_i^s/RT)}{\sum_{s=0, I, II, III} (r_i^s)^2 \exp(-\mathfrak{U}_i^s/RT)}. \quad (4-14)$$

This equation gives us the percentages of different configurations of the ion-fixed-charge pair at a particular value of  $c$ .

## B. CALCULATION—PART II. THE CALCULATION OF THE ASSOCIATION ENERGIES OF VARIOUS IONS AT DIFFERENT $c$ -VALUES

The problem of ionic association is always ambiguous because it depends intimately upon the definition of a state of association and the definition of a state of dissociation. The problem becomes even less clear when we deal with a fixed-charge system in equilibrium with a free solution. In this case, the dissociation of a counterion-fixed-charge pair could mean the assumption of a new position by the dissociated counterion either inside or outside the fixed-charge system. If it migrates out of the fixed-charge system, the process is complicated by the development of surface potentials (see Section 10.2 on cellular potential). Further, the association energy depends, in general, on both the spatial position of the counterion in the fixed-charge system and the history of the fixed-charge system itself. To eliminate these complications, we regard as associated any counterion that assumes one of configurations 0, I, II, or III. The fixed-charge system with its complete assortment of counterions is then brought from  $0^\circ\text{K}$  to the ambient temperature. We now define the association energy,  $\Delta E$ , to be the difference between the energy of the associated counterion and the energy of the first

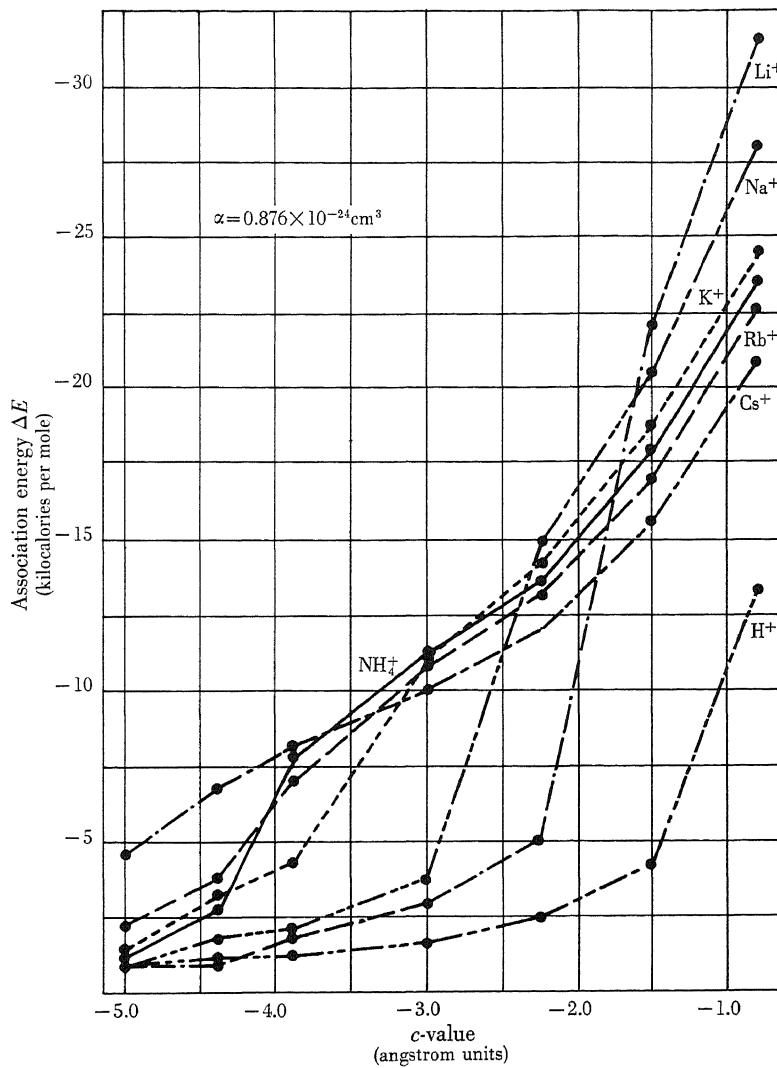
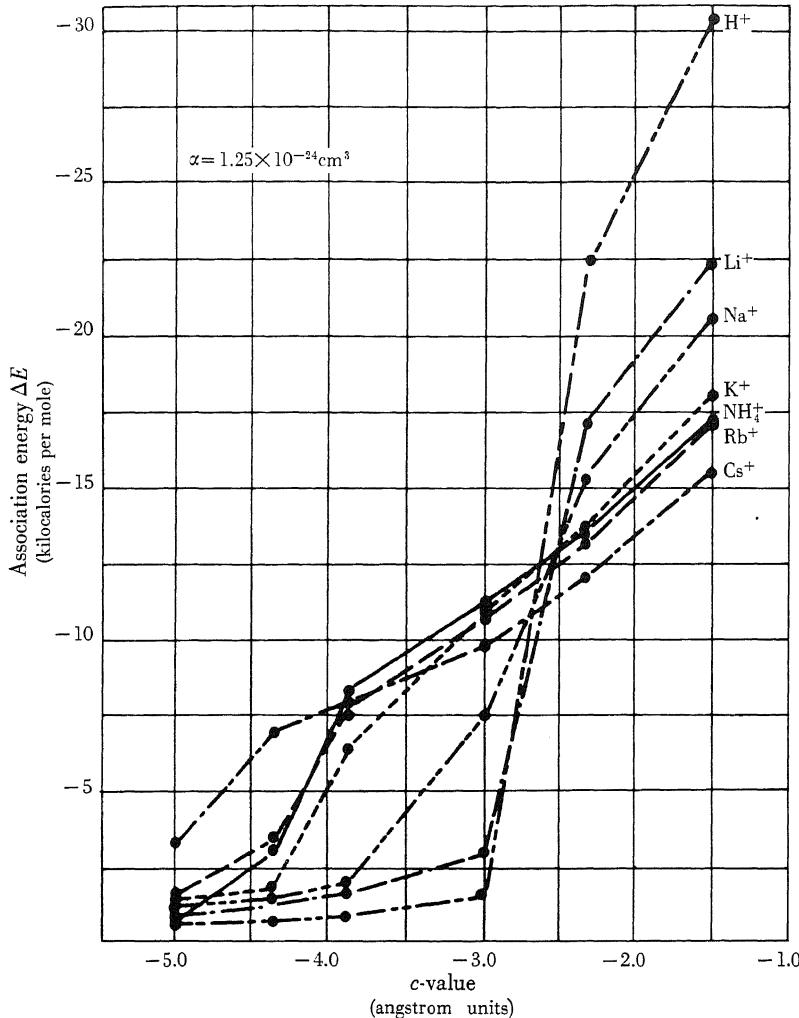


Figure 4.9. THE RELATION BETWEEN THE CALCULATED ASSOCIATION ENERGY  $\Delta E$  OF VARIOUS CATIONS AND THE  $c$ -VALUE OF THE ANIONIC GROUP. The polarizability  $\alpha$  is  $0.876 \times 10^{-24} \text{ cm}^3$ .

counterion that diffuses out of the fixed-charge system to an infinite distance in an infinitely dilute aqueous phase. This energy is not significantly different from that obtained if we compare the associated counterion in configuration 0, I, II, or III with a similar counterion in a hypothetical "beyond III" configuration within the fixed-charge system. The limit of association (equivalent to  $r_2$  of Section 2.4A) in this case refers to the radius at which there is a sharp



**Figure 4.10.** THE RELATION BETWEEN THE CALCULATED ASSOCIATION ENERGY  $\Delta E$  OF VARIOUS CATIONS AND THE  $c$ -VALUE OF THE ANIONIC GROUP. The polarizability  $\alpha$  is  $1.25 \times 10^{-24} \text{ cm}^3$ .

increase in the dielectric coefficient of the medium such that a volume of water which is not dielectrically saturated intervenes between the two charged particles (see Ling, 1952, inset of Figure 6).

Taking into account the freezing-in of the water molecules between the fixed ion and its counterion (Section 4.2B), we calculate the association energy by evaluating the work performed when the counterion is brought from infinity through a medium having the dielectric properties of water into a microcell at

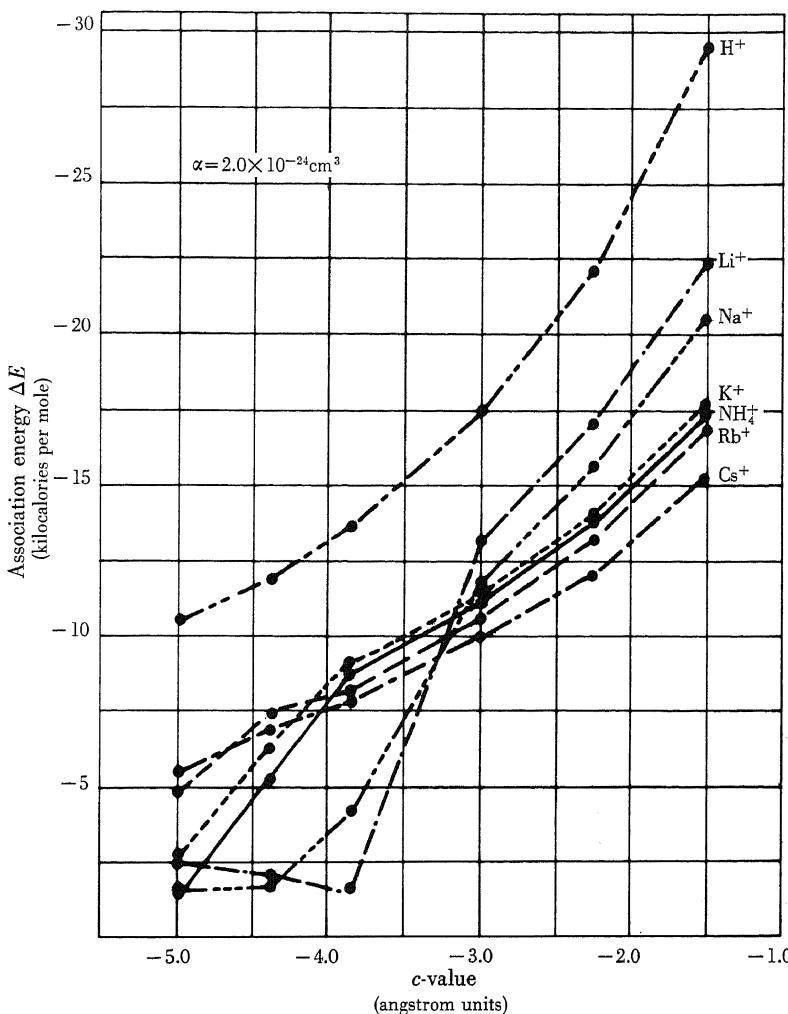


Figure 4.11. THE RELATION BETWEEN THE CALCULATED ASSOCIATION ENERGY  $\Delta E$  OF VARIOUS CATIONS AND THE  $c$ -VALUE OF THE ANIONIC GROUP. The polarizability  $\alpha$  is  $2.0 \times 10^{-24} \text{ cm}^3$ .

the equilibrium distance which that particular ion assumes. Instead of using a macroscopic dielectric coefficient, we take Debye's value for the dielectric coefficient  $D(r)$  at a distance  $r$  from the center of a univalent ion.\* In the present case, however, the sharp increase of dielectric coefficient with increase of  $r$  does not begin until  $r$  corresponds to a distance three water molecules away from the

\* No significant variation would be created if the curve of Grahame (1950) or of Hasted *et al.* (1948) were used for the rising part of the effective dielectric coefficient.

anion. Thus, within a region of  $8.1\text{\AA}$  ( $3 \times 2.7\text{\AA}$ ) between the cation and anion in configuration III, we assume a uniform dielectric constant of three.

The energy of association of each ion at each  $c$ -value is then

$$\Delta E = \sum_{s=0,I,II,III} \frac{n_i^s}{\sum_{s=0,I,II,III} n_i^s} \int_{r_i}^{\infty} -\frac{\epsilon^2}{D(r)r^2} dr \quad (4-15)$$

where  $n_i^s / \sum_{s=0}^{III} n_i^s$  is given by equation (4-14). In Figures 4.9 to 4.11, the computed

association energies of the various cations against anions of differing  $c$ -values are presented. For Figure 4.9, the polarizability of the oxyacid functional group is taken to be  $0.876 \times 10^{-24} \text{ cm}^3$ ; for Figure 4.10, as  $1.25 \times 10^{-24} \text{ cm}^3$ ; and for Figure 4.11, as  $2.0 \times 10^{-24} \text{ cm}^3$ . We believe that Figure 4.9 gives the closest approximation to ion interaction with biological fixed-charge systems; most of this discussion will be based on this figure.

#### 4.4. Discussion and Comment on the Present Model

##### A. THE TENTATIVE NATURE OF THE ABSOLUTE $c$ -VALUE AND ASSOCIATION ENERGIES

The adoption of the present linear model is a concession to the forbidding complexity associated with a three-dimensional model. Since transforming the linear model to a three-dimensional model would involve symmetrical changes only, one would not expect the  $\Delta E$ -versus- $c$ -value plot to involve any disorderly disturbance of the relations calculated, but only changes of absolute magnitude.

The linear model does discriminate against the higher configurations. Thus the calculation of the total energies of the 0-configuration would be virtually the same in the linear as in a three-dimensional model. On the other hand, for configurations I, II, and III, the energies calculated are a great deal less in the linear model than they would be in a three-dimensional one; in these higher configurations, a large part of the energy arises from the interactions between the ions and water molecules and these water molecules are obviously coordinated in three dimensions. In the linear model, therefore, only a fraction of the coordinated water molecules is taken into account; the higher the configuration the greater the discrepancy.

Another aberration is created by the adoption of the Born charging method for the determination of association energies. The assumption that the total hydration energy of the ions remains the same in the dissociated and associated states is implicit in this procedure. This assumption is closer to the truth for ions in the higher configurations than for ions in the 0-configuration. If we neglect

the partial loss of ion-water interaction in the 0-configuration the calculation overestimates the 0-configuration energy. Consequently, (1) the aberration from the first part of the calculation is accented and this causes the crossover points to fall on lower  $c$ -values than they should, and (2) the dissociation energy at high  $c$ -values is overestimated.

When the three-dimensional model is developed, we do not expect the shape of the  $\Delta E$ -versus- $c$ -value plot to be changed significantly; but the  $c$ -values should be much higher than the present tentative values which we have calculated. One must remember that the  $c$ -value, as defined, is not a spatial location of a particular electron; thus, comparison of its value with, say, the diameter of an oxygen atom is quite meaningless and leads to erroneous conclusions.

The heat of hydration in kcal/mole is 114.6 for  $\text{Li}^+$ , 89.7 for  $\text{Na}^+$ , 73.5 for  $\text{K}^+$ , 67.5 for  $\text{Rb}^+$ , and 60.8 for  $\text{Cs}^+$  (Latimer *et al.*, 1939). The coordination numbers of these ions range from 6 for  $\text{Li}^+$  to 2 for  $\text{Cs}^+$  (B. E. Conway, 1952, Table III, p. 53). The overestimation of the energy of the 0-configuration thus consists essentially of one-sixth to one-half of the heat of hydration, a value that ranges from -20 to -30 kcal/mole. Therefore, the corrected association energies for the alkali-metal and ammonium ions at high  $c$ -values should have values no greater than -20 kcal/mole. For a rough quantitative estimation involving these association energies, we halve the calculated association energies. Figure 4.12 shows the distribution ratios (with  $\text{K}^+$  as the reference ion) for an anion polarizability equal to  $0.876 \times 10^{-24} \text{ cm}^3$ . (Compare with Figure 4.13, where the uncorrected values were used.)

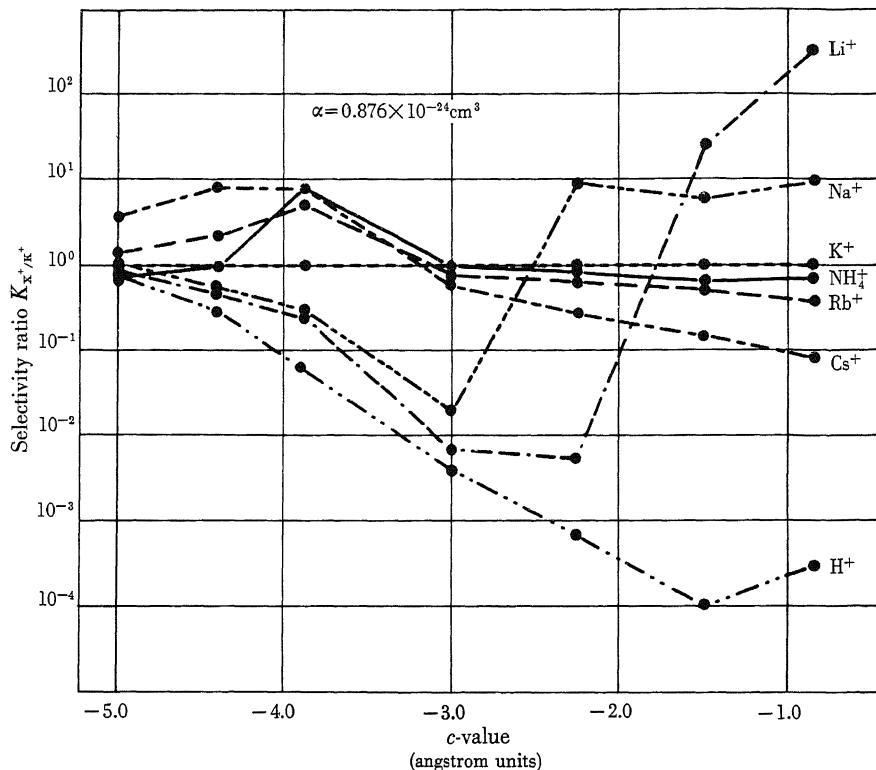
### B. INTERNAL ENERGY COMPARED WITH FREE ENERGY

Calculated as work, the association energy  $\Delta E$  is equivalent to a standard free energy of association in a dilute salt solution. It does not correspond to the standard free energy of association in a fixed-charge system because a large positive entropy of dissociation that is not included in the free energy found by the Born charging method contributes greatly to the true free energy of adsorption. This large entropy change arises from the configurational- and rotational-entropy gain experienced by a counterion when it is taken from the associated state in a fixed-charge system to the dissociated state in free solution. The free energy that we obtained approximates the association energy  $\Delta E$  more closely than the free energy of association  $\Delta F$ . Since we are interested primarily in the relative values of the association energies with reference to other counterions, we do not need the absolute  $\Delta F$  and have not attempted to calculate it.

### C. THE HYDROGEN ION

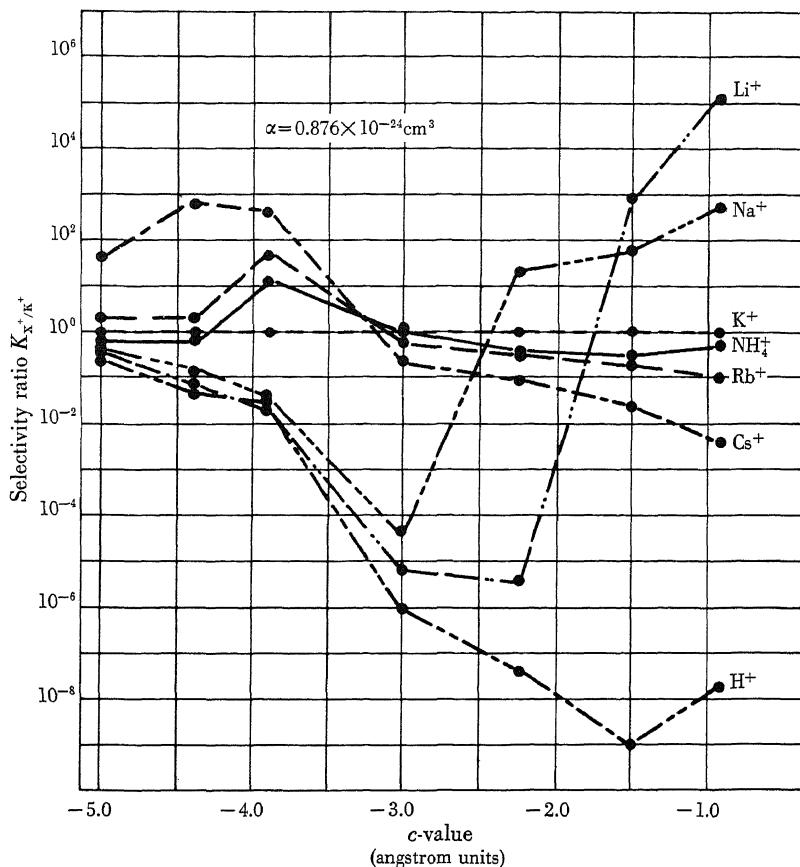
Although the O—H bond in an alcoholic group is almost entirely nonionic, resonance greatly increases the ionic nature of the O—H bond in the carboxylate

ion (Pauling, 1948; Branch and Calvin, 1941). In the present treatment, we do not take account of the covalent contribution to the bond between the  $H^+$  ion and the carboxyl oxygen; we assume that it is constant with respect to variation in *c*-value. This is justified. The *pK*'s of a list of oxyacids vary from less than



**Figure 4.12. THE RELATION BETWEEN THE SELECTIVITY RATIOS OF VARIOUS CATIONS AND THE *c*-VALUE.** The  $K^+$  ion is taken as unity and selectivity ratios are calculated from the association energies given in Figure 4.9, divided by 2 (see Section 4.4A).

1 ( $HIO_3$ ) to 11 ( $HPO_4$ ). Kossiakoff and Harker (1938) showed that variation in the nonexchange electrostatic part of the interaction energy alone can produce this wide *pK* variation. Thus the dissociation of an oxyacid with a *pK*-value between 1 and 11 may involve little additional contribution due to variation of the exchange energy. Bregman (1953) has shown that the sulfonic ion exchange resin (which has an average *pK*-value of about 1.6 for its sulfonate radicals) selects alkali-metal ions in the order  $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$ , whereas a carboxylic resin (with a *pK*-value of about 6.0) selects them in the reverse



**Figure 4.13. THE RELATION BETWEEN THE SELECTIVITY RATIOS OF VARIOUS CATIONS AND THE  $c$ -VALUE.** The  $\text{K}^+$  ion is taken as unity and the selectivity ratios are calculated from the association energies given in Figure 4.9. Here, the anionic polarizability is  $0.876 \times 10^{-24} \text{ cm}^3$ . The assumption is made that all ions are completely associated and that there is no difference among the partition functions of the various adsorbed states (configurations) other than the configurational entropy.

order,  $\text{Li}^+ > \text{Na}^+ > \text{K}^+$  (see Chapter 9). This shows that the range of  $c$ -value variation corresponding to  $p\text{K}$ -value variation from 1.6 to 6.0 includes roughly the entire range of  $c$ -value variation for which we have made calculations. The wider range of variation that Kossiakoff and Harker demonstrated to be dependent only on electrostatic variation assures us that it is safe to assume that variation of the covalent contribution has very little effect on variation of the acid dissociation within the  $p\text{K}$  range of interest to us.

The constancy of the covalent contribution does not reflect its magnitude.

Although this constancy makes comparison between  $\Delta E$ -values calculated at varying  $c$ -values valid, the absolute value for  $\Delta E$  may not be so reliable. Neglecting the exchange energy has two untoward effects: (1) the 0-configuration is de-emphasized, and (2) the association energy is reduced. Thus, if we were to evaluate it properly for a particular ionic group, the exchange energy would be added to the 0-configuration both in the calculation of the total energies of the system (step I) and in the electrostatically calculated association energy (step II). The net result of adding the exchange-energy contributions may be simulated by increasing the polarizability value  $\alpha$  of the anionic group, a change which primarily affects the 0-configuration. The result of this manipulation differs little from the change brought about by transposing the  $H^+$ -ion curve from Figure 4.10 (or even from Figure 4.11, with the higher polarizability) to Figure 4.9, without disturbing the values of the curves of the other ions.

#### D. THE STATISTICAL INTERPRETATION OF THE MEANING OF “HYDRATED IONIC RADII” IN THE CLASSICAL LYOTROPIC SERIES

At the lowest  $c$ -value, the free energy of association follows the same order as the classical lyotropic (Hofmeister) series,  $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$ ; this order was found to depict the relative effectiveness of the action of these ions on colloidal systems as well as their mobilities in dilute aqueous solution (Hofmeister, 1888; Höber, 1945). From the point of view of the present theory, this is due entirely to the statistical nature of the number of water molecules found between the cation and the fixed anion. Thus if the same number of water molecules intervenes between the different cations and the same anion, the cation with the smallest crystal radius will always have the highest free energy of association. In fact, however, at the lowest  $c$ -value, the largest ion ( $Cs^+$ ) has the highest energy of association because, statistically, it prefers to have fewer water molecules between it and the anion.

The water molecules between the cation and the anion clearly do not belong, as hydration water, to either the cation or the anion, exclusively. If we arbitrarily assign these water molecules to the cation only, the statistical nature of the water of hydration would explain the fact that a hydrated ion may have a radius only a fraction of an angstrom unit larger than its crystal radius although the water molecule has a diameter of  $2.7\text{\AA}$ . To calculate the hydrated diameter of a cation, one must include the crystal radius of the cation plus the statistical number of water diameters. For  $c = -5.0\text{\AA}^*$  and  $\alpha = 0.876 \times 10^{-24} \text{ cm}^3$ , given  $2.7\text{\AA}$  as the diameter of each water molecule, the theoretical curve would give the hypothetical hydrated cation diameters:  $Li^+$ ,  $8.57\text{\AA}$ ;  $Na^+$ ,  $7.10\text{\AA}$ ;  $K^+$ ,  $6.67\text{\AA}$ ;  $Rb^+$ ,  $5.64\text{\AA}$ ; and  $Cs^+$ ,  $3.52\text{\AA}$ .

\* The hydrated radii of dissociated cations in free solution correspond to the case in which  $c \rightarrow -\infty$ . To approximate this condition the lowest value of  $c$  equal in our calculation to  $-5.0\text{\AA}$  has been chosen for illustration.

The order of this series is the same as that in the classical lyotropic series. As the *c*-value changes, the relative preference for different counterions also changes. This variation includes both a reversal of preference for one or another of a pair of cations (for example, a system may go from a class for which  $\text{Na}^+/\text{K}^+ < 1$  to a class for which  $\text{Na}^+/\text{K}^+ > 1$ ), and a wide variation in the selectivity coefficient within any class of preferences. Thus, in a mixture of  $\text{Na}^+$  and  $\text{K}^+$  at a given *c*-value (let it fall within the range that gives  $\text{Na}^+/\text{K}^+ < 1$ ) the counterion population would be represented by both  $\text{K}^+$  ions and  $\text{Na}^+$  ions in all configurations. However,  $\text{K}^+$  ions would be selectively accumulated because statistically more  $\text{Na}^+$  ions would assume higher configurations than  $\text{K}^+$  ions and consequently the  $\text{K}^+$  ion would have a higher association energy. Conversely, there are high *c*-values at which there is no water between most of the  $\text{Na}^+$  ions and the fixed anions (configuration 0), while  $\text{K}^+$  ions remain mostly in configurations I and II. The selectivity coefficient for  $\text{Na}^+/\text{K}^+$  would be very much higher ( $\text{Na}^+/\text{K}^+ \gg 1$ ) than at the *c*-values discussed above. At very high *c*-values, both  $\text{K}^+$  and  $\text{Na}^+$  assume the 0-configuration and the absolute energies of association are much higher; however, the  $\text{Na}^+/\text{K}^+$  ratio again decreases because the interconfigurational advantages have vanished. But the ratio cannot fall to unity because the crystal radius of  $\text{Na}^+$  is smaller than that of  $\text{K}^+$ .

#### E. THE IMPORTANCE OF THE PHYSICAL PROPERTIES OF WATER

It is perhaps obvious that the physical properties of the water molecules intervening in the various configurations are of critical importance because of their effect on short-range interactions. Thus the substitution of deuterium oxide ( $\text{D}_2\text{O}$ ) for water ( $\text{H}_2\text{O}$ ) affects acid dissociation constants, as mentioned in the introduction, and has a profound effect on the *c*-value versus total-potential-energy plots of the various ions in Figures 4.6 to 4.8. Such changes in interaction energies are the basis of the functional and morphological changes of cells kept in  $\text{D}_2\text{O}$  media, discussed on page xxviii.

#### F. THE CRITICAL IMPORTANCE OF AN OPTIMAL MICROCELL SIZE

Very high selectivity ratios have been calculated for the ratios of  $\text{K}^+$ ,  $\text{NH}_4^+$ ,  $\text{Rb}^+$ , or  $\text{Cs}^+$  to  $\text{Na}^+$  or  $\text{Li}^+$  at various *c*-values. The reason is that at these *c*-values,  $\text{Li}^+$  and  $\text{Na}^+$  ions prefer high configurations, whereas  $\text{K}^+$ ,  $\text{NH}_4^+$ ,  $\text{Rb}^+$ , and  $\text{Cs}^+$  ions prefer low configurations. A high configuration subsumes a sufficient microcell volume to permit a sizable number of water molecules around the ion pairs. If the fixed ionic sites are too close together, the preference for the higher configurations by, say,  $\text{Na}^+$  or  $\text{Li}^+$ , is lost and the selectivity ratio falls. If the microcell is excessively large, the counterions will be dissociated and there will then be a loss of ionic selectivity. Thus, for optimal selectivity, a microcell is needed in which, for the particular association energies involved, there will be both

a high degree of ionic association and sufficient water to allow the assumption of the higher configurations. It seems that nature has anticipated this need by providing living cells with an average microcell 20 $\text{\AA}$  in diameter, and by providing effective charge fixation with a profusion of anchoring sites in the form of H-bonding and ionic groups.

### G. THE OPTIMAL *c*-VALUE FOR MAXIMUM SELECTIVITY

Given an optimal microcell size, one can calculate the theoretical selectivity ratios for a particular pair of ions from the association energies given in Figures 4.9 to 4.11. Results of such calculations are illustrated in Figure 4.13, which demonstrates that for the maximal ratio of, say,  $\text{Na}^+/\text{K}^+$ , a particular range of optimal *c*-values exists. Variation in *c*-value beyond this range in either direction causes a decline in selectivity.

## B I B L I O G R A P H Y

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# 5

## THE TRANSMISSION, AMPLIFICATION, AND MIXING OF BIOLOGICAL SIGNALS AT THE MOLECULAR LEVEL

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### **5.1. The *F*-Effect**

In the definition of the *c*-value (Section 4.2A), attention was drawn to the inductive *I*-effect and the direct *D*-effect, together called the *F*-effect. These effects determine the change in the *c*-value of a substituent group of a molecule pro-

Acid	Log <i>K</i> , observed	Log <i>K</i> , calculated
Cl <sub>3</sub> CCOOH.....	strong	-0.4
F <sub>2</sub> CHCOOH.....	-1.1	-1.2
ClCH(COOH) <sub>2</sub> .....	-1.4	-1.4
Cl <sub>2</sub> CHCOOH.....	-1.3	-1.4
CH <sub>3</sub> CBr <sub>2</sub> COOH.....	-1.5	-1.7
COOHCHBrCHBrCOOH.....	-1.4	-1.8
(CH <sub>3</sub> ) <sub>2</sub> <sup>+</sup> NHCH <sub>2</sub> COOH.....	-1.9	-1.9
(CH <sub>3</sub> ) <sub>3</sub> <sup>+</sup> NCH <sub>2</sub> COOH.....	-1.8	-1.9
Cl <sub>3</sub> CCH(OH)COOH.....	-2.3	-2.0
C <sub>6</sub> H <sub>5</sub> CHClCOOH.....	-2.4	-2.4
NCCH <sub>2</sub> COOH.....	-2.4	-2.4
COOHCH <sub>2</sub> CHBrCOOH.....	-2.3	-2.4
C <sub>6</sub> H <sub>5</sub> CHBrCOOH.....	-2.5	-2.6
FCH <sub>2</sub> COOH.....	-2.7	-2.6
COOHCH(OH)CH(OH)COOH (racemic).....	-3.0	-2.7
ClCH <sub>2</sub> COOH.....	-2.8	-2.7
C <sub>6</sub> H <sub>5</sub> CH <sub>2</sub> CHClCOOH.....	-2.6	-2.7
CH <sub>2</sub> (COOH) <sub>2</sub> .....	-2.8	-2.8
CH <sub>3</sub> CH(COOH) <sub>2</sub> .....	-3.0	-2.9
C <sub>6</sub> H <sub>5</sub> CH <sub>2</sub> CHBrCOOH.....	-2.8	-2.9
BrCH <sub>2</sub> COOH.....	-2.9	-2.9
CO <sub>2</sub> CHBrCHBrCOOH.....	-2.8	-2.9
(CH <sub>3</sub> ) <sub>2</sub> C(COOH) <sub>2</sub> .....	-3.2	-3.0
C <sub>6</sub> H <sub>5</sub> OCH <sub>2</sub> COOH.....	-3.1	-3.1
ICH <sub>2</sub> COOH.....	-3.0	-3.2
C <sub>6</sub> H <sub>5</sub> CH(OCH <sub>3</sub> )COOH.....	-3.1	-3.3
H <sub>2</sub> C=CHCH(OH)COOH.....	-3.3	-3.3
S(CH <sub>2</sub> COOH) <sub>2</sub> .....	-3.3	-3.4
HOCH <sub>2</sub> CH(OH)(COOH).....	-3.6	-3.5
CH <sub>3</sub> COCH <sub>2</sub> COOH.....	-3.6	-3.5
NO <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> COOH.....	-3.8	-3.6
HOCH <sub>2</sub> COOH.....	-3.8	-3.7
H <sub>3</sub> N <sup>+</sup> CH(CO <sub>2</sub> )CH <sub>2</sub> COOH.....	-3.8	-3.8
COOHCH <sub>2</sub> CH <sub>2</sub> COOH.....	-4.2	-3.9
HSCH(C <sub>2</sub> H <sub>5</sub> )COOH.....	-3.7	-3.9
Cl(CH <sub>2</sub> ) <sub>2</sub> COOH.....	-4.0	-4.0
COOHCH(CH <sub>3</sub> )CH(CH <sub>3</sub> )COOH (m.p. 209°C).....	-3.8	-4.0
BrCH <sub>2</sub> CH <sub>2</sub> COOH.....	-4.0	-4.1
<i>m</i> -ClC <sub>6</sub> H <sub>4</sub> CH <sub>2</sub> COOH.....	-4.1	-4.1
<i>p</i> -ClC <sub>6</sub> H <sub>4</sub> CH <sub>2</sub> COOH.....	-4.2	-4.2
C <sub>6</sub> H <sub>5</sub> OCH <sub>2</sub> CH <sub>2</sub> COOH.....	-4.3	-4.2
CH <sub>3</sub> COCH <sub>2</sub> CH <sub>2</sub> COOH.....	-4.6	-4.3
C <sub>6</sub> H <sub>5</sub> CH <sub>2</sub> COOH.....	-4.3	-4.3
CO <sub>2</sub> CHBrCH <sub>2</sub> COOH.....	-4.4	-4.3
COOHCH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> COOH.....	-4.3	-4.4

Table 5.1 (Continued on facing page)

Acid	Log $K$ , observed	Log $K$ , calculated
$\text{CH}_2=\text{CHCH}_2\text{COOH}$ .....	-4.4	-4.4
$\text{Cl}(\text{CH}_2)_3\text{COOH}$ .....	-4.5	-4.6
$\text{Br}(\text{CH}_2)_3\text{COOH}$ .....	-4.6	-4.6
$(\text{C}_6\text{H}_5\text{CH}_2)_2\text{CHCOOH}$ .....	-4.6	-4.6
$m\text{-ClC}_6\text{H}_4\text{CH}_2\text{CH}_2\text{COOH}$ .....	-4.6	-4.6
$p\text{-ClC}_6\text{H}_4\text{CH}_2\text{CH}_2\text{COOH}$ .....	-4.6	-4.6
$\text{C}_6\text{H}_5\text{CH}_2\text{CH}_2\text{COOH}$ .....	-4.7	-4.6
$\text{H}_2\text{C}=\text{CHCH}_2\text{CH}_2\text{COOH}$ .....	-4.7	-4.7
$\text{CH}_3\text{CO}(\text{CH}_2)_3\text{COOH}$ .....	-4.7	-4.7
$\text{CH}_3\text{CH}_2\text{COOH}$ .....	-4.9	-4.9
$(\text{CH}_3)_3\text{CCOOH}$ .....	-5.0	-5.1
$\bar{\text{CO}_2}\text{CH}_2\text{CH}_2\text{COOH}$ .....	-5.5	-5.3
$\bar{\text{CO}_2}\text{CH}(\text{CH}_3)\text{CH}(\text{CH}_3)\text{COOH}$ .....	-5.9	-5.5
$\bar{\text{CO}_2}\text{CH}_2\text{COOH}$ .....	-5.7	-5.6
$\bar{\text{CO}}\text{CH}(\text{CH}_3)\text{COOH}$ .....	-5.8	-5.7
$\text{H}_3\overset{+}{\text{N}}(\text{CH}_2)_4\text{COOH}$ .....	-4.2	-4.7
$\bar{\text{CO}_2}(\text{CH}_2)_4\text{COOH}$ .....	-5.5	-5.2
$\text{COOHC}(\text{C}_2\text{H}_5)_2\text{COOH}$ .....	-2.1	-3.0
$\bar{\text{CO}_2}\text{C}(\text{C}_2\text{H}_5)_2\text{COOH}$ .....	-7.3	-5.9
$\text{H}_3\overset{+}{\text{N}}\text{CH}_2\text{COOH}$ .....	-2.3	-1.9
$\text{CH}_3\text{SO}_2\text{CH}_2\text{COOH}$ .....	-2.3	-0.8
$\text{HCOOH}$ .....	-3.8	-4.4

**Table 5.1.** DISSOCIATION CONSTANTS OF FATTY ACIDS. The experimentally derived constants ( $-\log K = pK$ ) are compared with theoretical values calculated on the basis of an induction model. Table from Branch and Calvin (1941).

duced when another spatially separated substituent is varied. In this chapter, we shall develop the theory that an *F*-effect, produced not by substitution, but by adsorption, forms the foundation for the transmission and amplification as well as for the mixing of biological signals at the molecular level.

As early as 1911, Derick\* calculated the theoretical dissociation constants of fatty acids, applying the concept that a substituent group creates a change in the acid dissociation constant. The magnitude of this change is specific for the substituent group and decreases proportionally as more atoms separate the group from the acid. The success of this approach is illustrated by an extension of the formulation of Derick made by Branch and Calvin (1941). The data of Branch

\* G. N. Lewis (1916, 1923), Lucas and Jameson (1924), Ingold (1953), and others more explicitly developed the induction theory.

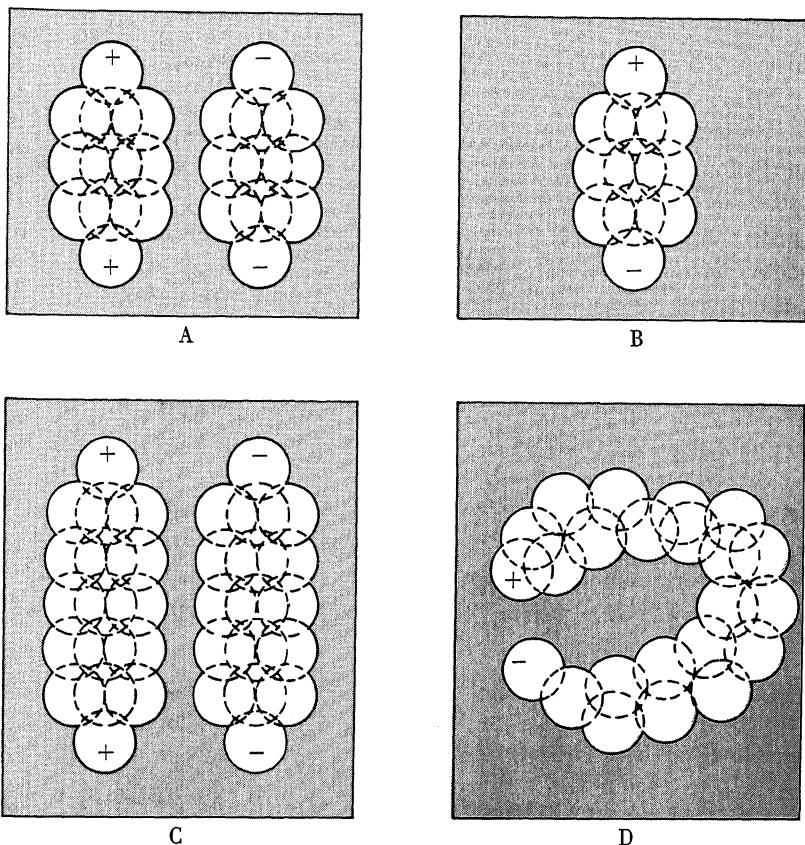
and Calvin (Table 5.1) show that of 67 fatty acids and derivatives studied, only eight yielded a difference exceeding 0.3 pH units between the experimentally and theoretically derived *pK* values.

More detailed studies have been made of the effect of the substitution of a second acid group onto a fatty acid; that is, a dicarboxylic acid. Bjerrum (1923b) suggested that the ratio of the two dissociation constants of a dicarboxylic acid is determined by a statistical factor and an electrostatic effect. The electrostatic effect was calculated on the basis of the simplifying assumption that both the proton and the carboxyl groups are immersed in a homogeneous medium with a dielectric constant of 81. The ratio of the two dissociation constants thus calculated agreed very well with the observed values for molecules in which two acidic groups are far apart; but it was too low in shorter molecules. Kirkwood and Westheimer (1938a,b) then developed a model based on a different interpretation of the dielectric constant of the medium.

A dielectric decreases the field strength between charged condenser plates immersed in it because the electric field, acting on the dielectric molecules, induces dipoles (distortion polarization) and aligns the permanent dipoles (orientation polarization), thereby creating a counter electric field. The net field strength acting on a test charge placed between the condenser plates is the vector sum of the original field and the counterfield. Water with its high dipole moment and polarizability has a dielectric constant of 81 and creates a high counter electric field; the effective field strength of a charged condenser plate or ionized molecule immersed in water is reduced to  $\frac{1}{81}$  of the effective field strength of the same plate or ion in a vacuum.

In the Kirkwood and Westheimer model, a cavity of low dielectric constant encloses both the carboxyl group and the ionizing proton; the chosen dielectric constant of 2.00 is close to that of liquid paraffin hydrocarbons. On the basis of this model, these authors were able to make better predictions than Bjerrum had obtained for small molecules such as aliphatic dicarboxylic acids and short-chained chloroacids. The treatment of Kirkwood and Westheimer differs from that of Bjerrum in that it assumes that for small molecules the space of shortest separation through which the substitution effect is transmitted can be regarded as filled with hydrocarbon; that is, transmission occurs through the carbon chains themselves. The relevance of this model decreases as the distance separating carboxyl groups increases and the chain is better able to bend on itself; when a flexible molecule bearing a positive charge on one end and a negative charge on the other is placed in a medium of low ionic strength the electrostatic attraction between the two ends causes the molecule to assume its most probable curved configuration.

In such a curved configuration, the space of shortest separation is no longer necessarily that enclosing the liquid hydrocarbon dielectric (see Figure 5.1). The substitution effect may be transmitted primarily through the medium; in



**Figure 5.1. THE EFFECT OF THE BENDING OF MOLECULES ON THE MINIMUM DISTANCE OF SEPARATION BETWEEN POLAR GROUPS.** For short-chain molecules bearing similarly or oppositely charged groups at either end (A, B) and for long-chain molecules bearing similar charges at either end (C), there is justification for the assumption that the direct electrostatic effect is mediated through a medium of dielectric constant corresponding to that of the chemical constitution of the middle portion of the molecule (indicated by the unshaded space). In the case of a flexible long-chain molecule bearing opposite charges at either end, the assumption is no longer valid, due to the bending of the molecule (D).

this case, the degree of bending and the proximity of the charged groups should greatly influence the acid dissociation constant. Because this bending and approximation are electrostatic in origin, the experimenter can vary them by changing the ionic strength of the medium. Such changes in ionic strength should be followed by significant changes in the dissociation constants. However, in Section 5.2, we demonstrate that large variations of the ionic strength of the medium have little effect on the relative values of the dissociation constants of certain large amphoteric molecules, polyglycine, for example. These results indicate

that the applicability of the Kirkwood and Westheimer model is limited to molecules in which the distance separating the interacting groups is small.

It should now be clear that we have adopted the concept of the *F*-effect, which includes both the direct electrostatic *D*-effect and indirect inductive *I*-effect, because it leaves nothing unaccounted for, whichever condition may prevail. In later chapters, we shall show that although the *D*-effect is significant in the semifixed-charge system of protein solutions, the inductive component uniquely distinguishes biological interactions from those of the nonliving world.

Discussions of the *I*- and *D*-effects in organic chemistry have usually concerned themselves with the consequences of alterations of molecular structure through group substitution, addition, or removal; these are all changes within the molecule itself. A study of the basic mechanism of the *F*-effect, however, indicates that any group which becomes closely associated with a molecule can have a similar effect. The theory developed for protein interaction in this monograph involves the notion of adsorptive induction; this is an extension of the Lewis-Ingold concept of inductive and direct effects to protein macromolecules. To understand this mechanism, it is necessary to recognize the significance of the proposition that charge fixation leads to greatly increased ionic association. Without ionic association, adsorptive induction is not possible.

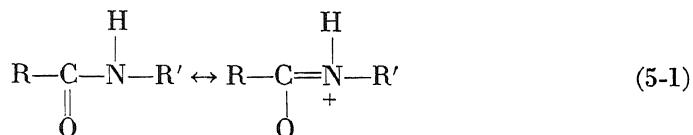
## 5.2. The Transmission of the *F*-Effect from One Functional Group to Another Within a Protein Molecule

We have noted that a charged substituent group can effect a change in the acid dissociation constant of a fatty acid. In the contrasted cases, trichloroacetic acid and acetic acid, one carbon atom separates the substituted chlorine atoms from the affected  $\text{COO}^-$  group. The transmission of the *F*-effect is not limited to such short distances; it may be transmitted over much longer chains of saturated hydrocarbons. As an example we examine the data of Peek and Hill (1951), who measured the acid dissociation constants of dicarboxylic saturated fatty acids in 20 per cent methanol in water.\* They found that tetradecanedioic acid with 14  $\text{CH}_2$  groups intervening between the two carboxyl groups has *pK* values of 5.91 and 5.10, the dissociation of the first proton being easier than that of the second. Thus the addition of a proton to a carboxyl group significantly alters the dissociation energy of a second carboxyl group 14  $\text{CH}_2$  groups removed. After the subtraction of an entropy-term contribution (Bjerrum, 1923b), this alteration corresponds to a change in the dissociation energy of the second carboxyl group of about 0.29 kcal/mole. Similarly, the titration of one  $\text{NH}_3^+$  group on octylene diamine,  $\text{NH}_3^+(\text{CH}_2)_8\text{NH}_3^+$ , changes the dissociation constant of the

\* Peek and Hill interpreted their results as support for the Kirkwood-Westheimer theory.

second  $\text{NH}_3^+$  group by about 0.41 kcal/mole (Rometsch *et al.*, 1951). In Chapter 4, we demonstrated that a difference of this magnitude in the free energy of binding could significantly alter the relative affinity of the carboxyl group for cations like  $\text{K}^+$  and  $\text{Na}^+$  if these acid groups are part of a fixed-charge system.

Peptide linkages resonate according to the following scheme:



(Mizushima, 1954, Chapter 6). The delocalized  $\pi$ -electrons of the resonating polypeptide backbone give rise to a high polarizability so that changes are transmitted more easily from one part of the molecule to another than is possible in an aliphatic hydrocarbon chain (see Edsall in Cohn and Edsall, 1943, p. 134). Edsall and Blanchard (1933) and Zief and Edsall (1937) have shown that the acid dissociation constant  $pK$  of unionized glycyl glycine,  $\text{NH}_2\text{CH}_2\text{CONHCH}_2\text{COO}^- + \text{H}^+$ , is 3.46, while the  $pK$  of the ionized glycyl glycine,  $\text{NH}_3^+\text{CH}_2\text{CONHCH}_2\text{COO}^- + \text{H}^+$ , is 3.14. This example suggests that the effect of the introduction of a proton or its removal from the amino group can be transmitted (1) through one short length of aliphatic hydrocarbon chain, (2) through a resonating peptide linkage, and then (3) through another short length of aliphatic hydrocarbon chain to effect a change of the acid dissociation constant. Stiasny and Scotti (1930) measured the  $pK$  values of both the acid and amino groups of glycine and its peptides. Their data, with the assignment of the acid and base dissociation reversed according to the modern Zwitterion concept (Adams, 1916; Bjerrum, 1923a) are listed in Table 5.2 with the later data of Czarnetzky and Schmidt (1931) on glycine. This table also includes another group of data on the peptides published by Stiasny and Scotti at a different time and quoted by Cohn and Edsall (1943). Preliminary data of Ling and Kushnir on  $pK$  values measured in 1M NaCl are presented in the third and sixth columns of the table.

The substitution of a COOH by a CONHCH<sub>2</sub>COOH group brings about a change in the strength of the amino group, although the latter is separated from the substituent group by one CH<sub>2</sub> and four peptide linkages. It can be argued that since one end of a polyglycine is positively charged and the other end negatively charged, the molecule can bend upon itself. The demonstrated effects might thus be due exclusively to a *D*-effect acting through the short distance of separation of the ends of the bent molecule rather than primarily to the *I*-effect. If this were so, the  $pK$  of the amino group would be high in glycine and would decline with the addition of more glycine residues as long as steric factors limited the bending of the peptide chain. A further increase of glycine residues would bring about a rise of the  $pK$  to a maximum, and more or less

constant value; this constant value, however, would vary greatly as the ionic strength of the medium was varied. Table 5.2 shows that the *pK* values of amino groups decline monotonically, although the carboxyl *pK* values have some up and down variation.\* The *pK* values of both the amino and the carboxyl groups in the higher polyglycine molecules approach a limiting value; this indicates a minimal interaction. Furthermore, the relative *pK* values in glycine and in glycine peptides are not materially altered when they are measured in 1*M* NaCl.

	Amino group		Carboxyl group		Water	1 <i>M</i> NaCl
	Water	1 <i>M</i> NaCl	Water	1 <i>M</i> NaCl		
NH <sub>2</sub> CH <sub>2</sub> COOH	9.70	9.60*	9.49	2.42	2.34*	3.02
NH <sub>2</sub> CH <sub>2</sub> CONHCH <sub>2</sub> COOH	8.20	8.13	8.07	3.13	3.06	3.33
NH <sub>2</sub> (CH <sub>2</sub> CONH) <sub>2</sub> CH <sub>2</sub> COOH	8.00	7.91	7.83	3.00	3.26	3.39
NH <sub>2</sub> (CH <sub>2</sub> CONH) <sub>3</sub> CH <sub>2</sub> COOH	7.75	7.75	7.93	3.05	3.05	3.50
NH <sub>2</sub> (CH <sub>2</sub> CONH) <sub>4</sub> CH <sub>2</sub> COOH	7.70	7.70		3.05	3.05	
NH <sub>2</sub> (CH <sub>2</sub> CONH) <sub>5</sub> CH <sub>2</sub> COOH	7.60	7.60		3.05	3.05	

**Table 5.2. THE DISSOCIATION CONSTANTS OF THE CARBOXYL AND AMINO GROUPS OF GLYCINE PEPTIDES.** The results of titration in water and in 1.0*M* sodium chloride in aqueous solution are given. The data in water is from Stiasny and Scotti (1930), who give two sets of values. Those values indicated by an asterisk are from Czarnetzky and Schmidt (1931). The results of titration in 1.0*M* sodium chloride are from Ling and Kushnir (to be published).

We use these observations to argue against the concept that the *D*-effect alone acts in a looping molecule and to support our contention that an *F*-effect is effectively transmitted through one CH<sub>2</sub> group and a number of peptide linkages. We note here that insertion of a CONHCH<sub>2</sub> group produces asymmetrical effects on the carboxyl group at one end of the molecule and on the amino group at the other end of the molecule. Thus the marked changes of the *pK* value of the amino groups between tetraglycine, pentaglycine, and hexaglycine are not paralleled by a similar change in the *pK* value of the carboxyl groups. This indicates the importance of the nature of the functional group in determining the transmitted effect and suggests the importance of the inductive component in the *F*-effect. The direct electrostatic effect alone would produce symmetrical free-energy changes at both the cationic and the anionic groups and is thus insufficient to explain the data of Stiasny and Scotti.

\* In our preliminary measurements we could not observe this fluctuation.

### 5.3. The Modes of Transmission and Amplification of the Direct F-Effect

*F*-effects which are transmitted through peptide linkages are examples of *direct F-effects*. There is another type of signal transmission which, though fundamentally dependent on the direct *F*-effect, involves secondary changes which allow propagation over much longer distances. We shall call this an *indirect F-effect* and the process, the *indirect F-process*. These two mechanisms underlie features that are unique to biological phenomena.

#### A. THE DIRECT *F*-EFFECT AND THE PRINCIPLE OF ADDITIVITY

Let us define  $(c_0)_0$  as the *c*-value of site  $f_0$  in the middle of a protein chain when neighboring sites bear no counterions. If now an ion  $p$  is introduced as a counterion on the neighboring site  $f_i$ , which is  $(n_0 + n_i)$  CH<sub>2</sub> groups and  $m_{0i}$  COCHNH groups removed from site  $f_0$ , a change of *c*-value at  $f_0$  is produced equal to  $\Delta_{0i}(p)$  and the *c*-value of site  $f_0$  is now  $c_0$ , given by

$$c_0 = (c_0)_0 + \Delta_{0i}(p) \quad (5-2)$$

where

$$\Delta_{0i}(p) = \pi_0 \chi^{(n_0+n_i)} \omega^{m_{0i}} I_{0i}(p) + \theta_{0i}(p). \quad (5-3)$$

In this expression,  $\theta_{0i}(p)$  is a direct electrostatic term;  $\pi_0$  may be regarded as a constant specific to each polar group which depends on its polarizability, its net charge, and other characteristics of the group;  $\chi$  and  $\omega$  represent transmissivity factors for a CH<sub>2</sub> and for a CONHCH group, respectively, and are also constants;  $I_{0i}(p)$  is the inductive factor of the adsorbent  $p$  on site  $f_i$ , and is either positive or negative, depending on the signs of the charges of sites  $f_0$  and  $f_i$ . The magnitude of the inductive factor  $|I_{0i}(p)|$  is specific to the site-adsorbent pair and not specific to either the site or the adsorbent alone. If the adsorbent  $p$  is monatomic, the inductive factor is equal to the adsorption energy ( $\Delta E_p$ )<sub>*i*</sub> on the site  $f_i$  after the appropriate sign has been added,

$$I_{0i}(p) = \beta_{0i}(\Delta E_p);$$

where  $\beta_{0i}$  is equal to +1 if  $f_0$  and  $f_i$  are of the same sign (both cationic or both anionic sites), and is equal to -1 if they are of opposite sign. If the adsorbent  $p$  has more than one locus of adsorption,  $I_{0i}(p)$  corresponds to the contribution of the association on  $f_i$  to the total adsorption energy of the species  $p$ . Taking into account all neighboring sites, each with its counterion  $p_i$ ,

$$c_0 = (c_0)_0 + \sum_i \Delta_{0i}(p_i). \quad (5-4)$$

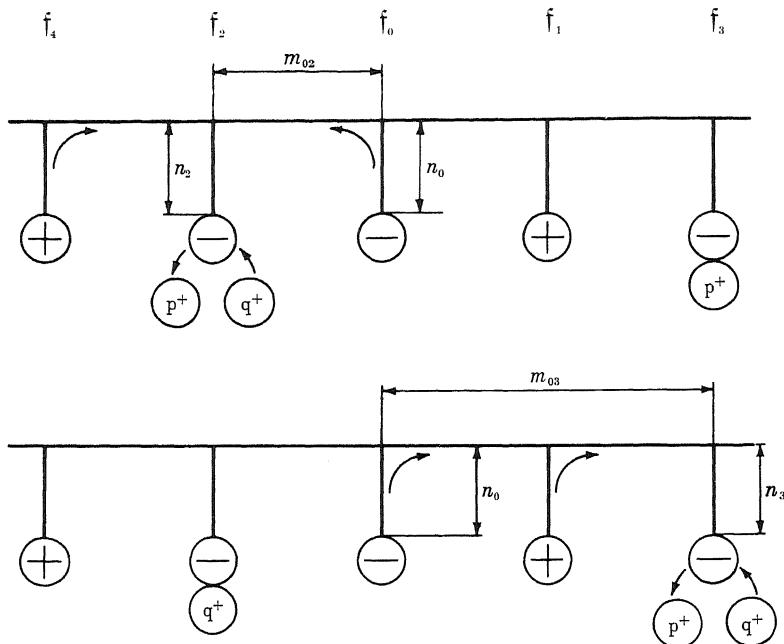
Let

$$\tau_{0i} = \pi_0 \chi^{(n_0+n_i)} \omega^{m_{0i}} \beta_{0i}. \quad (5-5)$$

Then

$$c_0 = (c_0)_0 + \sum_i \tau_{0i}(\Delta E_{pi})_i + \sum_i \theta_{0i}(p_i). \quad (5-6)$$

Equation (5-6) thus defines the additivity of the  $c$ -value shift produced by various adsorbents on different neighboring sites; it defines the  $F$ -effect, which lies at the foundation of the present theory of biological interaction.



**Figure 5.2.** A MODEL OF A SEGMENT OF PROTEIN CHAIN ILLUSTRATING THE OPERATION OF THE DIRECT  $F$ -EFFECT.

In a semifixed-charge system, the  $\theta$  term is of considerable importance. In a fixed-charge system with high  $\Delta E$  values, almost all fixed anions and cations are associated with counterions. Thus, in a fixed-charge system such as a living cell, ionic interaction usually involves the exchange of counterions rather than their complete dissociation. Such exchanges involve no net gain or loss of positive or negative particles and thus no change of the  $\theta$  term; thus in a fixed-charge system, the effect of an exchange on a neighboring site is due entirely to the inductive effect.

To illustrate the direct  $F$ -effect, let us imagine a protein molecule (Figure 5.2) bearing an anionic residue  $f_0$  with nearest neighboring ionic residues  $f_1$  and  $f_2$ , next nearest neighbors  $f_3$  and  $f_4$ , and so on. Now let us consider the effects on

site  $f_0$  of counterion exchanges at these neighboring sites. We introduce into this system a strongly bound counterion  $q$  which may couple with any one of the anionic sites. Let us suppose that the free energy of adsorption of  $q$  is such that when in limited supply it will adsorb predominantly onto site  $f_2$ , displacing a less electronegative ion  $p$ , which is originally adsorbed at this site. Electrons will be drawn away from  $f_0$  by the  $I$ -effect and the value of  $c_0$  will thus be decreased. The change of  $c$ -value at site  $f_0$  due to the interchange of counterion  $q$  for  $p$  at site  $f_i$  is given by

$$\Delta_{0i}(q|p) = \Delta_{0i}(q) - \Delta_{0i}(p) = \tau_{0i}[(\Delta E_q)_i - (\Delta E_p)_i]. \quad (5-7)$$

If more  $q$  becomes available, it will be adsorbed on other sites, say  $f_3$ , with further drawing of electrons from neighboring groups, and further decrease the  $c$ -value of  $f_0$ . Letting  $c_0^{(1)}$  and  $c_0^{(2)}$  represent the  $c$ -value of site  $f_0$  before and after the introduction of  $q$ , we have,

$$c_0^{(2)} = c_0^{(1)} + \sum_i \tau_{0i}[(\Delta E_{qi})_i - (\Delta E_{pi})_i]. \quad (5-8)$$

For a relatively small change of  $c$ -value, the  $\Delta E$ -versus- $c$ -value relation is linear (Figures 4.9 to 4.11), and we can write

$$(\Delta E_{qi})_i = A_{qi}c_i + B_{qi}$$

where  $A_{qi}$  and  $B_{qi}$  are constants. Equation (5-8) may now be written as

$$c_0^{(2)} = c_0^{(1)} + \sum_i \tau_{0i}[(A_{qi} - A_{pi})c_i + (B_{qi} - B_{pi})]. \quad (5-9)$$

Since more than one spatially separated group may be affected by a single exchange adsorption, the direct  $F$ -effect produces both amplification and transmission. The magnitude of the amplification and the effective range of transmission brought about by the direct  $F$ -effect are, however, relatively small when compared with those of the indirect  $F$ -effect. The direct  $F$ -effect, therefore, is regional in character.

## B. THE INDIRECT $F$ -EFFECT AND ITS ZIPPERLIKE ALL-OR-NONE ACTION

A more varied and extensive effect may be brought about by the indirect  $F$ -process (Figure 5.3). Let us assume that a protein fixed-charge system is in equilibrium with a solution that contains two species of free anions  $R^-$  and  $Y^-$  and three species of cations  $A^+$ ,  $B^+$ , and  $G^+$ . Let us also assume that this protein fixed-charge system contains segments of polypeptide chain on which a succession of polar groups is found and that the spacing of these polar groups permits the exchange of the counterion on one site to influence the  $c$ -value of at least the next nearest neighbor. Such an array of sites will be called a "gang" of sites. At

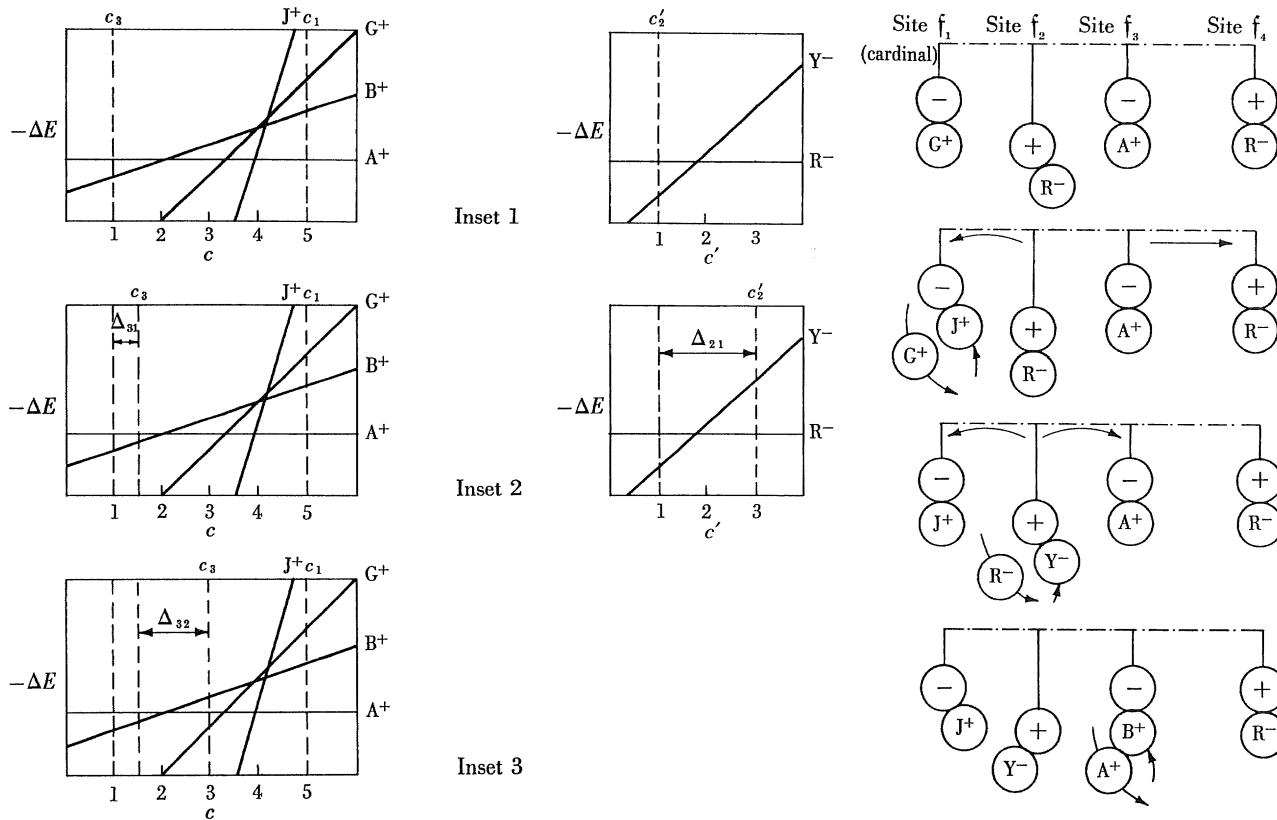


Figure 5.3. A MODEL OF A LENGTH OF PROTEIN CHAIN ILLUSTRATING THE OPERATION OF AN INDIRECT F-PROCESS.

the beginning, the fixed cationic and anionic sites ( $f_2$  and  $f_3$ ), with  $c$ - and  $c'$ -values shown in inset (1) of Figure 5.3, are coupled with  $R^-$  and  $A^+$ , respectively. Now a strongly adsorbed cation  $J^+$  is brought into the system and displaces  $G^+$  originally adsorbed on site  $f_1$  of high  $c$ -value; this causes a shift of electronic charge in the nearby region. By the direct  $F$ -effect, a  $c'$ -value shift from the value of 1 to 3 occurs at site  $f_2$ . This will shift the fixed group  $f_2$  from preferring  $R^-$  over  $Y^-$  to preferring  $Y^-$  over  $R^-$  as the  $\Delta E$  change shown in inset (2) of Figure 5.3 shows. In consequence the fixed cationic group  $f_2$  will now give up its counteranion  $R^-$  and take up  $Y^-$ . We may expect that the binding of  $Y^-$ , with its increased interaction energy, will lead to a further increase of  $c'$ -value of site  $f_1$ , reinforcing the effect of the original  $J^+|G^+$  exchange,\* and to an increase in the  $c$ -value of fixed anionic site  $f_2$  changing its  $c$ -value from 1 to 3. Site  $f_3$  will give up its counterion  $A^+$  in exchange for  $B^+$  and increase the  $c$ -value of a cationic site  $f_4$ . The process may then go on a number of steps beyond this.

The indirect  $F$ -effect has four distinct and important characteristics:

- (1) The distance of propagation and hence the transmission and amplification factors are not limited as are those of the direct  $F$ -effect. Its propagation proceeds in a zipperlike fashion.
- (2) Its action is all-or-none.
- (3) In its mechanism, an interchange of counterions involves successive jumps over activation-energy barriers; therefore, it is temperature sensitive.
- (4) For the same reason, it is also time dependent.

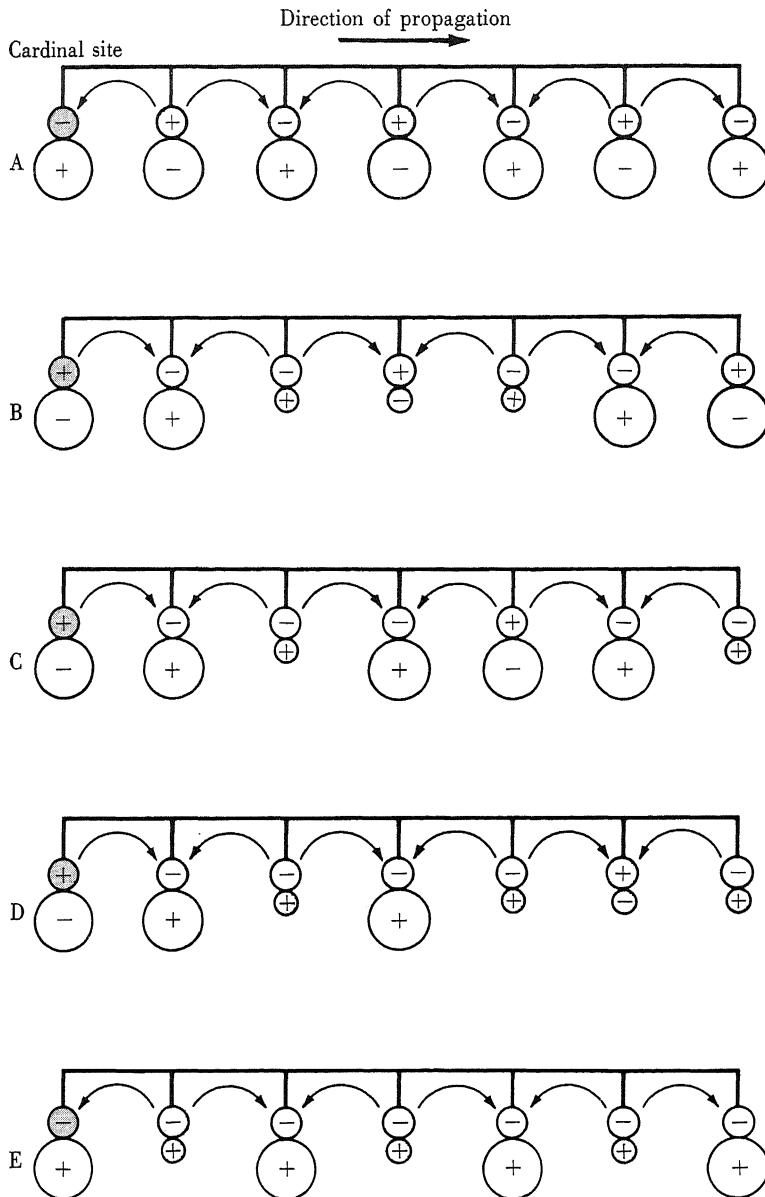
If, in the initial state, a polar group of one sign forms a salt linkage with a polar group of opposite sign on another peptide chain, or if there are backbone-to-backbone H-bonds between neighboring protein molecules, then the indirect  $F$ -effect should be transmitted to a neighboring protein molecule.

### C. VARIATIONS OF THE DIRECT AND INDIRECT $F$ -EFFECTS

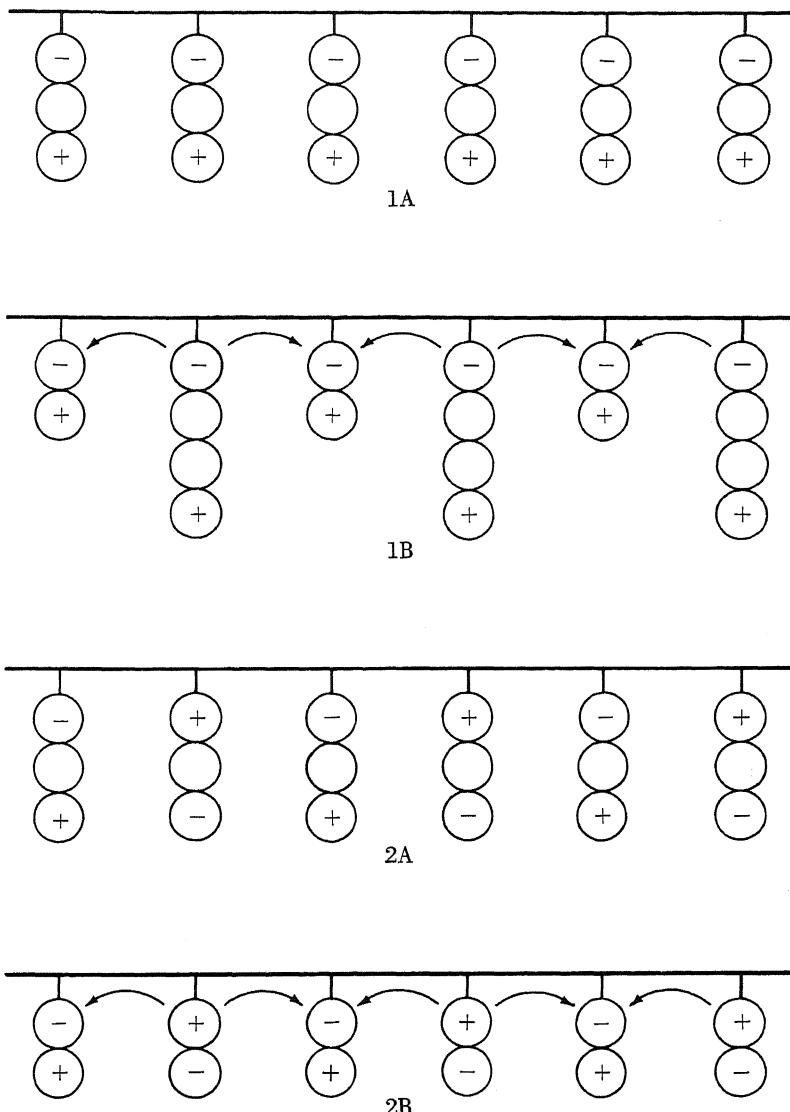
If we liken the 21 or more common amino acids to the 26 letters of the alphabet, we can infer that the variety in living phenomena, like the variety in language and expression, must be due to the large number of possible permutations of these amino-acid residues in the protein chain, as well as to the variety of possible combinations.

Figure 5.4 presents several hypothetical polypeptide chains containing polar groups separated from each other by not more than the maximum distance for the transmission of an  $F$ -effect. Such a chain of sites, called a gang of sites, may constitute a whole protein molecule or a part of it. Let us assume that the polypeptide chains are in equilibrium with a large array of free anions and cations other than those coupled with the polar side chains and that, for every site, the

\* The exchange at site  $f_2$  may act reciprocally on site  $f_1$  by doing more than just reinforcing the original  $J^+|G^+$  exchange. This is fully discussed in Section 6.3B.



**Figure 5.4.** MODELS OF HYPOTHETICAL GANGS OF SITES. It is assumed that the gang of sites is in equilibrium with a large variety of free anions and cations and that for every site every *c*-value change is sufficient to bring about an exchange of counterions.



**Figure 5.5. MODELS OF A SEGMENT OF PROTEIN CHAIN ILLUSTRATING THE OPERATION OF AN INDIRECT *F*-PROCESS.** Two models are presented: 1A and 1B represent a purely anionic chain; 2A and 2B, an alternately cationic and anionic chain. In each case, only one species each of counterion and counteranion (those with high *c*- and *c'*-value analogues) are present. The empty circles represent intervening water molecules.

$c$ - or  $c'$ -value change is sufficient to bring about an exchange of counterions. From the principle of the indirect  $F$ -effect, we theorize that:<sup>\*</sup> (A) If alternate sites bear different signs, an increase of  $c$ -value in one will tend to increase all  $c$ - and  $c'$ -values; a lowering will tend to decrease all  $c$ - and  $c'$ -values. (B) If the pattern is  $+ - - + - - +$ , an increase of  $c$ -value by the trigger adsorption of a strongly adsorbed anion to the first cation will lead to (1) a decreased  $c'$ -value in half of the remaining cationic groups with an increased  $c$ -value in the other half, and (2) an increased  $c$ -value in half of the anionic groups with a decrease in the other half. (C) If the pattern is  $+ - - - + - - - +$ , a similar trigger adsorption will lead to (1) an increase of the  $c'$ -value of all the fixed cation groups, (2) an increase of  $c$ -value in two-thirds of the anionic groups and a decrease in one-third of the anionic groups. (D) If the pattern is  $+ - - - - + - - - -$ , the trigger adsorption will lead to (1) a fall in half of the  $c'$ -values and a rise in the other half, (2) a  $c$ -value fall in half of the fixed anionic charges and a rise in the other half. (E) If successive sites all bear the same charge, a trigger adsorption will cause half of the neighboring charges to undergo an increase in  $c$ - or  $c'$ -value; the other half will suffer a decrease in  $c$ - or  $c'$ -value.

This analysis is an example of the variety of responses to a trigger adsorption made possible by differences in the order of arrangement of positively and negatively charged ions. The same trigger action may lead to a  $c$ - and  $c'$ -value rise or fall in some polar groups, with a concomitant fall or rise in others. In the cases analyzed, all may be reduced to pairs and triads with such succession as:  $--$ ,  $++$ ,  $- - -$ ,  $++ +$ , on the one hand, or  $- + -$ ,  $+ - +$ , on the other.

As Figure 5.4 shows, one criterion that determines the feasibility of propagation is the presence and availability of free counterions of both higher and lower adsorption energies than those already in association with the sites in question. In fact, this requirement does not necessarily exist. Under certain conditions, a single species of ion, like  $\text{Li}^+$  or  $\text{Na}^+$ , may, by assuming different configurations (see Section 4.2B), have the choice of a variety of adsorption energies and thus provide a means of expression of the indirect  $F$ -effect as though different ions were present. The mode of operation of a single ion species is shown in Figure 5.5.

#### 5.4. Autocooperative and Heterocooperative Interactions

We have shown that a change of the signs of polar side chains in a gang of sites can produce variations of the indirect  $F$ -process. This is due to the fact that the sign of the polar group of a neighboring site is one of the major factors that determine the nature of the direct  $F$ -effect. Thus, in a gang of uniformly dis-

\* We have considered the initial step as a change for a more strongly bound counterion; the converse exchange will bring about changes in the opposite direction.

tributed sites the adsorption of an entity A on one site may change the relative preference of a neighboring site for entities A and B in favor of A. We say that this type of interaction is autocooperative. In another system the adsorption of A on one site changes the relative preference of a neighboring site for entities A and B in favor of B. This we shall designate as a heterocooperative interaction. Both types of interaction are illustrated below.

#### A. HETEROOPERATIVE INTERACTION

If a gang of sites bears similar  $c$ -values and charges in an environment that contains a large variety of possible counterions, the basic operation is heterocooperative (Figure 5.4E). The adsorption of a very strongly bound entity A decreases the  $c$ - or  $c'$ -value of its neighboring sites. At the lowered  $c$ - or  $c'$ -value, the site will prefer B more than before.

#### B. AUTOCOOPERATIVE INTERACTION

When the sites are alternately positive and negative, the system tends to become occupied either entirely by  $A^+$  and  $A^-$ , preferred at lower  $c$ - and  $c'$ -values, or entirely by  $B^+$  and  $B^-$ , preferred at higher  $c$ - and  $c'$ -values. If one regards a pair of immediately neighboring positive and negative sites as one site, the adsorption at one such site of  $A^+A^-$  or of  $B^+B^-$  will tend to cause its neighboring site also to prefer  $A^+A^-$  or  $B^+B^-$ , respectively. This is an autocooperative interaction.

We shall discuss autocooperative and heterocooperative phenomena in greater detail in Chapter 7 with regard to adsorption onto and denaturation of proteins. Since all possible site arrangements are various combinations of small groups of homogeneous cationic or anionic sites (heterocooperative), and alternatingly cationic and anionic sites (autocooperative), we can understand the properties of all types of gangs by studying the simple cases of uniformly charged gangs and gangs with alternatingly cationic and anionic sites.

### 5.5. The Interpretation of Adsorption Isotherms

As a practical example of the use of the concepts of autocooperative and heterocooperative interaction, let us briefly consider the interpretation of adsorption isotherms. In various fields of study of adsorption phenomena, an empirical relationship of the following form is frequently observed:

$$\left[ \frac{[A]_{\text{ads}}}{[B]_{\text{ads}}} \right]^{\frac{1}{n}} \left[ \frac{[B]_{\text{free}}}{[A]_{\text{free}}} \right] = K' \quad (5-10)$$

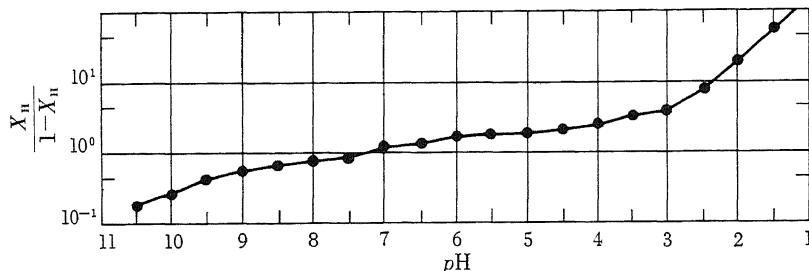
or, alternately,

$$\ln \left[ \frac{[A]_{\text{ads}}}{[B]_{\text{ads}}} \right] = n \ln \left[ \frac{[A]_{\text{free}}}{[B]_{\text{free}}} \right] + n \ln K'. \quad (5-11)$$

This equation was first applied to the study of ion exchanges in soils by Rothmund and Kornfeld (1918). It is the same as the empirical equation for the oxygen-hemoglobin combination curve derived by R. Hill and Wolvekamp (1936). We shall use this equation in a modified form that focuses attention on a single species of adsorbent,

$$\ln \left[ \frac{X_A}{1 - X_A} \right] = n \ln [A]_{\text{free}} + \text{const.} \quad (5-12)$$

where  $X_A$  and  $(1 - X_A)$  denote the mole fractions of sites occupied by A and sites otherwise occupied, respectively. The concentrations of all free species



**Figure 5.6.** A LOG-LOG PLOT OF THE THEORETICAL “TITRATION CURVE” FOR EGG ALBUMIN. This curve has been calculated from the usual  $pK$  values for the trifunctional amino acids in the protein without taking any interaction into account. Theoretical titration curve after Simms (1928). Recently Dr. Simms kindly communicated to us that the wrong value was used for the  $pK$  of arginine in the calculation of this curve; correction would lead to a slight shift of the curve in the region above  $pH$  7. Because this curve is used only to illustrate a general principle, that is, the value of  $n$  in the absence of interaction, changes in the absolute value of the points are not relevant to our argument.

except A are kept constant. When  $n$  is less than one, it may be interpreted as being due to heterogeneity in the affinity of the population of ionic adsorbing sites (see Walton, 1943; Eisenman and co-workers, unpublished; also Section 7.4A of this book). The first adsorbed A is taken up by the sites of highest adsorption energy, followed by adsorption on less preferred sites. Thus  $n < 1$  signifies that each adsorption is less favorable than the one that preceded it. When the sites belong to a few groups with sharply defined and well-separated affinities, more or less sharp bends occur in the log-log plot of the adsorption data. To illustrate this, we have made a log-log plot of the “titration curve” of Simms (1928), a theoretical curve calculated from the trifunctional amino-acid constituents of egg albumin, and the  $pK$  values usually given for these amino acids in

proteins, derived without taking interaction into account. It is obvious from Figure 5.6 that at no place does a slope exceeding one ever occur.

When  $n = 1$ , equation (5-11) reduces to the mass action relation and in some but not all cases may be interpreted to mean that each adsorption is independent of other adsorptions. By considering interaction between adsorption sites, one may conceive of an established homogeneous population in which  $n$  is not equal to, but less than, unity. This indicates that the adsorption of A decreases the free energy of adsorption of other A's on similar sites (heterocooperative interaction). On the basis of  $n$ -values alone, one cannot distinguish this type of interaction from the case in which  $n < 1$  is due to site heterogeneity. However, when  $n > 1$ , heterogeneity cannot be the cause and interaction must be indicated (see Wyman, 1948). We suggest that this type of interaction is due to the  $F$ -effects (autocooperative interaction). An explicit equation in terms of the interaction energy between neighboring functional groups will be published separately in conjunction with Dr. C. N. Yang. In anticipation, we may say that when half of the sites adsorb A and the other half adsorb B, the slope  $n$  is a function of the interaction energy. At other points, it is not. Nevertheless, whenever the interaction is autocooperative,  $n$  will always be greater than one, and whenever the interaction is heterocooperative,  $n$  will be less than one.

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# 6

## MODULATION AND CONTROL OF PHYSIOLOGICAL ACTIVITY

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We have thus far concentrated on the fundamental mechanisms, the direct and indirect *F*-effects, and their relation to adsorption on proteins. Next we must determine how these mechanisms can form a basis for the more complex phenomena of physiological activity. Before doing this, we should state what—according to the association-induction theory—underlies the basic mechanisms of physiological behavior in general.

### 6.1. A Comparison of the Magnitude of Reversible Adsorption Energies and “High-Energy” Phosphate-Bond Energy

Physiologists and biochemists have long recognized that a living organism must derive the energy necessary for its various functions from the food it consumes. The advances made in the knowledge of the metabolic pathways during the past few decades have pointed out the pivotal position held by compounds that contain labile pyrophosphate bonds; ATP is one of these (Lipmann, 1941). The chemical energy of this “high-energy” phosphate bond is utilized in biological synthesis and chemical transformations within living cells. The demonstration that the “eventual” metabolic product ATP is also important to muscular contraction (Engelhardt and Lyubimova, 1939; Szent-Györgyi, 1951) led physiologists to suspect that the chemical energy contained in the labile pyrophosphate bond of ATP may be utilized directly in contractile and other physiological processes. There is now a large body of experimental facts which contradict this hypothesis (see Section 7.1A footnote and Chapters 8 and 15).

We suggest an alternative to the direct participation of “high-energy” phosphate-bond energy in biological work performance: This work is the expression of electronic changes—losses, gains, and above all, redistributions within the protein fixed-charge system and its adsorbents through the operation of the *F*-effects. In 1930, Meyer and Mark advanced the concept that adsorption is an important process underlying muscular contraction (see Chapter 15); Morales and Botts (1953, 1956) have followed a similar line of thought.

We have shown that adsorption permits an electrostatically active entity to be anchored and thus be made to exert an effect (*D*-effect). The significance of our thesis is that the adsorption process and the inductive effects created by adsorption form the molecular bases for a large class of physiological phenomena; muscular contraction is one example. In Chapters 8 to 18, we shall present a large and representative collection of evidence in various fields of physiological studies to support this statement. In anticipation of this, we shall demonstrate that the total storage of free energy as adsorption energy in any living cell greatly exceeds that which is present as useful chemical energy (“high-energy” phosphate-bond energy).

The classical interpretation of ionic behavior is based on the limiting law of Debye-Hückel; thus it is usually assumed that the electrostatic energy of proteins  $U_{\text{elect}}$  is given by

$$U_{\text{elect}} = \frac{Z_1 Z_2 \epsilon^2}{Dr} \quad (6-1)$$

where  $Z_1$  and  $Z_2$  are the valences;  $\epsilon$  is the electronic charge; and  $D$ , the dielectric constant of the medium, is given the macroscopic value of 81. If we assume no direct ion-ion association,  $r$  is related to the Debye radius of the ion cloud and

	Type of bond	Type of interaction	Absolute energy, kcal/mole	Relative energy, kcal/mole	Source
K <sup>+</sup> -carboxyl group	Coulombic <sup>a</sup> van der Waals <sup>a</sup>	Charge-charge interaction Charge-induced dipole (Debye energy) Charge-permanent dipole Permanent dipole-induced dipole London dispersion energy	-66.9 -2.80 -0.25 -0.14 -0.30 <hr/> Sum - 3.49 + 5.0	-14.0 <sup>b</sup>	
H-bond	H-bond energy		-4 to -8		Kortüm and Bockris, 1951
ATP	ΔH° of dephosphorylation of high-energy phosphate bond			- 4.7	Podolsky and Morales, 1956
	ΔF° of dephosphorylation of high-energy phosphate bond			- 9.2 - 7.9 - 5.6	Burton and Krebs, 1953 Levintow and Meister, 1954 Vladimirov <i>et al.</i> , 1957
	ΔF° adsorption on actomyosin			- 9.45 <sup>c</sup>	Nanninga and Mommaerts, 1957
	ΔH° adsorption on G-actin			-24	Asakura, 1961
	ΔF° adsorption on G-actin			-11 to -12	Asakura, 1961

**Table 6.1. A COMPARISON BETWEEN THE MAGNITUDES OF ADSORPTION ENERGIES AND HIGH-ENERGY PHOSPHATE-BOND ENERGY.** The absolute energy is the total interaction energy between the cation and the anion. The relative energy is the energy needed to effect a separation of the ion pair in an aqueous medium. Coulombic bonds, van der Waals' bonds, and even H-bonds are basically electrostatic in nature although the term "electrostatic energy" is often used to refer to Coulombic charge-charge interaction energy alone.

<sup>a</sup> Various interaction energies between a K<sup>+</sup> ion and an anion (*e*-value = -2.25 Å,  $\alpha = 0.876 \times 10^{-2}$  cm<sup>3</sup>) calculated in Section 4.3A.

<sup>b</sup> Largely due to the Coulombic term, see Section 4.3B.

<sup>c</sup> According to the original authors, this value may be underestimated.

is of the order of magnitude of 10 Å in a 0.1M solution such as Ringer's solution. If  $Z_1 = -Z_2 = 1$ , then  $U_{\text{elect}} = -0.4$  kcal/mole. This is a relatively small energy. It is less than the average kinetic energy possessed by a mole of single particles at room temperature (0.9 kcal/mole) and constitutes a mere fraction of the ΔH° value of a high-energy phosphate bond; this is -4.7 kcal/mole (see Table 6.1).

In Chapter 2 we showed that site fixation increases ionic association. Proteins in solution, where they are semifixed-charge systems, and particularly in living cells, where they are true fixed-charge systems, associate with ions to a high degree. For this reason, the most important electrostatic energy is not that between the ion clouds and the macroscopic electronic charge of a protein molecule, but rather, the energy that acts between each fixed ion and its counterion. In Chapter 4, we calculated the equilibrium distance between the ionic particles (equivalent to  $r$ ) and outlined the mechanism of dielectric saturation. We also calculated detailed values of other non-Coulombic electrostatic energies, which are usually lumped together as van der Waals' energies. Table 6.1 presents illustrative examples taken from these data and, for comparison, the H-bond energy and the heat of dephosphorylation of ATP. This table shows that, per "bond," the Coulombic electrostatic energy far exceeds the chemical energy of the pyrophosphate linkage of an ATP molecule when the effect of dielectric saturation is taken into account. In fact, the  $-4.7$  kcal/mole  $\Delta H^\circ$  of the phosphate linkage is no higher than that of a single H-bond.

One kilogram of living cells contains about 150 millimoles of fixed ionic site-counterion pairs and fixed ionic site-fixed ionic site pairs plus three to six moles of backbone H-bonding sites. The net content of reversible adsorption energy thus exceeds what is at best equivalent to 100 millimoles of creatine phosphate ( $\Delta H$  about equal to that of ATP) and ATP phosphate bond energy (see Table 11.2). Perhaps as significant is that the free energies of adsorption of ATP on myosin (underestimated according to authors Nanninga and Mommaerts, 1957) and on G-actin are at least twice as high as its chemical heat content.\*

Having shown that the adsorption energies in quantity are competent to perform the function that has been ascribed to the chemical-bond energy, we present some physiological behavior patterns which, on the basis of the direct and indirect  $F$ -effects, should be expected.

## 6.2. Bioregulants, Cardinal Sites, and Their Interactions

We have discussed the effect that a change of adsorbent on one site may be expected to have on the adsorption at neighboring sites. The magnitude of such an effect depends on the magnitude of the change in an inductive factor  $I_i(p)$  involved [equation (5-3)]. The inductive factor is related to the adsorption energy  $\Delta E$ . From Figures 4.9 through 4.11, it is obvious that the adsorption energy at a high  $c$ -value will generally be greater than that at a lower  $c$ -value; this is also true of the  $c'$ -value and of the  $c$ -value analogue of the counterion. Figures 4.6 to 4.8 show that at high  $c$ - and  $c'$ -values interacting particles tend to assume

\* In Chapter 15, we shall develop the concept that ATP affects the contraction of muscle models by an adsorption mechanism.

the 0-configuration (the configuration with no intervening water molecule). This greatly facilitates the transmission of *F*-effects. It follows that if occasional sites possessing higher *c*- and *c'*-values than those of other sites are distributed along a polypeptide chain, these sites will control neighboring sites which belong to the same gang (those which interact cooperatively). These high *c*-value sites are designated as *cardinal sites*.

When cardinal sites on one peptide chain associate preferentially with cardinal sites on a neighboring chain, their high *c*- and *c'*-values cause them to assume the 0-configuration; an *F*-effect affecting one such site will change the *c*-value of its partner, providing a means for intermolecular transmission.\*

We shall develop the thesis that hormones, carcinogens, drugs, some vitamins, the H<sup>+</sup> ion, and ATP affect the living system through interaction at cardinal sites. These categories of substances are all biologically active at extremely low external concentrations. Acting in small amounts, they control and regulate whole protein molecules, whole cells, whole organisms. For simplicity, we shall call substances in this class *bioregulants*. Since the bioregulants either attract or repel electrons, we classify them into two categories: (1) electropositive bioregulants which repel electrons and are exemplified by strongly adsorbed anionic reactants, and (2) electronegative bioregulants which attract electrons and are exemplified by strongly adsorbed cations.

### 6.3. Models of the Mechanics of Control of Physiological Activity

The interaction of living systems with molecules and ions leads to characteristic variations in physiological responses; these are known as activation and inhibition, synergism and antagonism. Enzymologists have generally accepted the theory of Michaelis and Menten (1913). These authors considered the system



in which E represents the enzyme, S the substrate, and P the product. Relating the rate of enzymatic activity *V* to the concentration of the enzyme-substrate complex [ES], they derived

$$V = k_3[ES] \quad (6-3)$$

$$= \frac{k_3[E]_0}{\frac{K_s}{[S]} + 1}. \quad (6-4)$$

\* On the basis of the preceding discussion, there are theoretically four modes of transmission of *F*-effects from one peptide chain to another: (1) through disulfide bridges, (2) through phosphate and pyrophosphate linkages (G. E. Perlmann, 1955), (3) through H-bonds formed between neighboring proton-donating and proton-accepting sites, and (4) through cardinal salt bridges.

Here  $[E]_0$  is the total enzyme concentration,  $K_s$  is the Michaelis-Menten constant equal to  $(k_2 + k_3)/k_1$  and  $k_1$ ,  $k_2$ , and  $k_3$  are the three specific rate constants for the reaction. Clark's theory of drug action (1926, 1933) is derived from the same principle. Clark related the effect of a drug or pharmacon (A) to the concentration of the drug-receptor complex (RA). In the terminology of Ariëns *et al.* (1956, 1957), the effect  $F_A$  of a pharmacon (A) is derived according to the equations,



$$K_A = \frac{k_2}{k_1} \quad (6-6)$$

$$F_A = \zeta [RA] \quad (6-7)$$

$$= \frac{\zeta [R]_0}{\frac{K_A}{[A]} + 1}. \quad (6-8)$$

Here  $[R]_0$  is the total receptor-site concentration and  $\zeta$  is a proportionality constant. Ariëns and co-workers introduced the term "affinity" for  $1/K_A$  and "intrinsic activity" for  $\zeta$  (in their publication they used the symbol  $\alpha$ ).

The theories of Michaelis and Menten on enzyme action and of Clark on drug action explain the direct competitive type of interaction but do not explain the abundant and important noncompetitive types of interaction (see Woolley, 1946, and Gaddum, 1957). By assuming changes of  $K_s$  [equation (6-4)] and  $K_A$  [equation (6-6)], investigators in the field of pharmacology gained important insights into the basic mechanisms (A. J. Clark, 1933; Raventos, 1937; Ariëns *et al.*, 1956, 1957).

The association-induction hypothesis for proteins offers a general theory for both competitive and noncompetitive interactions at the molecular level. The mechanism is based on the direct and indirect  $F$ -effects. The nature and magnitude of a specific response depends on the spatial distribution and  $c$ - and  $c'$ -values or their analogues of reactive groups. To illustrate the type of theoretically predictable interactions (bell-shaped curves and curves showing competitive and noncompetitive inhibition and activation) we present a few typical examples.

In consideration of the various types of interaction, we assume that a physiological process may be quantitatively represented by the mole fraction of a particular set of fixed sites occupied by a particular adsorbent. Thus the physiological effect  $\Phi$  may be represented as\*

$$\Phi = k X_{\mathfrak{X}}^f. \quad (6-9)$$

\* This assumption was made by Michaelis and Menten and by Clark. Equation (6-9) is the same as equation (6-3) of Michaelis and Menten for enzyme action and equation (6-7) of Clark for drug action. While Michaelis and Menten's equation is explicit in its mechanism, equation (6-7) of drug action applied to smooth muscle contraction is assumed *a priori* and no mechanism is proposed.

Here  $k$  is a constant, and  $X_{\mathfrak{X}}^{f_0}$  is the mole fraction of fixed sites  $f_0$  occupied by the specific adsorbent  $\mathfrak{X}$ . We emphasize that not all physiological activity may be directly represented by such a simple equation; when direct application of the fundamental equation is not valid, variations may fit the experimental conditions better. The meaning and validity of this assumption will be discussed in Chapters 8 to 18, where various physiological activities are considered.

Certain physiological processes are related to the concentration rather than the mole fraction of sites  $f_0$  adsorbing  $\mathfrak{X}$ ; equation (6-9) may then be reformulated

$$\Phi = k' X_{\mathfrak{X}}^{f_0} [f_0] \quad (6-10)$$

where  $[f_0]$  is the total concentration of sites  $f_0$ . Take enzyme action as an example;  $\Phi$  represents the rate of enzyme activity and  $X_{\mathfrak{X}}^{f_0}[f_0]$  represents the concentration of the enzymatically active sites adsorbing the substrate which forms the activated complex.

In this example, the site involved in the physiological event is an individual site. The theoretical models to be presented are directly applicable to this type of physiological activity because the fundamental operation of the direct  $F$ -effect on single sites is straightforward. However, many physiological phenomena express the behavior of groups of neighboring sites, that is, gangs of sites. In these phenomena, because of the indirect  $F$ -process, all the sites of a gang will be found either in one state or in an alternative state; the cardinal site controls this all-or-none change in a gang of sites. Such a system can thus be modulated through variation of the adsorbent at the cardinal site. Therefore, a physiological event which involves the "mole fraction" of gangs of sites rather than the mole fraction of single sites may be quantitatively expressed in terms of the mole fraction of cardinal sites binding either one of two cardinal adsorbents. The control of protein behavior through cardinal adsorption will be more fully developed in Chapter 7; in anticipation of this, we note that the bulk of physiological activity, such as muscular contraction, nerve conduction, and ionic accumulation, may be interpreted in this way.

#### A. THE ONE-RECEPTOR SYSTEM AS A MODEL FOR COMPETITIVE INTERACTION

First, let us consider a gang of two sites, one physiologically active site (0) at a certain  $c$ -value and another site (I) at a higher  $c$ -value (site I is a receptor or a cardinal site).\* We shall assume that an exchange at site I produces a significant  $c$ -value shift at site 0; exchanges at site 0 produce no significant change at site I. We also assume that two species of ions, G and J, may react with site I;

\* The choice of signs for the fixed sites and counterions in this and the following models is not unique; in each case, the same curve or set of curves would result if the signs of all charges were reversed. This point will be implicit in references to the models in later chapters.

the mole fraction of receptor sites occupied by G and J are  $X_G^{fr}$  and  $X_J^{fr}$ , respectively, and their values can be calculated. Let the  $c$ -value at site 0 be

$$c_0 = (c_0)_0 + \Delta_{0I}(G) \quad (6-11)$$

when site I is coupled with G. The  $c$ -value of site 0 when site I is coupled with J would be  $c_0 + \Delta_{0I}(J|G)$ , from equations (5-4) to (5-7). Then

$$\Phi = kX_J^{f_0} = k \left[ \frac{[\mathfrak{X}] \frac{(\text{p.f.})_{\mathfrak{X}}^{\text{ads}}}{(\text{p.f.})_{\mathfrak{X}}^{\text{ins}}} \exp ([A_{\mathfrak{X}}(c_0 + \Delta_{0I}(J|G)) + B_{\mathfrak{X}}]/RT)}{1 + \sum_p [p] \frac{(\text{p.f.})_p^{\text{ads}}}{(\text{p.f.})_p^{\text{ins}}} \exp ([A_p(c_0 + \Delta_{0I}(J|G)) + B_p]/RT)} X_J^{fr} \right. \\ \left. + \frac{[\mathfrak{X}] \frac{(\text{p.f.})_{\mathfrak{X}}^{\text{ads}}}{(\text{p.f.})_{\mathfrak{X}}^{\text{ins}}} \exp ([A_{\mathfrak{X}}c_0 + B_{\mathfrak{X}}]/RT)}{1 + \sum_p [p] \frac{(\text{p.f.})_p^{\text{ads}}}{(\text{p.f.})_p^{\text{ins}}} \exp ([A_p c_0 + B_p]/RT)} X_G^{fr} \right] \quad (6-12)$$

where the summations are taken over all counterions p which may adsorb on  $f_0$ ;  $(\text{p.f.})^{\text{ads}}$  and  $(\text{p.f.})^{\text{ins}}$  are the partition functions of the subscripted species in the associated and dissociated states, respectively;  $[\mathfrak{X}]$  and  $[p]$  represent the concentrations of the indicated species;  $\Delta_{0I}(J|G)$  is given by equation (5-3). Ariëns and co-workers, in their analyses of drug action, represent the physiological effect due to this type of competitive interaction by the expression

$$F_{GJ} = \zeta_J X_J^{fr} + \zeta_G X_G^{fr}. \quad (6-13)$$

Their intrinsic activity  $\zeta_J$  is, therefore, equal to

$$k \frac{[\mathfrak{X}] \frac{(\text{p.f.})_{\mathfrak{X}}^{\text{ads}}}{(\text{p.f.})_{\mathfrak{X}}^{\text{ins}}} \exp ([A_{\mathfrak{X}}(c_0 + \Delta_{0I}(J|G)) + B_{\mathfrak{X}}]/RT)}{1 + \sum_p [p] \frac{(\text{p.f.})_p^{\text{ads}}}{(\text{p.f.})_p^{\text{ins}}} \exp ([A_p(c_0 + \Delta_{0I}(J|G)) + B_p]/RT)}. \quad (6-14)$$

Writing  $[R]$ ,  $[A]$ , and  $[RA]$  for the concentrations of free receptor sites R, the adsorbent A, and the complex RA, respectively, we define their dissociation constant for A as

$$K_A = \frac{[R][A]}{[RA]} \quad (6-15)$$

which is obviously equal to

$$\exp (\Delta F_A/RT) = \frac{(\text{p.f.})_A^{\text{ins}}}{(\text{p.f.})_A^{\text{ads}}} \exp (\Delta E_A/RT) \\ = \frac{(\text{p.f.})_A^{\text{ins}}}{(\text{p.f.})_A^{\text{ads}}} \exp ([A_A c_R + B_A]/RT) \quad (6-16)$$

where  $\Delta E_A$  and  $\Delta F_A$  are the energy and free energy of association of A with R.

To illustrate the operation of the  $F$ -effect, we shall suppose that there are five free cations and four free anions. Their adsorption energies  $\Delta E$  (in kcal/mole) on the fixed anionic site (described by  $c$ ) and fixed cationic site (described by  $c'$ ) are given by

$$\begin{array}{ll} -\Delta E_A = 10 & -\Delta E_{A'} = 10 \\ -\Delta E_B = 0.7c + 6 & \\ -\Delta E_G = 1.6c & -\Delta E_{G'} = 1.6c' \\ -\Delta E_J = 3.0c - 12 & -\Delta E_{J'} = 3.0c' - 12 \\ -\Delta E_{\mathfrak{X}} = 0.3c + 8.4 & -\Delta E_{\mathfrak{X}'} = 0.3c' + 8.4. \end{array}$$

For simplicity, the partition function ratios  $(p.f.)_p^{ads}/(p.f.)_p^{ins}$  are given the value of unity.\* In consequence, the free energy of adsorption  $\Delta F$  is equal to the adsorption energy  $\Delta E$ . We also assume that receptor and effector sites are separated by a total of  $(1 + 2)$   $CH_2\uparrow$  groups and 2  $NHCOCH$  groups. In the initial state the system contains reactants A, B, and  $\mathfrak{X}$  at activities 0.03, 0.1, and  $0.01M$ , respectively. The ion species G and J are introduced. The receptor site I is at a  $c$ -value of 9.0; the  $c$ -value at the effector site is 7.0. From the inset in Figure 6.1 it is obvious that J will adsorb primarily onto site I and very little onto site 0. The species G will react with both site I and site 0. However, we shall assume that the reaction of G with site 0 is prevented for steric reasons.

The displacement of B by G at site I will produce a  $c$ -value shift at site 0,  $\Delta_{0I}(G|B)$ . From equation (5-3),

$$\Delta_{0I}(G|B) = \pi_0 \chi^{n_0+n_I} \omega^{m_{0I}} \beta_{0I} ([\Delta E_G]_I - [\Delta E_B]_I).$$

Taking

$$\pi_0 = 50, \quad \chi = \frac{1}{3}, \quad \omega = \frac{2}{3}, \quad \beta_{0I} = +1,$$

we find that

$$\Delta_{0I}(G|B) = -1.73 \quad \text{and} \quad \Delta_{0I}(J|B) = -2.22.$$

By substituting in the given values [ $\mathfrak{X}$ ], [B], and [A], and giving different values to G and J, we obtain from equation (6-12) the family of curves in Figure 6.1.

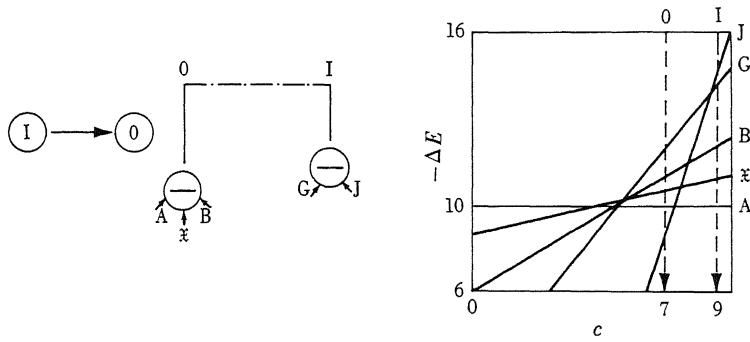
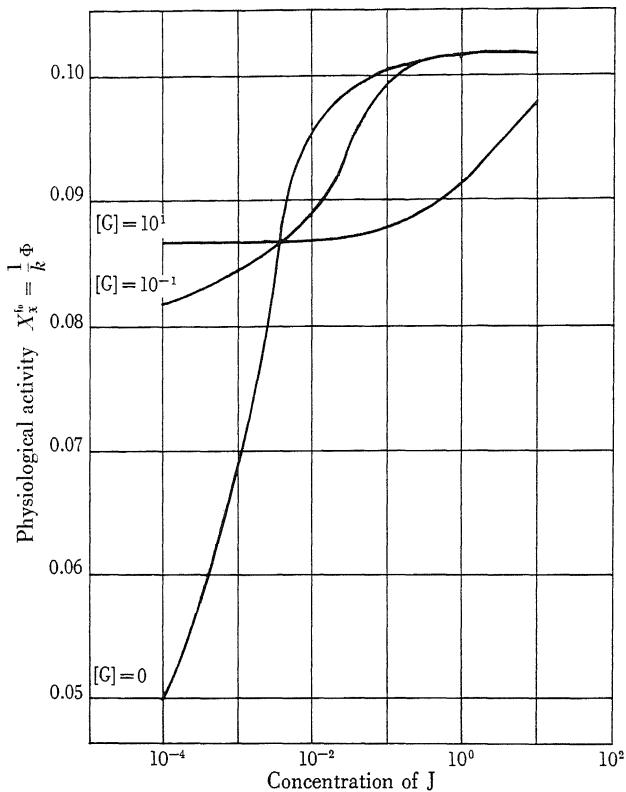
## B. MODELS WITH TWO RECEPTORS AS AN EXPLANATION FOR THE BELL-SHAPED CURVE AND NONCOMPETITIVE ACTIVATION AND INHIBITION

### (1) The bell-shaped curve

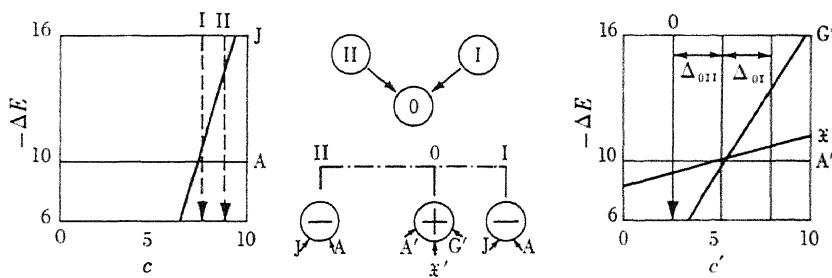
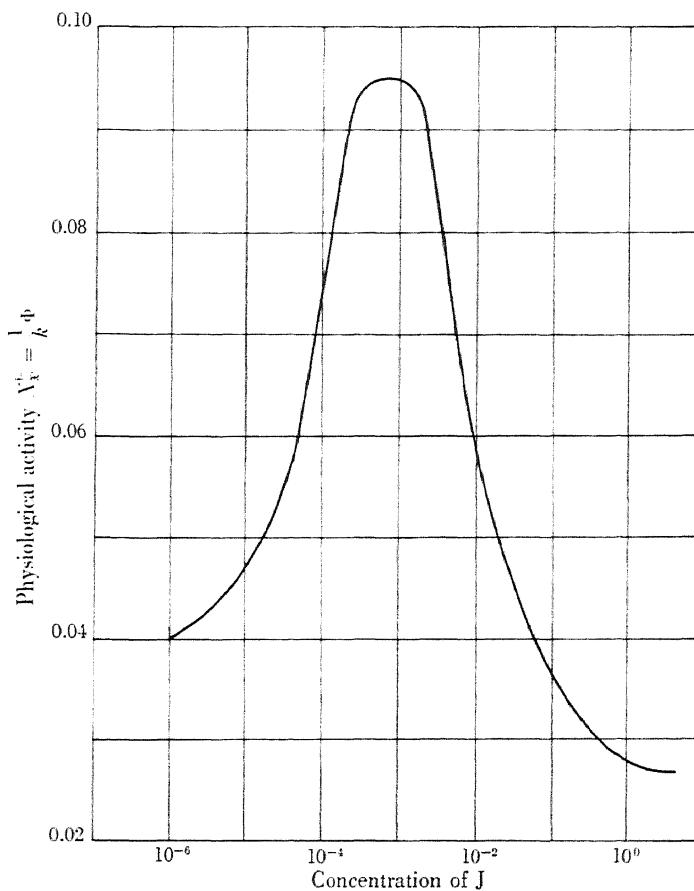
Figure 6.2 was constructed for a two-receptor system with receptor sites I and II. Both sites can affect the  $c$ -value of effector site 0, but they are incapable of

\* This assumption is not correct. The differences introduced are, however, readily compensated by changing the arbitrary  $-\Delta E$  values; this is what we have chosen to do.

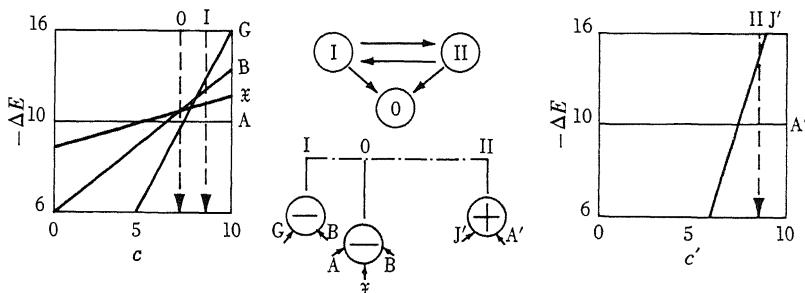
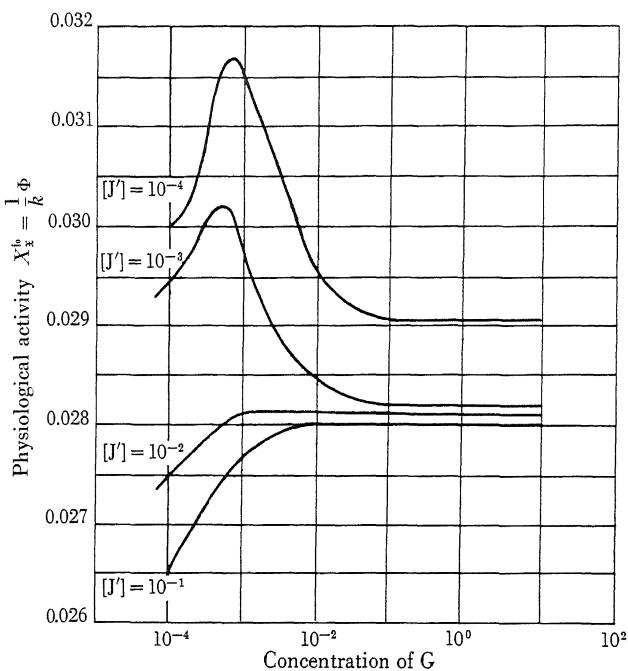
† For simplicity, a  $CH$  group is counted also as a  $CH_2$  group.



**Figure 6.1. VARIATION OF PHYSIOLOGICAL ACTIVITY  $\Phi$  WITH VARIATIONS IN THE CONCENTRATIONS OF CARDINAL ADSORBENTS J AND G IN A ONE-RECEPTOR SYSTEM.** The physiological activity in question is assumed to be proportional to the mole fraction of sites 0 adsorbing  $\bar{x}$ . The concentration of A is  $0.03M$ ; that of B,  $0.10M$ ; and that of  $\bar{x}$ ,  $0.01M$ .

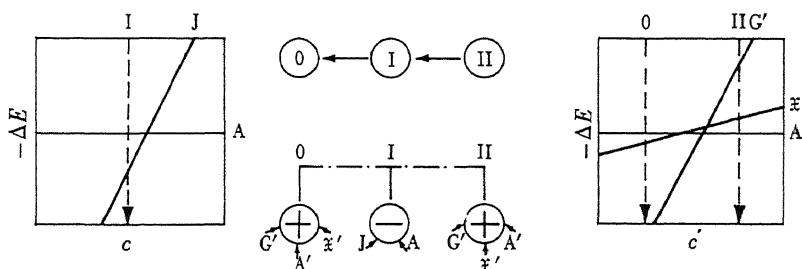
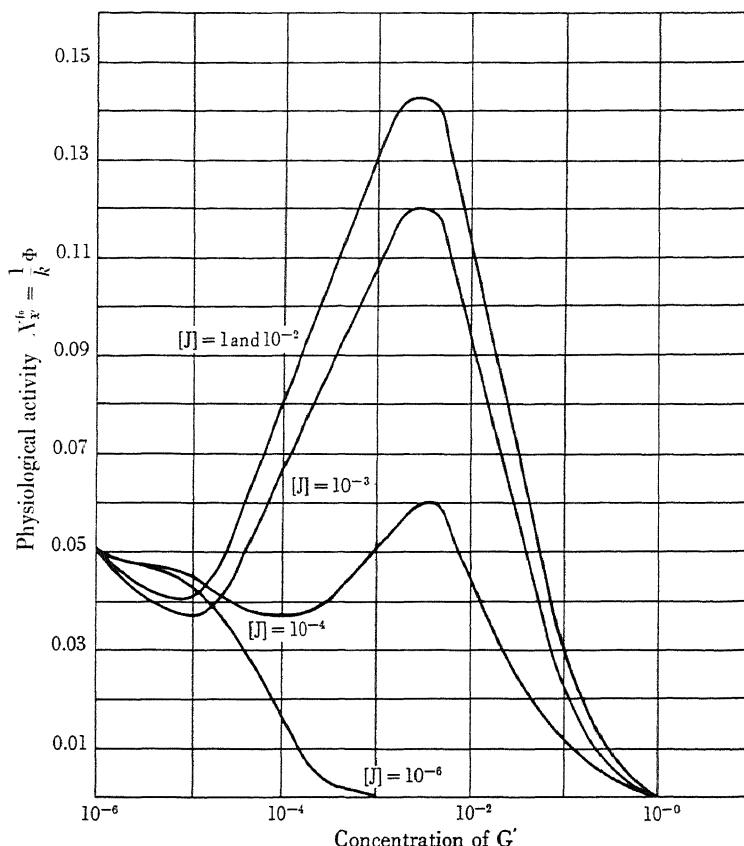


**Figure 6.2.** VARIATION OF PHYSIOLOGICAL ACTIVITY  $\Phi$  WITH VARIATION OF A CARDINAL ADSORBENT  $J$  IN A TWO-RECEPTOR SYSTEM. There is no interaction between the two receptor sites I and II. The physiological activity is proportional to the mole fraction of sites 0 adsorbing  $\mathfrak{X}'$ . The concentration of  $A'$  is  $0.10M$ ; that of  $\mathfrak{X}'$ ,  $0.01M$ ; that of  $G'$ ,  $0.01M$ ; and that of  $A$ ,  $1.00M$ .



**Figure 6.3. VARIATIONS OF PHYSIOLOGICAL ACTIVITY  $\Phi$  WITH VARIATION IN THE CONCENTRATION OF TWO CARDINAL ADSORBENTS  $J'$  AND  $G$  IN A TWO-RECEPTOR SYSTEM.** Interaction between the two receptor sites is present. The physiological activity is directly proportional to the mole fraction of sites 0 adsorbing  $\bar{x}$ . The concentration of A is  $0.30M$ ; that of  $\bar{x}$ ,  $0.01M$ ; that of B,  $0.10M$ ; and that of  $A'$ ,  $1.00M$ .

influencing each other because they are too far apart. In this case, the effector site 0 bears a charge opposite to that of the two receptor sites. Given the cardinal adsorbent J, two steps are involved in the production of a bell-shaped curve. As the concentration of J is continuously increased, the substitution of J for A at site II produces a  $c$ -value shift at the effector site  $\Delta_{\text{off}}(J|A)$ . This changed  $c$ -value of the effector site is more favorable to the adsorption of  $\bar{x}'$ . Also, with



**Figure 6.4. VARIATIONS OF PHYSIOLOGICAL ACTIVITY  $\Phi$  WITH VARIATION OF TWO CARDINAL ADSORBENTS IN ANOTHER TWO-RECEPTOR SYSTEM.** This figure differs from Figure 6.3 in the spatial arrangement of the fixed charges and the initial  $c$ - and  $c'$ -values. The physiological activity is directly proportional to the mole fraction of sites 0 adsorbing  $\tilde{x}'$ . The concentration of A is  $0.03M$ ; that of  $\tilde{x}'$ ,  $0.01M$ ; and that of  $A'$ ,  $0.10M$ .

the saturation of the exchange of J for A at site II, further increase of J leads to a J for A exchange at site I. This produces a *c*-value shift  $\Delta_{01}(J|A)$ , which pushes the *c*-value of the effector site beyond the optimum for  $\mathfrak{X}'$  adsorption, and  $\mathfrak{X}'$  is once again in disfavor. We suggest that this "on optimum-off optimum" mechanism underlies a number of the bell-shaped curves observed in biology; for example, see the pH-versus-enzyme-activity curves in Chapter 14. At least two receptor sites are necessary for this type of bell-shaped curve. Another type of bell-shaped curve which owes its falling limb to the direct competition of the cardinal and critical adsorbents for the effector site is theoretically possible. In this case, only one receptor and one effector site are needed.

### (2) A type of noncompetitive facilitation and inhibition

In Figure 6.3, sites I and II, one cationic and one anionic, may influence both the anionic site 0 and each other. The family of curves shows that at  $J' = 10^{-4}$ , a G concentration above  $10^{-3}$  leads to noncompetitive inhibition. However, at  $J' = 10^{-1}$ , the effect of G is uniformly facilitative (activating).

### (3) A model showing noncompetitive activation and inhibition

In Figure 6.4, a somewhat different spatial arrangement and initial *c*- and *c'*-values produce a family of curves which resemble those in Figure 6.3, except that the maximum  $X_{\mathfrak{X}'}^{f_0}$  increases rather than decreases with increasing concentration of J. Although the critical adsorbent  $\mathfrak{X}'$  can and does adsorb onto both site 0 and site II, only site 0 is physiologically active. The right-hand falling limb is created by direct competition of G' with  $\mathfrak{X}'$  for site 0, while in Figure 6.3 both limbs of the bell-shaped curves are created noncompetitively by *F*-effects. Figure 6.4 also demonstrates that the two receptor sites do not have to flank the "effector" site, but may both be on the same side of the effector site.

## B I B L I O G R A P H Y

### REVIEWS AND ORIGINAL ARTICLES

Ariëns, E. J., van Rossum, J. M., and Simonis, A. M., A theoretical basis of molecular pharmacology. *Arzneimittel-Forsch.* 6 (1956), Part I, pp. 282-296; Part II, pp. 611-621; Part III, pp. 737-746. An extremely valuable series of papers presenting a more or less formal analysis of the mode of action of drugs. Experimental support for theoretically derived drug interaction is based primarily upon studies of enzyme activity and the shortening of frog *rectus abdominis* muscle.

Lipmann, F., Metabolic generation and utilization of phosphate bond energy. *Advances in Enzymol.* 1, 99-162 (1941). A comprehensive discussion of the "high-energy phosphate bond" in biochemical reactions and its possible role as a source of energy for biological work performance. Although the function of this group in chemical reactions is well established, the second role assigned to it is now in grave doubt.

# PART III

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ASSOCIATION AND INDUCTION

*IN VITRO*

During the past 50 years, extensive research into the chemistry of proteins has yielded a rich collection of exact data on the properties of proteins in aqueous solutions. Proteins in aqueous solutions are semifixed-charge systems. As such, their behavior does not necessarily reflect the entire scope of the behavior the same proteins exhibit in their more organized state within a living cell. Nonetheless, the *in vitro* studies have permitted extensive experimentation under conditions impossible to obtain within the cell and thus have revealed important basic characteristics of protein-water-salt systems. The results of these studies enable us to test the theoretical conclusions stated in Parts I and II; they also provide a solid path toward the understanding of the living state as represented by cells, organs, and entire organisms.

## 7

## THE THEORY OF PROTEINS

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The theory of proteins is built around two central themes: first, a specific protein may be defined as a functional unit in terms of its *c*-value ensemble; second, this functional unit is inherently able to exist in more than one cooperative state—an indirect *F*-process effects the transformation from one cooperative state to another.

### 7.1. The Characterization of a Protein by Its $c$ - and $c'$ -Value Ensemble; The Protein as a Functional Unit

Figure 7.1 shows the conspicuously different plots of pH versus apparent heat of ionization  $\Delta H'$  for four different proteins: myosin, wool, lysozyme, and oxyhemoglobin.\* Figure 7.2 shows the pH-versus- $\Delta H'$  plots of serum albumins from three

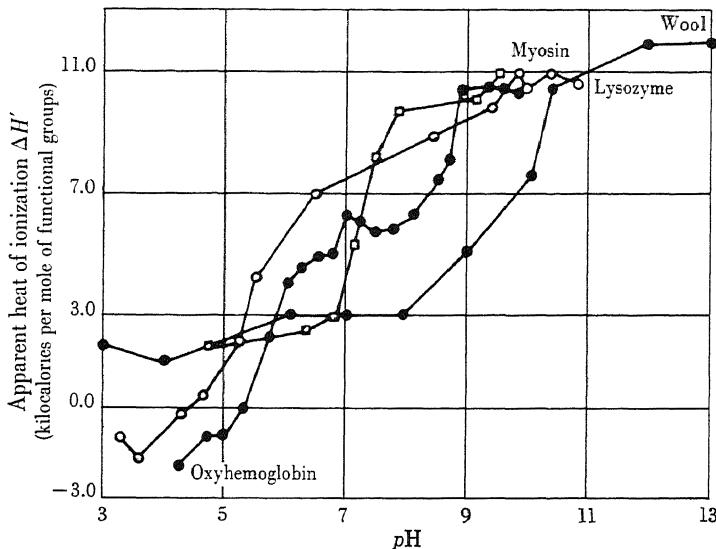


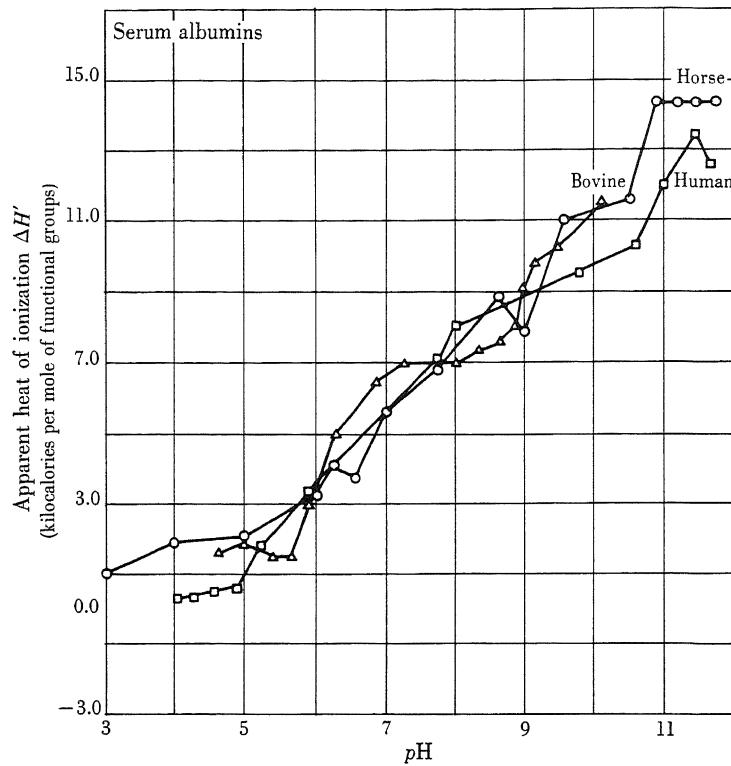
Figure 7.1. THE APPARENT HEAT OF IONIZATION  $\Delta H'$  OF IONIZING GROUPS OF VARIOUS PROTEINS PLOTTED AGAINST THE pH. Note the great diversity of the  $\Delta H'$  values in functionally different proteins. [Data from Mihályi (1950): myosin ( $25^{\circ}$ – $38^{\circ}$ C); from Wyman (1939): oxyhemoglobin ( $25^{\circ}$ – $37.7^{\circ}$ C); from Tanford and Wagner (1954): lysozyme ( $4^{\circ}$ – $25^{\circ}$ C); from Steinhardt *et al.* (1941): wool ( $0^{\circ}$ – $25^{\circ}$ C).]

different species. With respect to polar amino-acid composition, myosin and human serum albumin differ about as much as human serum albumin and bovine serum albumin (Table 7.1). The functional characteristics of these proteins indicate that there is no distinct difference between human and bovine serum albumin (for ion adsorption data; see Carr, 1952). But the tremendous functional difference

\* The apparent heat of ionization is defined as

$$\Delta H' = -2.303RT^2 \left( \frac{\partial p\text{H}}{\partial T} \right)_{\bar{X}}$$

where  $(\partial p\text{H}/\partial T)_{\bar{X}}$  refers to the change of the pH of a solution with temperature when the amount of base or acid (represented by  $\bar{X}$ ) is kept constant.



**Figure 7.2.** THE APPARENT HEAT OF IONIZATION  $\Delta H'$  OF IONIZING GROUPS OF THREE SERUM ALBUMINS PLOTTED AGAINST  $pH$ . Compare the plots of these functionally similar proteins with those of the functionally different proteins shown in Figure 7.1. [Data from Tanford (1950): human serum albumin ( $0^\circ$ – $25^\circ\text{C}$ ); from Tanford *et al.* (1955): bovine serum albumin ( $5^\circ$ – $25^\circ\text{C}$ ); from E. J. Cohn *et al.* (1943): horse serum albumin ( $5^\circ$ – $25^\circ\text{C}$ ).]

Protein	Amino-acid content							
	Tyr	Try	CySH	Arg	His	Lys	Asp	Glu
Wool	4.65	1.8	—	10.4	1.1	2.76	7.2	14.1
Myosin (rabbit)	3.4	0.8	—	7.36	2.41	11.92	8.9	22.1
Hemoglobin (horse)	3.03	1.7	0.56	3.65	8.71	8.51	10.60	8.50
Lysozyme	3.6	10.6	0	12.7	1.04	5.7	18.2	4.32
Bovine serum albumin	5.06	0.68	0.3	5.90	4.0	12.82	10.91	16.5
Human serum albumin	4.70	0.2	0.7	6.20	3.50	12.30	8.95	17.0

**Table 7.1.** THE TRIFUNCTIONAL AMINO-ACID COMPOSITION OF SEVERAL PROTEINS. [Data (given in grams of amino acid per 100 grams of protein) from Tristram (1953).]

between serum albumin and the contractile protein, myosin, needs no emphasis. We may thus draw the important conclusion that a  $pH$ -versus- $\Delta H'$  plot characterizes the properties of protein in its interactions better than a description of the amino-acid composition does.

#### A. THE HETEROGENEITY OF NOMINALLY IDENTICAL POLAR GROUPS ON PROTEINS

The work of Derick, Ingold, and others (discussed in Chapter 5) demonstrates that the affinity of a carboxylic group for a proton depends on the nature of the remainder of the molecule.\* It is well known that the acid-dissociation constants or  $pK$  values of the same  $\alpha$ -carboxyl groups on different amino acids differ from each other. The  $pK$  value of the carboxyl group of an amino acid is also changed when its amino group is linked to another amino acid to form a dipeptide (see Section 5.2; also Cohn and Edsall, 1943, p. 84). Similarly, the  $pK$  value of the side-chain polar group in a trifunctional amino acid (glutamic acid or lysine, for example) alters when the amino acid participates in the formation of a protein molecule. Such changes have usually been considered small (scarcely over 2 pH units). Many studies made in this field have emphasized uniformity in the characteristics of different proteins. However, a number of exceptions exist, notably, the work of Crammer and Neuberger (1943), who studied the phenolic group of the tyrosine residue; and the work of Karush and Sonenberg (1949), Teresi and Luck (1948), and Tanford and Wagner (1954), who studied  $\beta$ - and  $\gamma$ -carboxyl groups. All concluded that individual groups may have very different  $pK$  values.

For the present, we emphasize the heterogeneity manifested by the nominally identical carboxyl, amino, and other functional groups. We shall consider both their  $pK$  values (which are directly related to the free energies of dissociation of the protons  $\Delta F$ ), and their apparent heats of dissociation  $\Delta H'$ , which are closely related to their true heats of dissociation  $\Delta H$ .

##### (1) Carboxyl groups

The  $\Delta H'$  values usually given for carboxyl groups on various organic molecules vary between +1.5 and -1.5 kcal/mole (Cohn and Edsall, 1943). This variation represents true variation resulting from changes in the nature of the rest of the molecule. Thus, although the  $\alpha$ -carboxyl group of oxyvaline has a  $\Delta H'$  value of -1.30 kcal/mole, the  $\Delta H'$  value for the  $\beta$ -carboxyl group of aspartic acid is +2.10 kcal/mole and the  $\Delta H'$  value of the  $\gamma$ -carboxyl group of glutamic acid is +1.04

\* Derick, 1911; G. N. Lewis, 1923; Bjerrum, 1923b; Kirkwood and Westheimer, 1938a,b; Ingold, 1953; Branch and Calvin, 1941.

kcal/mole (Cohn and Edsall, 1943, Chapter 4, Table 6). Wyman (1939) has shown that within a given pH range\* the  $\Delta H'$  of protein molecules seems to depend solely upon the relative abundance of the various groups which ionize in that range.

In most large protein molecules, all the side-chain carboxyl groups belong to either glutamic- or aspartic-acid residues. If the  $pK$  and  $\Delta H$  values of the carboxyl residues in protein are only slightly different from the  $pK$  and  $\Delta H$  values of the corresponding groups in the free amino acid, the  $\Delta H'$  of all proteins of large molecular weight should be about +1.0 to +2.0 kcal/mole of carboxyl groups at a pH near 4. However, as we have indicated, the usual  $\Delta H'$  values assigned to protein carboxyl groups vary from -1.5 to +1.5 kcal/mole. In fact, the pH-versus- $\Delta H'$  plots of wool protein, myosin, and lysozyme, as well as Wyman's original plot for oxyhemoglobin, demonstrate an even wider variation (Figure 7.1). At pH 4, the  $\Delta H'$  value of the carboxyl group can be as high as +2.0 kcal/mole or lower than -2.0 kcal/mole. In proteins such as  $\beta$ -lactoglobulin, wool keratin, and myosin, plateaus in the curves indicate a uniformity in the  $pK$  values of the carboxyl groups; in proteins like oxyhemoglobin and lysozyme, the steepness of the slope indicates multiple  $pK$  and  $\Delta H'$  values. The possibility of multiple  $pK$  values for protein carboxyl groups has often been suggested recently. In the case of some small protein molecules, a high proportion of C-terminal residues ( $\alpha$ -carboxyl groups) may account for very low  $\Delta H'$  values; this appears to be the case with insulin (Tanford and Epstein, 1954). The shape of the pH-versus- $\Delta H'$  curve in Figure 7.1 for lysozyme (molecular weight, 14,700), which has one C-terminal residue and 10.5 dissociable carboxyl groups, indicates that the  $\Delta H'$  values for the  $\beta$ - and  $\gamma$ -carboxyl groups vary considerably.

## (2) Amino groups

It is probable that in the pH range 7.0 to 9.5, only the imidazole group of histidine and the  $\epsilon$ -amino groups of lysine significantly determine the measured  $\Delta H'$  of proteins. If these groups, as present in protein, possessed uniform  $pK'$  values, one would expect the pH-versus- $\Delta H'$  plots of lysozyme, myosin, and wool keratin to show that the  $\Delta H'$  values at the pH range follow the relative abundance of histidine as compared with lysine (for lysozyme, the histidine content is 18.2 per cent of the lysine content, Fromageot and deGarilhe, 1950; for myosin, it is 20.2 per cent; and for wool keratin, 39.8 per cent; see Table 7.1). However, the  $\Delta H'$ -versus-pH plot of these proteins (Figure 7.1) shows wide variation in this range; and the variation is not at all predictable on this simple basis. Bailey (1951), using the fluorodinitrobenzene method of Sanger, showed an absence of  $\alpha$ -amino groups in myosin; this indicates that the high  $\Delta H'$  value for myosin at pH 8 cannot be due to terminal  $\alpha$ -amino groups. A possible explanation is that the  $pK$  values of the  $\epsilon$ -amino groups in myosin are much lower than the  $pK$  values of 9.4 to 10.6 usually

\* See definition of Zwitterion in glossary.

given for  $\epsilon$ -amino groups in protein\* (Cohn and Edsall, 1943). This is certainly an admissible possibility since amino groups in simpler molecules are known to have  $pK$  values as low as 7.60 (hexaglycine, see Section 5.2), and 7.17 ( $\alpha$ -hydroxyasparagine, Cohn and Edsall, 1943, p. 84). Conversely, in wool protein, the  $pK$  of the  $\epsilon$ -amino groups of the lysine residue may be much higher than that usually given. We conclude that the  $pK$  and  $\Delta H'$  values of  $\epsilon$ -amino groups as well as those of carboxyl groups vary significantly when these groups are part of a protein molecule.

### B. THE $c$ -VALUE ENSEMBLE AS THE DETERMINANT OF THE FUNCTIONAL CHARACTERISTICS OF PROTEINS

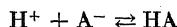
The apparent heat of ionization  $\Delta H'$  is usually considered virtually equal to the true heat of ionization  $\Delta H$ . In turn,  $\Delta H$  is related to the dissociation energy  $-\Delta E$  by the relation

$$\Delta H = -\Delta E + p\Delta V \quad (7-1)$$

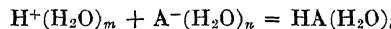
where  $p\Delta V$  represents the compressional work done on nearby water molecules during ionization. An estimate of this value, based on the treatment of Webb (1926), shows that  $p\Delta V$  contributes much less than the electrical work  $\Delta E$  does. Thus,  $\Delta H \approx -\Delta E$ .† Referring to Figures 4.9 to 4.11, one can see that the energy of

\* If a lower  $pK$  value for the  $\epsilon$ -amino groups of myosin is accepted, at least two interesting paradoxes can be resolved. (1) Dubuisson (1941) and Dubuisson and Hamoir (1943) compared the actual titration curve of myosin with a theoretical one constructed according to its known constituent amino acids, assigning the usual  $pK$  values to each of these groups. They found that there is an amino-acid residue which ionizes in the pH range from 7 to 8; but this is unknown from the chemical analysis. A reduction of the  $pK$  value of the  $\epsilon$ -amino-acid residue would fill in this gap. (2) A great discrepancy exists between the value of the heat of dephosphorylation of ATP given by Meyerhof and Lohmann (1932) as  $-12.0$  kcal/mole and the much smaller figure of  $-4.7$  kcal/mole determined by Podolsky and Kitzinger (1955) and Podolsky and Morales (1956). Podolsky and Morales suggested, in a footnote, that the difference originated from the heat of neutralization (equal to minus the heat of dissociation) of the imidazole group of histidine residues on muscle proteins present in the *Muskelsaft* used by Meyerhof and Lohmann. Bernhardt (1956), discussing the same general topic, mentioned that there is insufficient histidine residue in the intracellular fluid of muscle to make a significant contribution to the total heat change measured during ATP cleavage. Meyerhof and Lohmann's *Muskelsaft* could not contain a higher concentration of histidine than muscle itself; consequently, we suspect that histidine residues cannot account for all the extra heat. However, if we assume a low  $pK$  value for the  $\epsilon$ -amino group of myosin, the participation of these groups in the buffering capacity of muscle proteins near pH 8 should be expected. The concentration of  $\epsilon$ -amino groups is much higher than that of the histidine groups, and the  $\epsilon$ -amino groups also have a much higher heat of neutralization ( $-10$  to  $-12$  kcal/mole); thus they could easily compensate for the difference between Podolsky and Morales' figure and the larger one found by Meyerhof and Lohmann.

† Actually, the  $\Delta H'$  values measured from the change of pH with variation of the temperature are related to the  $\Delta E$  values but not quite as simply as equation (7-1) predicts. This is because the acid dissociation phenomenon is not really



but rather



and  $m$ ,  $n$ , and  $l$  vary with temperature (see Section 7.4C). This involvement of varying numbers of water molecules also accounts for the seemingly unreasonable negative values of  $\Delta H'$  found for some protein carboxyl groups.

dissociation of a proton at a definite temperature, pressure, and concentration is substantially determined by the *c*-value. Thus the *pH*-versus- $\Delta H'$  plot in the acidic range may be visualized as a "profile" of the *c*-value ensemble of a protein.\*

We have mentioned that the variation in the *pK* value of a specific polar group in a protein (which is related to the *c*-value and  $\Delta H'$  variation) is not large, and usually does not exceed two *pH* units. If each fixed carboxyl group could combine with a proton and nothing else, a *pK* shift of this magnitude would not be important. However, a *pK* shift of 2 units will result in an estimated difference of more than 2.8 kcal/mole in the free energy of dissociation of  $H^+$ ; Figures 4.9 to 4.11 illustrate that a change of dissociation energy of this order of magnitude will involve significant shifts of *c*-value. Consider, for example, the carboxyl groups: we may expect such a *c*-value shift to be very important when these anionic groups have cations of several species as possible counterions. Since all physiologically active proteins have both cationic and anionic side-chains (see Table 0.2), even in a salt-free solution, the carboxyl groups have the choice of associating with  $H^+$  ions from water or with the fixed positive groups on the protein molecule itself ( $\epsilon$ -amino group or guanidyl group, for example). In living cells, ions of the alkali metals, the alkaline earths, and others are always present. Thus, in general, the fixed anionic groups of proteins may combine with a variety of counterions. According to the present theory, small *pK* differences are sufficient to alter the preference for these counterions. This alteration will bring about profound changes in the physicochemical nature of the protein.

It should now be clear why the *pH*-versus- $\Delta H'$  plot of a protein characterizes its functional aspects better than its amino-acid composition does; this plot is a reflection of the *c*-value ensemble of the polar groups of the protein. The *c*-value ensemble is of basic importance in the interaction of a protein with its surroundings, (ions, water, and other proteins) and these interactions determine its functional characteristics. Let us now discuss the mechanism that produces the marked heterogeneity in the *c*-value, and, therefore, in the  $\Delta H'$  values and the *pK* values of the polar groups on a protein.

### C. THE ORIGIN OF THE HETEROGENEITY OF FUNCTIONAL GROUPS

The electrostatic effect of the ionization of charged groups on the dissociation of other side-chain polar groups is generally recognized. The Debye-Hückel theory of ionic solution was applied to proteins by Linderstrøm-Lang in his well-known electrostatic theory. In this theory, the protein molecule is treated as a sphere with its net electric charge uniformly smeared over its surface. The effect of the removal or addition of charges from or to such a body is a uniform one; it affects all charged groups in the molecule equally. This theory of proteins attributes no

\* This statement is usually true. For reservations, see Section 7.3B. The  $\Delta H'$ -versus-*pH* plots in the basic range are related to, but are not equivalent to, the *c*-value profile.

significance to the exact location of the ionizing or the affected groups or to the sequential order of arrangement of amino-acid residues on the protein polypeptide chain.

In theory, a direct electrostatic effect transmitted through space along a path of minimum length always exists.\* The weight of evidence to be presented shows that in molecules of high polarizability, like proteins, particularly in the form of true fixed-charge systems, neither the macroscopic nor the microscopic electrostatic effect plays as important a role as do the inductive effects. The combined *F*-effects acting through the resonating, highly polarizable polypeptide chain, produce changes on groups all along the molecule; these changes depend upon the relative position of the interacting groups and the molecular structure that intervenes between the affecting and the affected groups. An example of the variety thus produced in the polar groups is the amino-acid sequence of a particular protein. The structural analysis of  $\beta$ -corticotropin (Shepherd *et al.*, 1956) led to the complete identification of its amino-acid sequence. Part of the molecule may be represented as:

Try	Gly	Lys	Pro	Val	Gly	Lys	Lys	Arg	Arg	Pro
9	10	11	12	13	14	15	16	17	18	19

where the numbers designate the sequential position of the amino-acid residues (see Table 0.1). The present theory emphasizes the fact that the *pK* value of the 11-lysine determined by, say, electrometric titration would be considerably different from that of the 16-lysine, which is flanked by three other positively charged side chains. Further, the ionization of the 16-lysine would have a very different effect on the ionization of the 15-lysine than it would on the ionization of the 11-lysine. When we consider the  $\beta$ -corticotropin molecule as a whole, it is evident that the *pK* values of the lysine residues as well as those of many other polar side chains must be heterogeneous. Only in exceptional cases will they be the same.

Although we have focused our attention on the *pK* and  $\Delta H$  values of the fixed ionic groups, these are by no means the only groups whose interactions are affected by their neighboring amino-acid residues. Section 7.2 will deal with inductive effects on charge-bearing groups, and with the influence of such effects on H-bonding sites as well as on groups which participate in oxidation-reduction reactions.

## 7.2. The Nature of the Functional Groups of Proteins Influenced by Transmitted Effects

We have shown that the *F*-effect can be transmitted through at least a few peptide linkages to a functional group on another side chain. The discussion has been

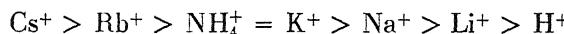
\* This microscopic electrostatic effect is not quite the same as the macroscopic electrostatic effect in Linderstrøm-Lang's theory.

limited to targets that were charged groups along the same peptide chain. We shall now demonstrate that *F*-effects affect not only ionic adsorption on charged groups, but also the chemical reactivity and H-bonding power of other functional groups, as well as the H-bonding power of the peptide chain itself.

#### A. THE INFLUENCE OF THE *F*-EFFECT: INDUCED CHANGES IN IONIC GROUPS—THE PRINCIPLE OF THE VARIABILITY OF THE PREFERRED COUNTERION

The work of Jenny (see Section 4.1), the transition orders of Bungenberg de Jong (1949), and the 11 orders of preference for the monovalent cations of Eisenman *et al.* (1957; see Isard, 1959) produced by differences in the anionic field strength all imply that the preferred adsorbents on similar functional groups may differ. The present author's introduction of inductive effects as the basis of long-range interaction (Ling, 1957, abstract), as well as Chapters 5 and 6 of this book, have emphasized that variation of the *c*-value through long-range *F*-effects enables a given functional group to change its counterion preference. The existence of an appropriate *c*-value is necessary for successful adsorption by a particular ion in competition with other ions. This is a crucial and more subtle factor that must be considered with such well-known limits as the electric sign of the reacting groups and steric hindrances. In a competitive situation, a protein site having a certain *c*-value will adsorb a specific ion; a shift of *c*-value at this site will lead to the desorption of this ion and the adsorption of an ion of a different species.

Although our theoretical calculations have been limited to seven monovalent cations, the applicability of the principle of the variability of the preferred counterion is far more general; the fundamental differences among monovalent cations in any group are due to their different *c'*-value analogues. Recalling the definition of the *c*- and *c'*-values, one may imagine that in order to approximate the properties of the small  $H^+$  ion, for example, the excess positive charge must be moved a considerable distance toward the interacting anion (using  $NH_4^+$  ion as a reference). This makes its *c'*-value analogue very high. On the other hand, to approximate the properties of the large  $Cs^+$  ion, the excess cationic charge must be moved in the opposite direction. A low *c'*-value analogue is thus obtained for  $Cs^+$  ion. Since the sizes of these ions follow the order,



their *c'*-value analogues must follow the reverse order,



The calculations in Chapter 4 show that a site with a very low *c*-value tends to prefer a low *c'*-value adsorbent; a site with a very high *c*-value tends to prefer a high *c'*-value adsorbent. The following examples provide further confirmation of this concept.

Since the  $H^+$  ion has a very high  $c'$ -value analogue, one might expect its order of preference for anions to follow the  $c$ -value analogues of the anions. This is in agreement with the fact that among the halide acids, the acid-dissociation constant is smallest for HF ( $7.4 \times 10^{-4}$ ), the constants for the other halide acids being much higher. Similarly, for the acetate series, the acid-dissociation constants follow the order, acetate < monochloroacetate < dichloroacetate < trichloroacetate; this is the reverse of their  $c$ -value order (see Section 4.2A). Since the  $NH_4^+$  ion has a relatively low  $c'$ -value analogue one would expect the amino exchange resin, which bears the functional group  $NH_3^+$ , to have a low  $c'$ -value analogue. This resin should then have an order of preference for anions which is the reverse of that of the  $H^+$  ion. This is the case (see Table 7.5) both for the halides in which the preference of the resin follows the order,



and for the acetate series, where trichloroacetate > dichloroacetate > monochloroacetate > acetate. Such reversals of order of preference are very difficult to explain unless we accept the notion that under certain conditions water intervenes between site and adsorbed ion to produce preferred configurations higher than the 0-configuration.

It should be remembered that interspersed water molecules belong as much to the cation as to the anion (oxyacid group in our model), and that configurational variation with  $c$ - and  $c'$ -value changes is applicable to all types of oxyacids and nonoxyacids. In an aqueous medium, anionic groups with no steric limitation will react with one cation rather than another, depending entirely upon the  $c$ - and  $c'$ -values of the interactants. This type of specificity adds an extra dimension to the reactions of compounds with fixed-charge systems.

#### B. THE INFLUENCE OF THE $F$ -EFFECT: INDUCED CHANGES IN THE PROTON-DONATING AND PROTON-ACCEPTING GROUPS OF THE PEPTIDE CHAIN

The NH and C=O groups of the peptide linkages of proteins form intramolecular H-bonds (see Ketelaar, 1953). The success of the hydrogen-bond concept (Latimer and Rodebush, 1920; Pauling, 1935, 1936) in explaining abnormal physical properties in solutions has led to the study of the relative strength of the proton-accepting and the proton-donating powers of a variety of reactive groups (Gordy, 1936; Gordy and Stanford, 1940, 1941; Tsuboi, 1951, 1952; Mizushima, 1954). Tsuboi came to a general conclusion: For X-H . . . Y, "the proton-donating power of the X-H group is determined by the tendency of the X atom to attract the electron of hydrogen to make the proton bare, and the proton-accepting power of the Y atom is determined by the tendency of this atom to attract electrons from the adjacent atoms or groups to increase its effective negative charge." (Tsuboi, 1951, 1952; Mizushima, 1954, p. 132.) Thus, *the substitution of chlorine for hydrogen atoms*

*in diethylether* (Gordy and Stanford, 1941) has an influence similar to the influence of the substitution of chlorine atoms into acetic acid (see Section 4.2A). Substitution of chlorine into diethylether decreases the effective negative charge on the ether oxygen; substitution of chlorine into acetic acid has the same effect on the oxygens of the carboxyl group. A decrease of negative charge is, of course, equivalent to a decrease of *c*-value. Thus, we should expect a *c*-value shift, produced by a change of counterion on a polar side chain, to affect not only the *c*- and *c'*-values of other polar groups, but also both the proton-donating power of the NH group and the proton-accepting power of the C=O group of the peptide linkages. Conversely, sufficiently large changes of interaction at the peptide linkages may be expected to change the *c*- and *c'*-values of polar side chains.

### C. THE INFLUENCE OF THE *F*-EFFECT: INDUCED CHANGES IN THE OXIDATION-REDUCTION POTENTIALS OF CHEMICALLY REACTIVE GROUPS

#### (1) Oxidation-reduction potentials

Modern chemists regard chemical reactions as electron transactions between molecules; hence the classification of reagents by Ingold (1933, 1953) as electrophilic or nucleophilic is widely accepted. All reagents used for the determination of protein reactive groups fall into one of these two categories. However, only certain nucleophilic reagents will react with a given electrophilic reagent and vice versa. The occurrence or nonoccurrence of such a reaction is an expression of the electron-donating and electron-accepting tendencies of the participating groups, that is, their oxidation-reduction potentials  $\mathcal{E}^{\circ}$ .

It has been shown on theoretical grounds (see Latimer, 1952, Chapter 2) that the oxidation-reduction potential of simple atoms like K and Na, in an aqueous solution is quantitatively determined by (1) the free energy of formation of the nonionized form, (2) the free energy of hydration of the ionized product, and particularly (3) the ionization potential for cations and the electron affinity for anions.

To illustrate this point, we have calculated (from these three parameters) theoretical values of  $\mathcal{E}^{\circ}$  for simple monovalent elements and compared them with the experimentally determined reduction potentials. Table 7.2 establishes the correspondence between  $\mathcal{E}^{\circ}$  and the ionization potential. Thus, oxidizing agents will attack the aldehyde group and ignore the paraffin end of a molecule like acetaldehyde  $\text{CH}_3\text{CHO}$ , since the ionization potential of one of the nonbonding electrons in the aldehyde group is considerably lower than that of the electrons in the paraffin end (by about 3 electron volts).

Since, conventionally, the oxidation-reduction potential is experimentally determined, rational discussion of a reaction in terms of oxidation-reduction potentials becomes meaningless in cases for which no procedure exists for its accurate determination. As a case in point, studies on the oxidation-reduction potential of thiols have, because of the high reactivity of the SH compounds with noble metal electrodes, given rise to great confusion. However, the oxidation-reduction potential is directly related to the ionization potential

Metal	Theoretical			Experimental		
	Free energy of hydration, $-\Delta F_{\text{hydration}}$ , kcal/mole	Free energy of formation, $-\Delta F_{\text{formation}}$ , kcal/mole	Ionization potential, kcal/mole	Theoretical absolute oxidation- reduction potential, kcal/mole	Experimental relative oxidation- reduction potential, v	Experimental absolute oxidation- reduction potential, kcal/mole
H.	250	48.58	311.6	110.18	0	110.18
Li	114	29.19	123.56	38.75	3.045	39.95
Na	88.7	18.67	117.96	47.93	2.714	47.58
K	71.7	14.62	99.49	42.41	2.925	42.72
Rb	66.3	13.35	95.82	42.87	2.925	42.71
Cs	59.4	12.24	89.16	42.00	2.923	42.76
Ag	93.7	59.84	173.7	139.84	-0.7991	128.61
Tl	68.5	35.05	139.9	106.45	0.3363	102.42

**Table 7.2. COMPARISON OF THEORETICALLY CALCULATED AND EXPERIMENTALLY DETERMINED ABSOLUTE OXIDATION-REDUCTION POTENTIALS FOR SIMPLE MONOVALENT ELEMENTS.** The experimental absolute oxidation-reduction potential has been calculated by adding the relative oxidation-reduction potential in kilocalories per mole to the theoretical value of 110.18 kilocalories per mole for H. [Data from Ketelaar (1953): free energy of hydration; from Rossini *et al.* (1952): free energy of formation; from "Handbook of Chemistry and Physics" (1956-57): ionization potential; from Latimer (1952): experimental oxidation-reduction potential.]

and this can be understood by a direct and simple theoretical model; thus we shall use the ionization potential as the point of reference in our discussion of  $\mathcal{E}^{\circ}$ .

Experimental studies on the oxidation-reduction potential of organic compounds have shown that a substitution changes  $\mathcal{E}^{\circ}$  so that acid-weakening groups reduce the potential and acid-strengthening groups increase it\* (Conant *et al.*, 1922; LaMer and Baker, 1922; Cohen *et al.*, 1924; Fieser, 1929; Fieser and Fieser, 1935; Preisler, 1939; Branch and Calvin, 1941, Chapter 7). These are primarily effects on the ionization potential or electron affinity of the reactive group. Furthermore, substitutions that would raise the *c*-value of anionic groups reduce the oxidation-reduction potential. As an illustration, we refer to Fieser and Fieser

\* The convention regarding the sign of the oxidation-reduction potential commonly employed by physical chemists in Europe and practical electrochemists and biologists in America is the reverse of that commonly employed by physical chemists in America (values quoted from Latimer in Table 7.2, for instance; this system was introduced by Lewis and Randall, 1961). Thus, the  $\mathcal{E}^{\circ}$  value of oxygen in pure water is -815mv according to Latimer (1952, p. 40) but +815mv according to K. G. Stern (1944, p. 898). We shall follow the biological convention, giving a more positive value to a better oxidant and a more negative value to a better reductant.

(1935):  $\mathcal{E}^{\circ}$  for unsubstituted 1,4-naphthoquinone is 0.484v, 2-substitution with the acid-weakening  $\text{CH}_3$  group lowers  $\mathcal{E}^{\circ}$  to 0.408v, and 2-substitution with the acid-strengthening Cl group raises it to 0.508v. These data are for a highly con-

Substituted group	Quinones, 25°C	Indophenols, 30°C
Nitro		
Chloro	712 (Chloroquinone)	663 ( <i>o</i> -Chloro phenol indophenol)
Bromo	715 (Bromoquinone)	659 ( <i>o</i> -Bromo phenol indophenol)
Hydrogen	699 (Benzoquinone)	649 (Phenol indophenol)
Methyl	645 (Toluquinone)	616 ( <i>o</i> -Cresol indophenol)
Ethyl		
Isopropyl		
t-butyl		
Isopro-methyl	588 (Thymoquinone)	592 (Thymol indophenol)

Substituted group	Aldehydes and ketones, 25°C
Nitro	152 ( <i>m</i> -Nitroacetophenone)
Chloro	277 (Trichloroacetaldehyde)
Bromo	129 ( <i>p</i> -Bromoacetophenone)
Hydrogen	257, 270 (Formaldehyde)
Methyl	226 (Acetaldehyde)      257, 270 (Formaldehyde)      118 (Acetophenone)
Ethyl	129 (Di-methyl ketone)      110 (Di-ethyl ketone)
Isopropyl	220 (Isobutylaldehyde)      100 (Di-isopropyl ketone)
t-Butyl	211 (Trimethylacetaldehyde)
Isopro-methyl	

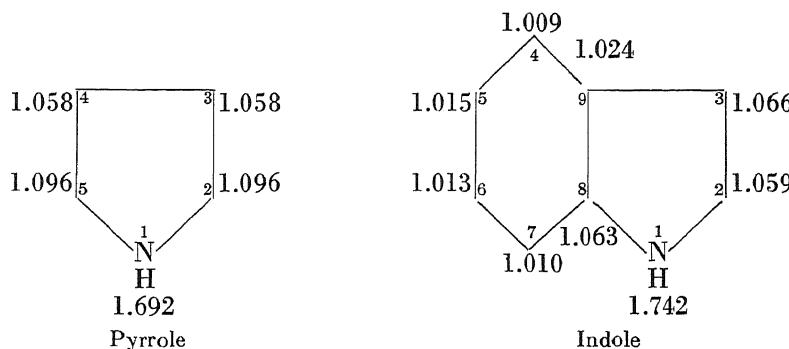
**Table 7.3. OXIDATION-REDUCTION POTENTIALS  $\mathcal{E}^{\circ}$  OF VARIOUS SUBSTITUTED COMPOUNDS.** The table illustrates the consistent effect of various substituent groups in modifying oxidation-reduction potentials. [Data from Adkins *et al.* (1949): aldehydes and ketones; from LaMer and Baker (1922): quinones; from Cohen *et al.* (1924): indophenols.] All values quoted are positive and in millivolts (see footnote on page 135).

jugated compound, but the same effect has been observed in simple alkyl derivatives. Such data are presented in Table 7.3. This table demonstrates that *the substitution of groups, which changes the c-value of an anion, has an essentially similar effect on  $\mathcal{E}^{\circ}$  values. Note, for example, that the substitution of three chlorine for H atoms on the  $\alpha$ -carbon (the conversion acetaldehyde  $\rightarrow$  trichloroacetaldehyde) increases*

the oxidation-reduction potential of the aldehydic group. This is an exact counterpart of the effect of the same substitution on the  $pK$  and hence the  $c$ -value of acetic acid, and on the proton-accepting power of ethyl ether. In a protein molecule, a particular functional group is generally separated from a variety of electron-accepting or electron-donating groups by a polypeptide chain, the resonating nature of which is particularly efficient in transmitting inductive effects. This indicates that nominally identical groups will have different oxidation-reduction potentials, depending on the nature of their neighboring groups. Consequently, a limited amount of a given chemical reagent will react differently with nominally identical functional groups at different loci. Furthermore, when the  $c$ -value ensemble varies, the individual  $\varepsilon^0$  values may be expected to vary correspondingly. A change of chemical reactivity could thus be brought about through the action of the  $F$ -effect.

## (2) Electron density

By the method of molecular orbitals, Longuet-Higgins and Coulson (1947) calculated the  $\pi$ -electron density of a number of N-containing heterocyclic compounds



**Figure 7.3. STRUCTURAL FORMULAS OF PYRROLE AND INDOLE.** Numerals within the ring structures indicate the numbering of the atoms in the ring. The numerals outside the ring structures indicate the  $\pi$ -electron densities. (Figure after Longuet-Higgins and Coulson, 1947.)

and showed that the greater the calculated  $\pi$ -electron density at a certain position, the greater the ease and rate of an electrophilic substitution at that position. On the other hand, at positions of lower  $\pi$ -electron density, nucleophilic substitutions become easier. Thus, for pyrrole, the  $\pi$ -electron density is greater at the 2- than at the 3-position. The reverse is true for indole (Figure 7.3). In agreement, the same electrophilic reagent attacks the 2-position of pyrrole and the 3-position of indole. (For reference and a more extensive list of similar examples see Badger, 1954.)

In an aromatic molecule like benzene, a  $\pi$ -electron may be considered as shared by all atoms. In the case of substituted aromatic molecules, the carbon atom at which the  $\pi$ -electron density is highest will have the highest affinity for electrophilic reactions. Equation (4-3) shows that the  $\pi$ -electron density, which is incorporated into  $\eta$ , is a component of the  $c$ -value. Thus the values of both  $\varepsilon^0$  and  $c$  are important in determining the chemical reactivity of aromatic molecules or groups. Although there is no simple relationship between ionization potential and  $\pi$ -electron density, the two tend to covary. Using the above reasoning, one can predict, for instance, that the phenolic groups of different tyrosine residues in a protein molecule will react differently toward chemical reagents; and that this difference will depend on both the  $\varepsilon^0$ - and the  $c$ -value of the phenolic group.

#### D. SUMMARY

To illustrate the inductive effect, we have used as our example the substitution of the electronegative chlorine atom into various molecules. We had previously shown that substituting chlorine for hydrogen in the acetate molecule greatly reduces the  $c$ -value (and, therefore, the  $pK$ ) of the carboxylic group. Since this change in  $pK$  is clearly obtained through an inductive effect (see Section 4.2A), the substitution of chlorine for the alkyl hydrogen in ethyl alcohol and in acetaldehyde must also work inductively to bring about the observed changes in H-bonding power and oxidation-reduction potential, respectively, of these molecules. This analogy provides a method for relating the  $c$ -value of the charged groups, the H-bonding power of the proton-donating and proton-accepting groups, and the  $\varepsilon^0$  value of oxidizable and reducible groups. Thus, if we can show that a particular agent can effect a change in any one of these parameters, we are theoretically justified in inferring that a change in the other two parameters may be effected in the same way. Having established the basis of the heterogeneity of the functional groups on proteins and the influence of transmitted effects on these groups, we are nearly ready to begin a discussion of the cooperative metastable states of protein molecules as a whole. There is one more fundamental aspect of proteins that we must discuss: the effect of polar-group fixation on interactions between proteins and ions or other proteins.

### 7.3. The Effects of Charge Fixation on Protein Behavior

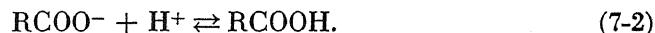
#### A. THE SIGNIFICANCE OF CHARGE FIXATION FOR SALT-LINKAGE FORMATION

According to the association-induction hypothesis, nearly complete association exists between fixed polar groups and counterions in a fixed-charge system. The reason is that the entropy gain of a hydrated ion dissociating within the fixed-

charge system is considerably less than the entropy gain enjoyed by an ion dissociating into free solution (Section 2.4). Within a fixed-charge system that contains both fixed cationic amino groups and fixed anionic carboxyl groups, the difference between the free energy of adsorption of a fixed cation (a fixed  $\epsilon$ -amino group, for example) and that of a "free" cation (a  $K^+$  ion, for example) is determined primarily by their dissociation energies. The  $K^+$  ion gains relatively little entropy when it dissociates; this is also true of the tethered  $\epsilon$ -amino group. However, once the amphoteric macromolecule is dislodged from its three-dimensional structure and brought into a dilute salt solution, it becomes a more or less linear polymer. Such degradation of a true fixed-charge system into a semifixed-charge system significantly alters the free energy of association of free ions. The fixed anion-counterion pair is now no longer surrounded by permanent energy barriers; as a result, the dissociating ion enjoys a much greater gain of configurational and rotational entropy. This favors its dissociation. But the fixed groups are tethered to the peptide chain; they are unable to gain the degree of freedom enjoyed by a free ion in free solution. Consequently, the value of their free energy of adsorption after the degradation of the system does not differ very greatly from its previous value and is, in general, favorable to adsorption and the formation of salt linkages. Thus, in a dilute solution of protein, more carboxyl groups will form salt linkages with fixed cationic groups than would form salt linkages if these cationic groups were on free monomeric amino acids and possessed comparable  $c'$ -values.

## B. THE EFFECT OF CHARGE FIXATION ON THE TITRATION OF POLAR GROUPS

We have provided reasons to believe that in a true fixed-charge system the fixed anionic and cationic groups are nearly always associated with free cations and anions like  $K^+$  and  $Cl^-$  or with each other in the form of salt linkages. In our discussion of the significance of charge fixation on protein behavior, we presented the hypothesis that proteins in solution will associate less with free cations and anions, but will nevertheless retain a high potentiality for forming salt linkages. It follows that the titration of a protein carboxyl group, for instance, will not always involve the simple acid dissociation



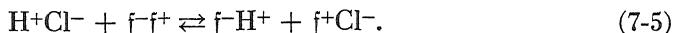
It will frequently involve the more complex reactions,



The degree to which one or the other of these schemes represents the total picture depends on more than one factor. Most important is the degree of fixation of the protein charged groups. The more limited the degrees of freedom for the dissoci-

ated ion pair, the greater the probability that equations (7-3) and (7-4) best approximate a description of the actual situation.

In a true fixed-charge system or in a semifixed-charge system, the titratable groups  $\text{RCOO}^-$  and  $\text{R}'\text{NH}_3^+$  may be represented as  $f^-$  and  $f^+$ . Then  $f^-f^+$  represents the salt linkage. If  $f^-$  were titrated\* with the strong acid HCl, equation (7-4) would assume the form:



In this case, we write four separate equations:



In terms of concentration (writing  $[\text{HCl}]$  for HCl concentration, and so on) we have

$$[\text{HCl}] = [\text{H}^+][\text{Cl}^-] \exp(-\Delta F_1/RT) \quad (7-10)$$

$$[f^-f^+] = [f^-][f^+] \exp(-\Delta F_2/RT) \quad (7-11)$$

$$[f^-\text{H}^+] = [f^-][\text{H}^+] \exp(-\Delta F_3/RT) \quad (7-12)$$

$$[f^+\text{Cl}^-] = [f^+][\text{Cl}^-] \exp(-\Delta F_4/RT). \quad (7-13)$$

The equilibrium constant  $K$  for equation (7-5) would be

$$K = \frac{[f^-\text{H}^+][f^+\text{Cl}^-]}{[\text{HCl}][f^-f^+]} = \exp[(\Delta F_1 + \Delta F_2 - \Delta F_3 - \Delta F_4)/RT]. \quad (7-14)$$

In the titration of a semifixed-charge system,

$$\exp[(\Delta F_1 + \Delta F_2 - \Delta F_3 - \Delta F_4)/RT] = \frac{[f^-\text{H}^+][f^+][\text{Cl}^-] \exp(-\Delta F_4/RT)}{[\text{H}^+][\text{Cl}^-] \exp(-\Delta F_1/RT)[f^-f^+]} \quad (7-15)$$

Taking the logarithm of both sides and rearranging, we have

$$\frac{\Delta F_2 - \Delta F_3}{2.3RT} = \text{pH} + \log \frac{[f^-\text{H}^+]}{[f^-f^+]} + \log [f^+] \quad (7-16)$$

and when  $[f^-\text{H}^+] = [f^-f^+]$  at the point of half titration,

$$\text{pH} = \frac{\Delta F_2 - \Delta F_3}{2.3RT} - \log [f^+] = \text{pK}. \quad (7-17)$$

This shows that if the titrated group of a soluble protein in a semifixed-charge system was originally joined in a salt linkage, the  $\text{pK}$  value determined as the  $\text{pH}$  at the half point of neutralization is not merely an expression of  $\Delta F_3$  as for equation (7-2); it also includes the  $\Delta F_2$  and the concentration of the fixed cations.

\* For simplification, we are assuming titration in the absence of salt so that equation (7-3) is applicable. Were salt present, as in equation (7-4), the analysis would be similar to equations (7-6) to (7-18), but much more complex.

In a true macroscopic fixed-charge system, an additional restriction exists: Macroscopic electroneutrality must be conserved within the system. Thus

$$[\text{Cl}^-] = [\text{H}^+]$$

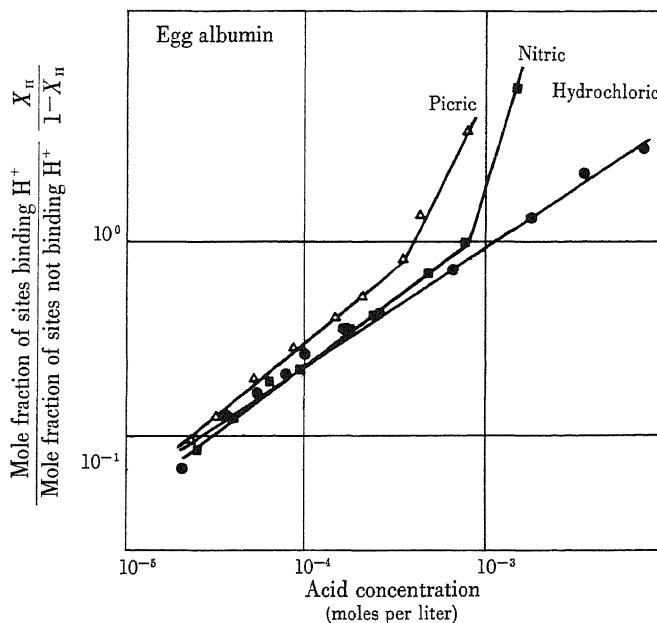
and

$$[\text{f}^-\text{H}^+] = [\text{f}^+\text{Cl}^-] = [\text{f}^-\text{f}^+] = \frac{1}{2}[\text{f}^-]_{\text{total}} = \frac{1}{2}[\text{f}^+].$$

One can derive from equation (7-15) that

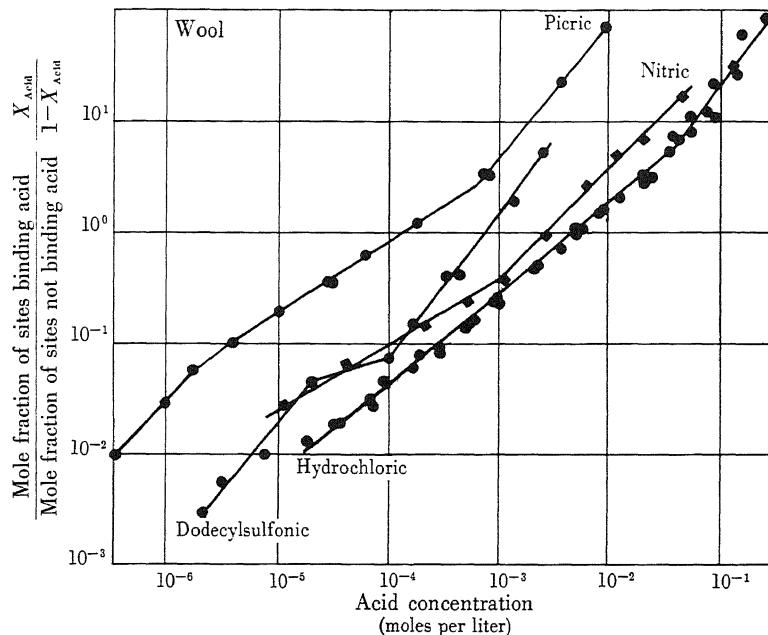
$$\text{pH} = \frac{1}{2}[(\Delta F_2 - \Delta F_3 - \Delta F_4)/RT] - \frac{1}{2} \log \left( \frac{[\text{f}^-]_{\text{total}}}{2} \right). \quad (7-18)$$

Thus, in a true fixed-charge system, the apparent  $pK$  is not merely a measure of  $\Delta F_3$  and  $\Delta F_2$ ; it also involves  $\Delta F_4$  and the concentration of fixed anions. In Section 7.3A we showed that, from a consideration of the entropy of dissociation, one might expect a high degree of salt-linkage formation in proteins in dilute solution. This being the case, titration of such proteins should show a dependence not only on the free energy of association of the fixed anion with the  $\text{H}^+$  ion ( $\Delta F_3$ ) but also on the free energies of association of the fixed anion and fixed cation ( $\Delta F_2$ ) and of



**Figure 7.4.** LOG-LOG PLOTS OF THE TITRATION CURVES OF EGG ALBUMIN WITH VARIOUS MONOVALENT STRONG ACIDS (22°C). The ratio of the mole fraction of sites binding the  $\text{H}^+$  ion to the mole fraction of sites not binding the  $\text{H}^+$  ion is plotted on a log scale against the  $\text{H}^+$ -ion concentration, also on a log scale. See equation (5-12) and Section 5.5. (Data from Steinhardt *et al.*, 1941.)

the fixed cation and free anion ( $\Delta F_4$ ). This prediction is borne out by the actual titration of certain proteins in solution. Figure 7.4 presents a log-log plot [see Section 5.5, equation (5-12)] of the data of Steinhardt *et al.* (1941, 1942) on acid titration of egg albumin, which is a semifixed-charge system. Figure 7.5 presents a similar titration curve for wool; this is a true fixed-charge system. The dependence



**Figure 7.5. LOG-LOG PLOTS OF THE TITRATION CURVES OF WOOL PROTEIN WITH VARIOUS MONOVALENT STRONG ACIDS (0°C).** For further explanation, see Figure 7.4 and Section 5.5. (Data from Steinhardt *et al.*, 1941.)

of the titration curves of wool with various strong acids on the affinity of the anions is clearly shown; the upward displacement of the curve in Figure 7.4 indicates a greater amount of acid bound (since these strong acids are completely dissociated in solutions at this concentration and pH range). Steinhardt and co-workers emphasized this fact. For egg albumin in solution (a semifixed-charge system) the relation is somewhere between that given by equation (7-2) and that given by equation (7-4), as shown by the first segment\* (acid concentration less than  $5 \times 10^{-4} M$ ) of the curves in Figure 7.4. Here the displacement of the curves produced by variation of the anion is relatively much less; this indicates only a

\* The sharp bends in the curves of Figure 7.4 beyond an acid concentration of  $5 \times 10^{-4} M$  mark the beginning of protein denaturation; we shall discuss them extensively in Section 7.4.

slight dependence on the nature of the anion. Following their realization that anion binding was of importance in the titration process, Steinhardt *et al.*, in their estimation of the free energy of acid binding, took into account the factors we have called  $\Delta F_3$  and  $\Delta F_4$ , but they did not consider the factor we call  $\Delta F_2$ . In their later work on the acid titration of ferrihemoglobin, however, Steinhardt and Zaisser (1955) arrived at the conclusion that salt linkages also participate in the titration of this protein.

It is clear that the  $\Delta H'$ -versus-pH plot for different proteins (Figures 7.1 and 7.2) may involve complex reactions as shown in equations (7-3) and (7-4). However, since the degree of anion participation as well as the extent of salt-linkage formation are both functions of the  $c$ -value, the  $\Delta H'$ -versus-pH plot in the acidic range remains essentially a function of the  $c$ -value "profile"; as such, it is a useful representation of the  $c$ -value ensemble of a protein.

#### 7.4. The Cooperative States of Protein and Their Transformation

Having defined a protein in terms of its amino-acid composition, the sequential arrangement of these amino acids in the macromolecule, and the  $c$ -,  $c'$ -, and  $\varepsilon^0$  values of its polar groups, we suggest that the properties of a particular protein in a particular environment are the expression of a *cooperative metastable state* (see Sections 5.4 and 5.5). Shifts from one cooperative state to another, as seen in protein denaturation, can be effected through alteration of the environment: changes in temperature, pH, and ionic composition of the medium, also addition of heavy-metal ions, and so on. We shall show that such shifts always involve changes in the adsorption of various ions and molecules onto the protein; thus, before beginning a detailed discussion of transition from one metastable state to another, we shall take up certain aspects of adsorption onto proteins.

##### A. ADSORPTION ONTO PROTEINS

The most striking characteristic of adsorptions of ions and molecules onto proteins is that they do not follow the laws of mass action. Two interpretations have been offered. The first interpretation may be called a heterogeneity theory. It states that all of the adsorbing sites do not have the same affinity for the adsorbent. As a consequence, the sites that have the highest preference for a particular adsorbent will be the first to combine, followed by the next-preferred sites. If the amount of adsorbent taken up by each mole of protein  $r_A$  is plotted against the concentration (or more accurately, the activity) of the external free adsorbent [A] and if  $N$  is the total number of binding sites per mole of protein, the relation

$$\frac{N}{r_A} = \frac{1}{1 - f(A)} \quad (7-19)$$

has been derived (Karush and Sonenberg, 1949). If one plots the data obtained from this equation in a log-log plot, one sees that the function  $f(A)$  is such that

$$\left( \frac{X_A}{1 - X_A} \right)^{\frac{1}{n}} \frac{1}{[A]} = K' \quad (7-20)$$

where  $X_A = r_A/N$  is the mole fraction of sites on the protein occupied by A; and the value of  $n$  is less than unity (see below). In logarithmic form, equation (7-20) is identical with equation (5-12).

Another explanation for the deviation of adsorptions on proteins from the law of mass action attributes the interaction to a macroscopic electrostatic effect. Thus, as  $H^+$  ions react with an isoionic macromolecule, a net excess positive charge will be built up causing a macroscopic repulsion of  $H^+$  ions reacting later. This electrostatic theory, which we owe to Linderstrøm-Lang (1924), Cannan *et al.* (1941), and Scatchard (1949), may, in its application to acid titration, be expressed by the equation:

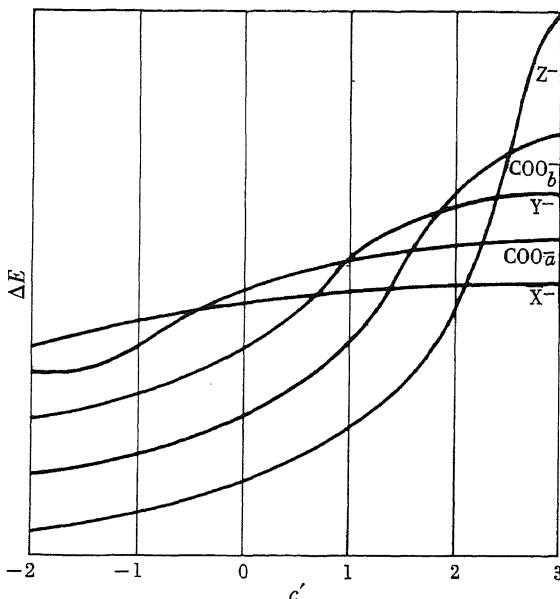
$$\log \frac{N - r_H}{r_H} = pH - pK + \frac{2\omega'r_H}{2.303} \quad (7-21)$$

Here  $r_H$  represents the number of  $H^+$  ions bound per mole of protein,  $N$  is the total number of binding sites per mole, and  $\omega'$  is a constant that applies for a particular protein in a particular medium. In studies using adsorbed entities that are highly soluble (acids or detergents), the maximum number of ions bound corresponds to the number of fixed cationic or anionic sites in the proteins. In some cases, such as the titration curves of egg albumin and  $\beta$ -lactoglobulin, the complete titration curves may be explained by the electrostatic theory (Cannan *et al.*, 1941, 1942). In other cases, if adsorption within the *lower* concentration ranges of the acid or detergent is considered, the observations are consistent with both the heterogeneity theory and the electrostatic theory. However, as higher concentrations of the adsorbents are reached, an abrupt change in the mechanism of adsorption apparently takes place and the protein denatures. In this range, neither the heterogeneity theory nor the electrostatic theory adequately describes the adsorption phenomenon.

We shall show that, by adding to the heterogeneity and electrostatic factors the critical and sometimes overwhelming contribution of the inductive effect, we can account for the entire range of the adsorption isotherms; and in doing so we can give an interpretation of the basic mechanism of the phenomenon of protein denaturation. Before we present a general interpretation, we shall discuss a specific case of adsorption to illustrate that the association-induction hypothesis can explain experimental findings that are not directly explicable on the basis of other theories.

Karush (1951) studied anionic dye binding on bovine serum albumin and found that the binding capacity of this protein for the anionic dye, methyl orange, undergoes a slight *increase* when the  $pH$  is increased from 6.4 to 7.6. This  $pH$  change,

according to Karush, should increase the total negative charge of the protein molecule from  $-8$  to  $-16$ . According to the electrostatic theory, such an increase of negative charge should cause a definite *decrease* in methyl-orange binding. Karush emphasized the discrepancy between the experimental findings and the prediction of the electrostatic theory. The results are explicable within the framework of the present theory. Referring to Figure 7.6, let us assume that, at pH 6.4,



**Figure 7.6. THE VARIATION IN ADSORPTION ENERGIES OF ANIONIC ADSORBENTS WITH THE  $c'$ -VALUE OF A FIXED CATIONIC GROUP.** Free anions are represented as  $X^-$ ,  $Y^-$ , and  $Z^-$ . Fixed anionic groups  $COO^-_a$  and  $COO^-_b$  represent carboxyl groups with  $c$ -values equal to  $c_a$  and  $c_b$ , respectively.

the fixed anionic groups  $COO^-_a$  have a  $c$ -value equal to  $c_a$ , and that the fixed cationic groups have a  $c'$ -value of  $-1$ . Under these conditions, most of the fixed cationic groups would be bound to and occupied by  $COO^-_a$  groups; only a few fixed cationic sites would be free to bind the anionic dye  $Y^-$  (methyl orange). An increase in the pH of the medium would result in the loss of protons from some neighboring acidic groups on the protein. The protons would be replaced by less strongly adsorbed cations like  $Na^+$ , by fixed cationic groups, or the sites would be left vacant. In any event, the direct  $F$ -effect could thereby increase the  $c'$ -value of site A from  $-1$  to  $+1$  with a resultant increase of methyl-orange ( $Y^-$ ) binding,

mainly at the expense of fixed carboxyl groups. This explanation predicts that pre-existing salt linkages will break and the protein molecules will unfold as a result of changing the pH in the presence of methyl orange. (For discussion of unfolding mechanism, see Section 7.4C.) That this actually happens was suggested by Klotz (1953) and by another series of experiments by Karush (1952; see also Lundgren *et al.*, 1943) in which he demonstrated that when an anionic azo dye is bound to bovine serum albumin, it facilitates the binding of a second optically isomeric molecule. Karush suggested ". . . that the initial binding of the dye causes the rupture of secondary intramolecular bonds leading to a partial and reversible opening-up and thereby making additional combining regions available." The present theory offers a mechanism for the rupture of these bonds. The essence of this mechanism is, of course, the *F*-effect. Its action brings about an alteration of *c*-value and this changes the preference of certain cationic sites from a fixed anion to methyl orange, demonstrating the principle of the variability of the preferred counterion. Other and more detailed features of adsorption on proteins will become evident as we pursue our discussion of the transformation of protein from one metastable cooperative state to another.

## B. PROTEIN DENATURATION

Having defined the cooperative metastable state of a protein in terms of the *c*-, *c'*-, and  $\varepsilon^0$ -value ensembles consistent with the particular environment, we suggest that *a shift of the c, c'; and  $\varepsilon^0$  ensembles from a natural (native) state to another stable or metastable state constitutes denaturation.\**

In Chapter 3, we demonstrated that all reactive proteins possess some 20 per cent of trifunctional amino-acid residues, approximately half cationic, half anionic. This means that a large percentage of these polar side chains must be separated from their nearest-neighbor polar side chains by a length of polypeptide chain no greater than the limit of the effective direct *F*-effect (see Section 5.2). Thus, in most reactive proteins we are likely to find either as isolated sites or as parts of gangs one or more of the following combinations of neighboring pairs: (1) — —, — — —, — — — —, + +, + + +, + + + +; (2) + — + —; (3) —, +, + —. As we stated in Chapter 5, adsorption on chains of similar charges as in (1) is heterocooperative; adsorption on chains bearing alternating positive and negative charges as in (2) is autocooperative; and adsorption to isolated or semi-isolated sites as in (3) is independent.

We shall present the view that, in proteins containing various proportions of these different arrangements of polar side-chains, the existence of a metastable

\* Earlier views on the definition and interpretation of the phenomenon of protein denaturation can be found in the following articles and reviews: Wu (1931), Bull (1941), Neurath *et al.* (1944), Anson (1945), Haurowitz (1950), Putnam (1953), and Joly (1955).

native state, and (under extreme conditions) its all-or-none transformation into a denatured state follows naturally from the present hypothesis.

### (1) The stability of the native metastable state of proteins

The ability of proteins to maintain considerable stability in their native state is a very striking feature, particularly when one considers the relative ease with which they can be "tripped" into another state, given the proper conditions. We suggest that this stability in the native state is due basically to two types of long-range cooperative phenomena: One is entropic in nature; the other, which will be emphasized in this chapter, has an electronic interpretation.

We have stated that the formation of salt linkages is favored by an entropy factor not enjoyed by the adsorption of free ions (Section 7.3A). The same applies to backbone-to-backbone H-bond formation within a given molecule or between protein molecules. The formation of one salt linkage or H-bond fixes the protein chains in space, decreases the entropy of dissociation of the next pairs of sites, and thus favors the formation of more salt linkages and more H-bonds. The networks and folded configurations thus achieved through a large number of cooperative adsorptions have the effect of stabilizing a unique native configuration.

The electronic type of cooperative stabilizing influence can be illustrated best with a discussion of the response of a native protein when it is brought into contact with a denaturant. Most chemical denaturants possess a component or components that react more strongly with proteins than with the substances they replace on the protein molecule. Referring to the  $\Delta E$ -versus- $c$ -value and  $\Delta E$ -versus- $c'$ -value plots (Figures 4.9 to 4.11), we conclude that these denaturing substances prefer sites with higher  $c$ - and  $c'$ -values. Consider HCl; the more important component is clearly the  $H^+$  ion ( $0.1N$  HCl is a very effective denaturant;  $0.1N$  NaCl is not); it is most preferred at the highest  $c$ -value.

In our discussion of the heterogeneity of sites, we suggested that the intrinsic  $c$ - or  $c'$ -value of a polar group on a protein is basically determined by the group's own nature and the nature of its immediately neighboring amino-acid residues, particularly the trifunctional ones. Thus an anionic site flanked exclusively by other anionic sites as in  $-- -$  will have a very high  $c$ -value; another anionic site flanked exclusively by cationic sites as in  $+ - +$  will have a much lower  $c$ -value. When present in low concentration, the  $H^+$  ion will react only with the type of site having a higher  $c$ -value (that is, a high  $pK$ ). An adsorption onto a linear chain of similarly charged sites is heterocooperative; that is, given adjacent anionic sites A and B, if an  $H^+$  ion adsorbs onto site B, displacing a less strongly adsorbed counterion (an amino group on a neighboring chain), then the direct  $F$ -effect will act to decrease the  $c$ -value of site A. This will cause site A to favor its original counterion (perhaps also an amino group) even more than before and the  $H^+$  ion

even less. Thus, a heterocooperative interaction has the effect of continuously decreasing the affinity of a protein for a potentially disturbing agent, in this case the H<sup>+</sup> ion; in so doing, it acts to preserve the original metastable state.

This type of interaction gives rise to an *n*-value less than unity in a log-log plot (Section 7.4A); this effect cannot be distinguished readily from that arising from the intrinsic heterogeneity of sites or from the macroscopic electrostatic effect. Heterocooperative interaction is, however, totally compatible with the results of most adsorption studies in low concentration ranges where the heterogeneity or the macroscopic electrostatic factors, alone or together, have been invoked and found to fit the experimental adsorption curves. Examples appear in studies of the adsorption of dyes onto serum albumin (for review, see Klotz, 1953). In these studies, due to the low water solubilities of the dyes used, the range of the adsorption isotherms investigated is usually limited. The results uniformly conform to a pattern of less-than-unity *n*-values in a log-log plot. Similarly, the rule applies to the lower concentration ranges of hydrochloric-acid binding by hemoglobin as studied by Steinhardt and Zaisser (1951, 1953, Figures 7.9, 7.10), and to the binding of the anionic detergent, sodium dodecylbenzenesulfonate (SDBS), on bovine serum albumin studied by Foster and Yang (1954, Figure 7.8). However, since both HCl and SDBS have high solubilities, these studies were not limited to low concentration ranges. At higher concentrations, the low *n*-value pattern abruptly changes to one of high *n*-value; the protein then denatures.

## (2) The all-or-none transformation into the denatured state

Although sites of high *c*-value react first and soften the initial onslaught of a denaturant through heterocooperative interaction, as the denaturant concentration increases, these buffering sites will eventually be exhausted. Next, the H<sup>+</sup> ion may react with the isolated sites. Aside from contributing to heterogeneity, this will have little effect on the protein as a whole. Thus, up to this point in the acid binding, the total adsorption curve typically shows a slope of less than unity in the log-log plot. However, as the concentration of denaturant increases further, sites with even lower *c*-values (those having oppositely charged groups as neighbors) will begin to react.

From the consideration of the entropy of dissociation of fixed ions (Section 7.3A), it follows that there is a marked tendency for fixed anions and cations to form salt linkages. Many may not participate in the formation of such salt linkages because they do not have oppositely charged fixed groups in their immediate vicinity. However, the low *c*- and *c'*-value sites under discussion are probably joined in salt linkages since it is the presence of neighboring sites of opposite charge that causes them to have low *c*-values. Let us assume the H<sup>+</sup> ion is preferred at high *c*-value (see Figures 4.9 to 4.11) and the Cl<sup>-</sup> ion at high *c'*-value. HCl thus reacts as B<sup>+</sup>Y<sup>-</sup> in Figure 5.3. Reaction of HCl as of B<sup>+</sup>Y<sup>-</sup> with chains of alter-

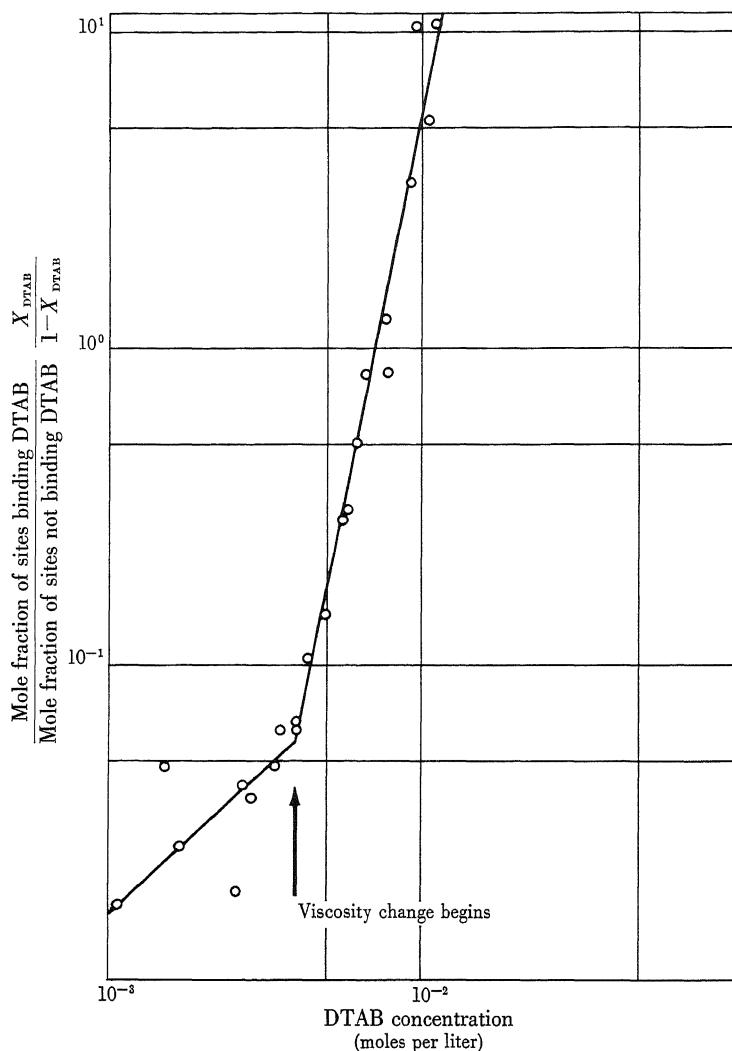
nating cationic and anionic sites is autocooperative: adsorption of an  $H^+$  ion favors the adsorption of a  $Cl^-$  ion on the next cationic site 2; this adsorption of a  $Cl^-$  ion on site 2 favors adsorption of an  $H^+$  ion on site 3. This indirect  $F$ -process continues until the whole gang of cooperative sites is occupied by  $H^+$  or  $Cl^-$  ions; as stated in Section 5.3B, the process will be all or none and will also be time and temperature dependent. In this way, the macroscopic conformation of the protein molecules, held together by the salt linkages, is transformed into a new denatured "unfolded" conformation.

To summarize, the hypothesis of direct and indirect  $F$ -effects leads to the following consequences. In one part of a protein molecule, one finds a chain or chains of sites bearing the same sign: (1) these sites will have high  $c$ - or  $c'$ -value (high  $pK$ , and hence large free energy of adsorption  $\Delta F$ ), and consequently they will always react first in a titration process; (2) since adsorption on these sites is heterocooperative, in the earlier part of such a titration the protein will tend to resist changes by the denaturant. In another part of the protein molecule, there will be a chain or chains of alternating cationic and anionic sites: (1) these sites will have low  $c$ - or  $c'$ -values (low  $pK$  and hence small free energy of adsorption  $\Delta F$ ) and consequently they will not react with the titrant until a higher concentration range is reached; (2) since they always have fixed sites of opposite charge nearby, they have a great tendency to form salt linkages, possibly contributing to the formation of helical or folded native configurations; (3) adsorption of denaturants on these sites is auto-cooperative, thereby bringing in the all-or-none indirect  $F$ -process that leads to dissolution of salt linkages and  $H$ -bonds and hence to unfolding and loss of the original native configuration.

(3) The change from heterocooperative to autocooperative interaction: an indicator of denaturation

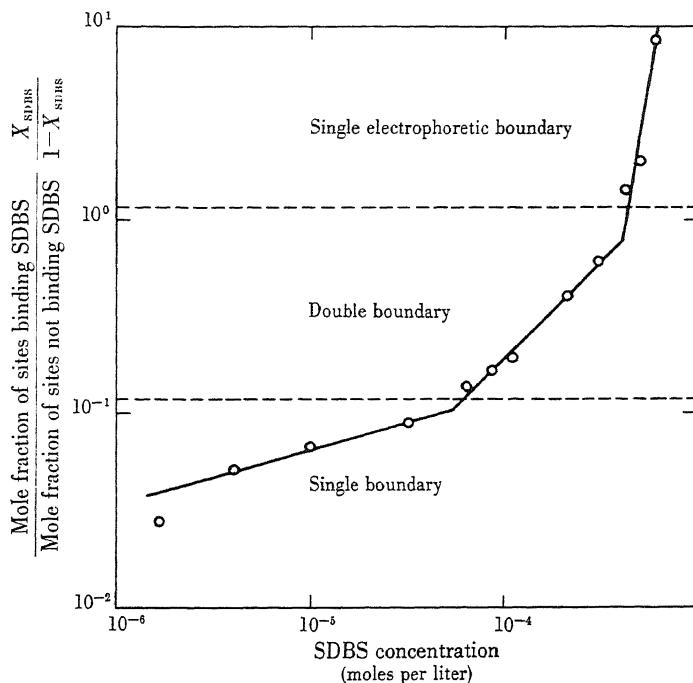
We have reasoned that the first reaction of a protein toward a denaturing agent is always one which tends to resist the disturbing effect. The heterocooperative interaction that underlies this response reveals itself as a less-than-unity  $n$ -value in a log-log plot. The indirect  $F$ -process that follows represents the denaturation process and gives rise to a greater-than-unity  $n$ -value. Our thesis, therefore, demands that, if the concentration of adsorbed denaturant is plotted against the free denaturant concentration in a log-log plot, the onset of denaturation should always be accompanied by an abrupt  $n$ -value change.

Lundgren *et al.* (1943) first observed by electrophoretic means that the anionic detergent, alkylbenzene sulfonate, causes a liberation of structurally unavailable groups in ovalbumin; these liberated groups then combine with the more anionic detergent. The existence of this phenomenon was verified and extended to other protein systems (Putnam and Neurath, 1945; Foster and Yang, 1954; Pallansch and Briggs, 1954; Few *et al.*, 1955). The liberation of previously unavailable



**Figure 7.7. THE BINDING OF THE CATIONIC DETERGENT, DODECYLTRIMETHYLMONIUM BROMIDE, BY BOVINE SERUM ALBUMIN (20°C).** The arrow indicates the concentration at which the relative viscosity of the mixture abruptly rises; this point, given by the authors, roughly coincides with the point at which the slope of the replotted data abruptly changes from 0.92 to 5.0. Dodecyltrimethylammonium bromide is represented as DTAB. The total number of binding sites, 109 per mole, is the maximum binding capacity given by the original authors. [Data (which are plotted on a log-log scale) calculated from those of Few *et al.* (1955).]

groups was shown to coincide with other changes in physical and chemical properties usually associated with denaturation, among them, optical rotation and viscosity changes. In Figure 7.7 we have plotted, according to equation (5-12), the

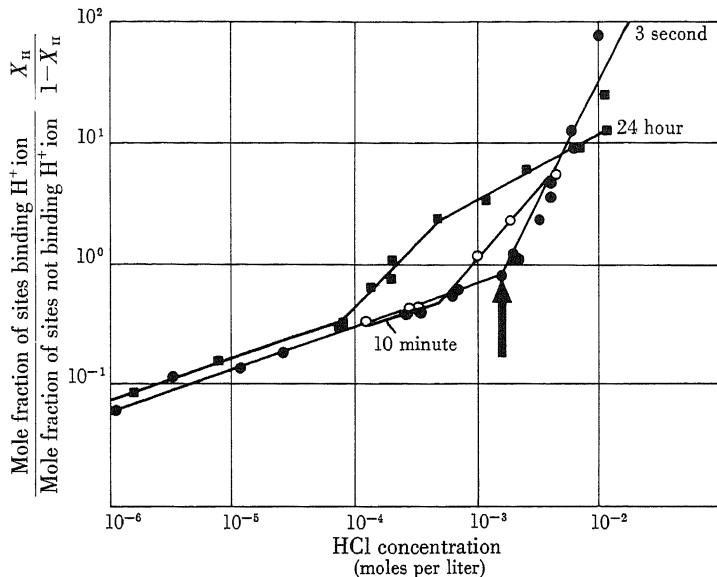


**Figure 7.8. THE BINDING OF THE ANIONIC DETERGENT, SODIUM DODECYLBENZENESULFONATE, BY BOVINE SERUM ALBUMIN (1-3°C).** The lower inflection point coincides roughly with the point (indicated by the lower dotted line) at which the single electrophoretic boundary splits into a double boundary; the upper inflection point coincides roughly with the point at which the electrophoretic boundary becomes single again (indicated by the upper dotted line). The dotted lines were introduced by the authors. SDBS represents sodium dodecylbenzenesulfonate. The total number of anion-binding sites, 101, is the sum of the number of arginine, lysine, and histidine residues per protein molecule (see Tristram, 1953, Table 17). [Data (plotted in this figure on a log-log scale) calculated from the experimental values of Yang and Foster (1953).]

data of Few *et al.* (1955) for the adsorption of dodecyltrimethylammonium bromide onto bovine serum albumin. The curve that describes this adsorption is best fitted by a theoretical curve consisting of a straight portion followed by an abrupt change of slope from 0.92 to about 5.0; this change occurs at approximately the same free-detergent concentration ( $4.3 \times 10^{-3} M$ ) as that at which viscosity changes begin ( $5.2 \times 10^{-3} M$ ).

The data of Yang and Foster (1953) on the effect of the anionic detergent,

sodium dodecylbenzenesulfonate, SDBS, on bovine serum albumin have been plotted on a log-log graph in Figure 7.8. The abrupt changes of  $n$ -value occurred at detergent concentrations corresponding to those which the authors gave as marking changes in the electrophoretic boundary. The first change was a split

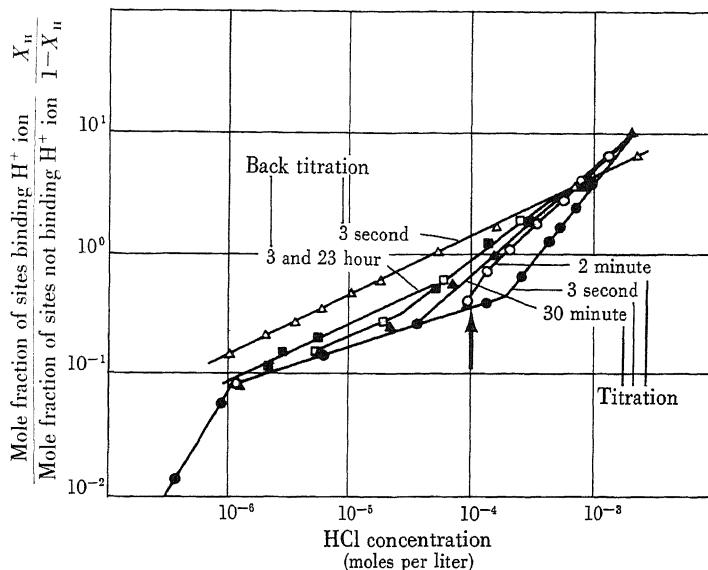


**Figure 7.9.** ACID BINDING BY CARBONYLHEMOGLOBIN ( $25^\circ\text{C}$ ). The data plotted on a log-log scale shows the time-dependent unmasking of acid-binding groups. The arrow points to the acid concentration beyond which the three-second titration value no longer represents instantaneous equilibrium; this point coincides with the sharp break in the slope of the experimental values. The authors give the total number of sites binding the  $\text{H}^+$  ion as 1.6 moles per kilogram of protein. The titration was performed in the absence of salt. [Data calculated from experimental values of Steinhardt and Zaiser (1951).]

from a single to a double boundary; the second was a fusion of the two boundaries to form a new single boundary.

Steinhardt and Zaiser (1951, 1953) presented their remarkable work on the acid denaturation of carbonylhemoglobin and ferrihemoglobin. The combination of hydrogen ions with a small number of trigger groups leads to the liberation of a much larger number of acid groups. This liberation is time dependent and, at room temperature, it takes hours to reach the new equilibrium. Figure 7.9 presents a log-log plot of the data of Steinhardt and Zaiser on the titration of carbonylhemoglobin in the absence of salts. The line of solid circles represents the pH value registered three seconds after mixing the carbonylhemoglobin and acid. At a pH of about 2.76, the slope of the curve changes abruptly from  $n = 0.36$  to  $n = 1.89$ .

This is also the point ( $\text{pH } 2.8$  to  $3.0$ )\* beyond which Steinhardt and Zaiser found that the three-second titration no longer represents an instantaneously reversible equilibrium. For ferrihemoglobin, the point of abrupt slope change is at  $\text{pH } 3.7$  (Figure 7.10), and the authors state that the curve could not be regarded as



**Figure 7.10.** REVERSIBLE ACID BINDING BY FERRIHEMOGLOBIN ( $25^\circ\text{C}$ ). These data, plotted on a log-log graph, show the reversibility of the time-dependent unmasking of acid-binding groups. The arrow indicates the approximate acid concentration beyond which the three-second titration no longer represents instantaneous equilibrium; this point coincides roughly with the sharp break of the slope. The authors gave the total number of acid-binding sites as 1.5 moles per kilogram of protein. [Data calculated from experimental values of Steinhardt and Zaiser (1953).]

instantaneously reversible "below about  $\text{pH } 4.0$ ." In other words, at higher acid concentrations, the denaturation process has already set in three seconds after mixing. This is another example of an abrupt  $n$ -value change marking the point at which denaturation occurs.

We emphasize that, although these data are all in agreement with the concept that denaturation is accompanied by a change in  $n$ -value, an abrupt  $n$ -value shift does not necessarily indicate an indirect  $F$ -process. This is clear from Figure 5.6. In the theoretically calculated "titration curve" of Simms, a more or less abrupt change of  $n$ -value is apparent. In this case, the  $n$ -value shift marks the beginning of the titration of carboxyl groups which brings about a change of slope from the flatter region where little buffering capacity exists.

\* In the 1953 paper the authors altered this limit to  $\text{pH } 3.2$ .

## C. THE MOLECULAR BASIS OF PROTEIN DENATURATION

The three most commonly recognized features of protein denaturation are changes of dimension and state of aggregation, the unmasking of reactive groups, and a decrease in solubility. Other features pertaining to changes in biologically specific properties such as immunological characteristics and enzymatic activity may be inferred from the discussion of each of these specific physiological topics in later chapters.

### (1) Aggregation and dimensional change in protein

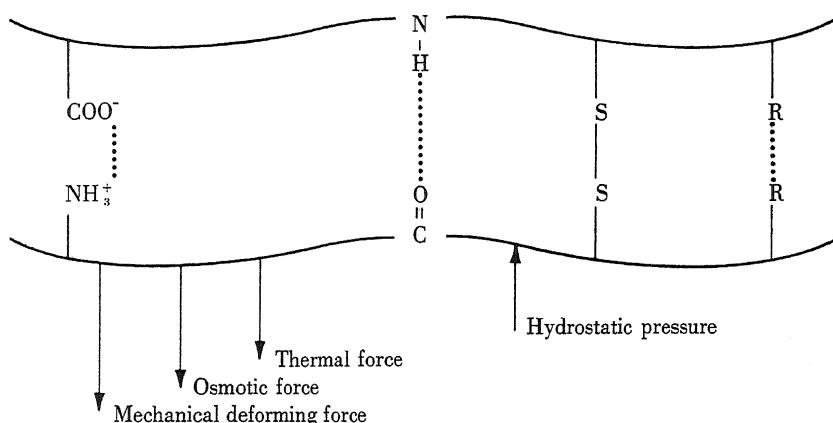
Protein molecules undergo changes of form without hydrolytic cleavage. This indicates that proteins must contain bonds which are stable under many conditions but which may be broken rather easily, given the proper circumstances (see Chapter 15 on contractile phenomena). We shall restrict our discussion to three types of such bonds, the salt linkage, the disulfide bond, and the hydrogen bond. That these three types are of major importance in stabilizing the structure of some proteins (synthetic fibers made from feather keratin, for example) has been known for some time (see Figure 7.11).\*

(a) *The salt linkages.* In free solution at 0.5M aqueous concentration, the dipolar ion, glycine, has an activity coefficient of 0.91 (see Smith and Smith, 1937; Scatchard and Prentiss, 1934); for eleven  $\alpha$ -amino acids in 0.5M aqueous solution, the average activity coefficient is greater than unity, that is, 1.05 (Smith and Smith, 1937). This shows that, at this concentration, there is no tendency for the  $\alpha$ -amino group to associate with the  $\alpha$ -carboxyl group. Protein contains polar groups at concentrations of less than 0.5M (see Chapter 3). Thus, without further support, the *a priori* assumption that a high degree of association exists between the amino groups and carboxyl groups of protein (Speakman and Hirst, 1931; Speakman and Stott, 1934) is not safe from criticism. However, a consideration of the differences in entropy that occur with charge fixation as discussed in Sections 2.4 and 7.3A enables us to deduce a mechanism for salt-linkage formation in significant quantity. The complete dissociation of amino and carboxyl groups in free amino-acid solutions is due to the high entropy gain on dissociation. In the fixed-charge system, a large entropy gain on dissociation does not occur; consequently, a high degree of association or salt-linkage formation is the result.

We can understand the mechanism whereby the salt linkage is formed and broken if we refer to Figures 4.9 to 4.11. If we assume that the ammonium ion can

\* A fourth type of interaction between protein molecules, the van der Waals' forces between nonpolar side chains, is shown in Figure 7.11. Notice that we have included such van der Waals' forces in the calculation of the energies of electrostatic and hydrogen bonds. The van der Waals' forces between the nonpolar groups on proteins are due to the London dispersion energy and are thus small (see Table 6.1). For this reason, we have not included this fourth type of bond in our discussion. For discussion of other types of hydrogen bonds, see Haurowitz, 1950; Benesch *et al.*, 1954; and G. I. Loeb and Sheraga, 1956.

serve as a prototype of the  $\epsilon$ -amino group or guanidyl group on proteins, the relation of the  $c$ -value to the relative affinity of the carboxyl group for  $\text{NH}_4^+$ ,  $\text{K}^+$ , and  $\text{Na}^+$  ions leads us to expect that the percentage of salt-linkage formation will also be a function of the  $c$ -value. As the  $c$ -value varies, there will be more or less salt-linkage formation within the same protein or with other proteins bearing suitable groups of the appropriate  $c'$ -values. This would lead to changes in dimension and



**Figure 7.11. THREE TYPES OF LIGANDS MAINTAINING THE STRUCTURE OF PROTEINS.** By employing complex solvent systems of alcohol and water containing reducing agents, Lundgren obtained evidence which demonstrated that the disulfide bonds, salt linkages, and hydrogen bonds are the major ligands maintaining the structure of synthetic fibers made from feather keratin. The other labels indicate other forces influencing the equilibrium state of the protein system. (Figure after Lundgren, 1949.)

the state of aggregation, usually described as folding and unfolding, aggregation and disaggregation, association and dissociation.

(b) *The disulfide linkage.* The concept that the S—S bonds of cystine may be responsible for linkages of protein molecules was introduced rather recently (Huggins *et al.*, 1951; Kauzmann and Douglas, 1956). However, as pointed out by Mazia (1954), S—S bonds cannot be the only type of intermolecular bond even in a protein as high in cystine content as wool keratin (more than 10% of  $\frac{1}{2}$  cystine residues) or complete reduction by thioglycolic acid in neutral solution would lead to complete dissociation of the protein; this does not occur (Patterson *et al.*, 1941; Speakman, 1936). In general, the cystine content of other proteins is much lower than that of wool keratin; thus the S—S linkages must be relatively unimportant (see Figure 0.2). In Chapter 8, we shall show that, if the sulphydryl-

to-disulfide reaction does proceed during denaturation, then the extent of the reaction will depend on the  $\varepsilon^0$  value of the SH groups; this is a function of the *c*-value. The S—S bond must make some contribution to intramolecular or intermolecular linkage, but we adopt the view that the most important role of S—S and SH groups in dimensional and aggregational changes is an indirect one. Since the reduction or oxidation of S—S or SH groups involves the gain or loss, respectively, of electrons, it leads to a profound alteration of the local *c*-value ensemble through the *F*-effect. This may influence the extent of salt-linkage and hydrogen-bond formation which brings about changes in dimension and state of aggregation.

(c) *The hydrogen bond.* An important type of intramolecular or intermolecular bond in crystalline dry protein is the direct peptide-to-peptide hydrogen bond (see Figure 2.10; Astbury, 1933, 1940; Pauling and Corey, 1951, 1953). In aqueous solution or as part of a living-cell fixed-charge system, the *c*- and *c'*-value analogues of the C=O and NH groups could determine whether direct peptide linkages or peptide-water linkages are statistically preferred. In other words, this type of bond will also vary its specificity according to the *c*-value ensemble of the protein.

The dimensions and state of aggregation of a protein are functions of the number of salt linkages, disulfide linkages, and peptide-to-peptide and other types of hydrogen bonds. These various linkages are dependent variables of the *c*-, *c'*-, and  $\varepsilon^0$ -value ensembles. The coincidence of dimensional changes and abrupt *n*-value changes may be interpreted as being due to the indirect *F*-effect (Figures 7.7 and 7.8); thus, we have used this phenomenon to support our hypothesis.

## (2) The unmasking of functional groups

The reactivity of a specific polar group may differ from protein to protein or even in the same protein in its native and denatured states (for reference, see Putnam, 1953). In denaturation, the process underlying the increase of the reactivity of such a group is commonly called an unmasking phenomenon.

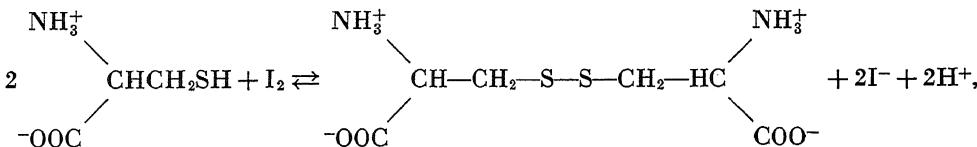
(a) *The unmasking of sulphhydryl groups.* We have discussed the proposition that a given functional group in different protein molecules may have different oxidation-reduction potentials just as given  $\beta$ - or  $\gamma$ -carboxyl groups may have widely different *c*-values. That *c*-value variations may occur in a sulphhydryl group as consequences of group substitution remote from the SH group is shown by the variability of the *pK* values of different thiols (the *pK* value of cysteine is 8.3; for cysteinylcysteine it is 7.3 and 10.8, respectively, Calvin, 1954, p. 9). Similarly, substitution changes the oxidation-reduction potentials of naphthoquinones (Fieser and Fieser, 1935), phenanthrenequinones (Fieser, 1929), disulfides (Preisler and Berger, 1947), and alkylaldehydes and ketones (Adkins *et al.*, 1949). A *c*-value shift at one functional group may be brought about by the direct and/or the indirect *F*-effect. Such a *c*-value shift, in the case of the thiol group,

will involve both a change of the dissociation constant and, we postulate, a change of oxidation-reduction potential. This concept is supported by the demonstration that the normal potential of free heme, another reactive group, which is  $-0.200\text{v}$  shifts strongly to  $0.150\text{v}$  when it combines with globin to form hemoglobin (K. G. Stern, 1938). We have suggested that a polar group on a protein can have a varying *c*-value and that the *c*-value ensemble is an important characteristic of a particular protein. Extended to the SH group, this means that there will be differences in the ionization potentials of the SH groups and, hence, in the oxidation-reduction potentials. Such differences will appear not only among the thiol groups of different protein molecules, but also among the thiol groups distributed on the same molecule.

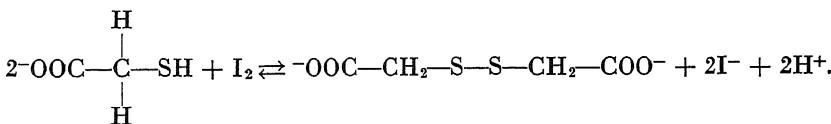
Thiol oxidation, unlike the oxidation of simple elements or of aldehydes, involves a bimolecular condensation. For oxidation by iodine,



In the case of thiols, the free-energy change involves four factors: (1) the ionization potential of the single-pair electron of the SH group, (2) the hydration-energy change, (3) the free-energy change upon dissociation of the H<sup>+</sup>, and (4) the energy of interaction between the two R residues. The first two factors have been discussed. The third factor is an expression of the SH-group *c*-value. We call these three factors collectively the intrinsic oxidation-reduction potential  $E_{\text{intr}}^{\circ}$ . The fourth factor is due primarily to an electrostatic interaction if R carries charges. Since, in a true fixed-charge system, all charged groups are coupled with a counterion, factor (4) is unimportant. But in the reaction of simple monomeric thiols, this factor is important. Thus, in the oxidation of cysteine,



this term is different from that in the oxidation of thioglycolic acid.



In a semifixed-charge system, this electrostatic factor is determined by the  $c$ - and  $c'$ -values of nearby polar groups as well as the specific environment. Thus, in this case, the effective oxidation-reduction potential  $\mathcal{E}^\circ$  is an expression of  $\mathcal{E}_{\text{intr}}^0$  and the  $c$ -value ensemble of the neighboring polar groups.

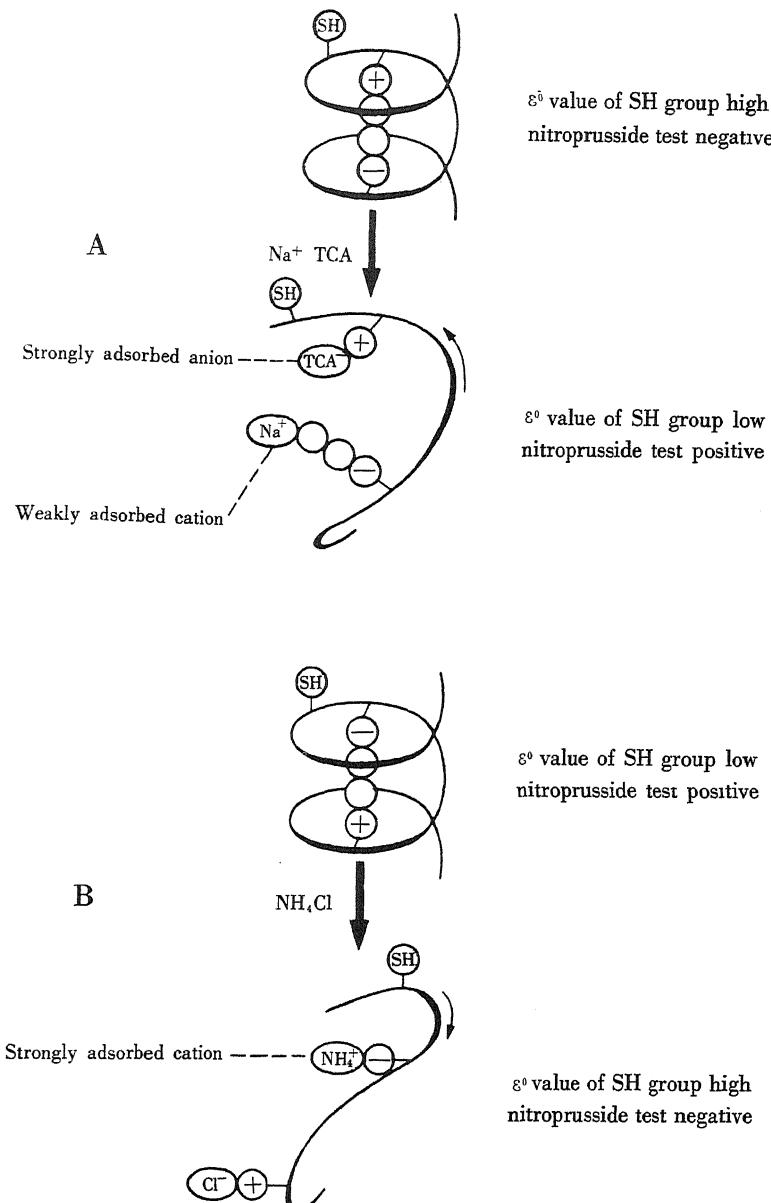
Since the different SH groups in a protein molecule may have different  $\delta^{\circ}$  values, in the presence of a mild oxidizing agent such as ferricyanide (Anson, 1939), porphyrindin (Greenstein, 1938), or iodosobenzoate (Hellerman et al., 1941), the fraction of the SH groups that has a high  $c$ -value and a low oxidation-reduction potential will

react with the oxidant, that is, Guzmann-Barron's (1951) freely reacting SH group, type-a SH group of Hellerman *et al.* (1943). *Sulphydryl groups of high  $\varepsilon^0$  value will hardly react, thus behaving as if they were masked* (Guzmann-Barron, 1951). On denaturation, the *c*-value ensemble is changed; some or all of the masked groups may experience a lowering of the oxidation-reduction potential  $\varepsilon^0$ . These previously masked groups now react with oxidizing agents with which they did not react in the native state (Figure 7.12).

Having discussed the general concept of the mechanism of sulphydryl liberation in denaturation, let us relate it to specific experimental facts. First, from our consideration of the limited extent of the direct *F*-effect, it is clear that the groups most likely to affect the oxidation-reduction potential of a sulphydryl group are its immediate neighbors, and in some cases, the next-nearest neighbors. Two types of neighbors are particularly important: polar side-chains that have high excess charge density, and peptide-chain H-bonding sites. Since the peptide-chain H-bonding sites are on the resonating backbone, they suffer a lower loss factor than the polar groups which must transmit the inductive effect through a side-chain. We shall demonstrate that both types of neighbors can contribute toward modifying the  $\varepsilon^0$  value of an SH group. But first let us discuss a possible mechanism underlying the denaturation of protein by urea.

In Section 5.4, we showed that a gang of alternatingly cationic and anionic sites interacts autocooperatively in an indirect *F*-process. In Section 7.2B, we also stressed that the C=O and NH groups of the polypeptide chain are essentially similar to the carboxyl and amino groups from which they were derived. The backbone is thus a chain of alternating "cationic" hydrogen-donating NH sites and "anionic" hydrogen-accepting C=O sites, differing from a gang of alternating cationic and anionic net-charge-bearing sites only in that the alternating sequence is more perfect. Like their net-charge-bearing counterparts, the *c*- and *c'*-value analogues of these H-bonding groups will determine what particular H-bonding partners the groups will choose. They have a number of possible choices. Prominent among these are water, urea, and other peptide groups that belong to the same or different protein molecules. We shall confine our discussion to a comparison of urea and water.

Tsuboi (1951, 1952; see also Mizushima, 1954) found that urea is both a stronger proton acceptor and a stronger proton donator than water. Thus the relation of urea to a peptide group is similar to the relation of HCl to a salt linkage (see Section 7.4C). If the concentration of urea is increased, a critical concentration will be reached, an indirect *F*-process will set in, and, in an all-or-none fashion, all the water molecules will be replaced by urea. Tsuboi, in determining the H-bonding strengths of water and urea relative to the peptide linkage, referred to a single unvarying peptide. In Section 7.2B we presented evidence that the hydrogen-donating or hydrogen-accepting power of an H-bonding group varies with the *c*- and *c'*-value ensembles of the protein. Thus, if the *c*- and



**Figure 7.12. THE EFFECT OF THE ADSORPTION OF A STRONG ANION ON THE REACTIVITY OF THE SULFHYDRYL GROUP.** A, the addition of the trichloroacetate ion leads to its successful competition with a second anion (in this case a fixed site) for a fixed cationic site. The adsorption of the stronger anion leads to an *F*-effect that decreases the  $\varepsilon^\circ$  value of the proximal SH group, increasing reactivity to the nitroprusside reagent. B, the introduction of  $\text{NH}_4^+$ , strongly preferred by this particular anionic site, causes a withdrawal of electrons from the SH group by a direct *F*-effect and thus increases its  $\varepsilon^\circ$  value and decreases its reactivity with the nitroprusside reagent. Empty circles represent  $\text{H}_2\text{O}$  molecules.

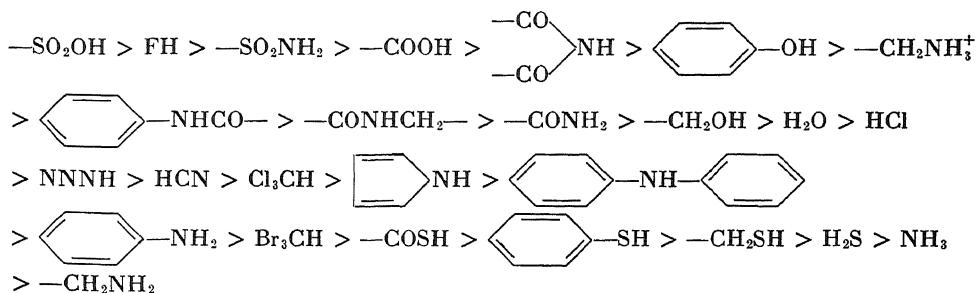
*c'*-value analogues of the peptide CONH are changed, the binding energy of urea relative to that of water will also vary. Such changes in the *c*- and *c'*-value analogues can be effected by changes in the degree of association or dissociation of polar groups. This was observed experimentally by Doty and Katz (1953; see also S. Katz, 1950) whose light-scattering measurements indicated that serum albumin in 8*M* urea preferentially binds 2000 molecules of urea at pH 8, and about 3000 molecules of water at pH 3. Similarly, Bresler (1949) concluded from sedimentation-constant measurements that, at pH 6.5, serum albumin binds 360 molecules of urea per protein molecule.

Table 7.4 presents the results of Tsuboi's study of the relative strengths of proton-accepting and proton-donating groups. It shows that an amide is a weaker proton donator than a simple peptide linkage (RNHCO > NH<sub>2</sub>CONH<sub>2</sub>). Conversely, an amide is a stronger proton acceptor than a simple peptide linkage (NH<sub>2</sub>CONH<sub>2</sub> > RNHCO). This suggests, and we postulate, that urea, a diamide, is generally stronger as a proton acceptor than as a proton donor. If so, when urea reacts with a protein at both the NH and C=O sites, it will have a stronger effect as a proton acceptor. This is equivalent to saying that urea will have a net electron-repelling effect. Thus, in a series of peptide linkages where the backbone exchanges its original partners (either water or perhaps another backbone) for urea, one expects a general rise of the *c*-value analogues and a fall of the  $\varepsilon^0$  values of the groups in the immediate vicinity.

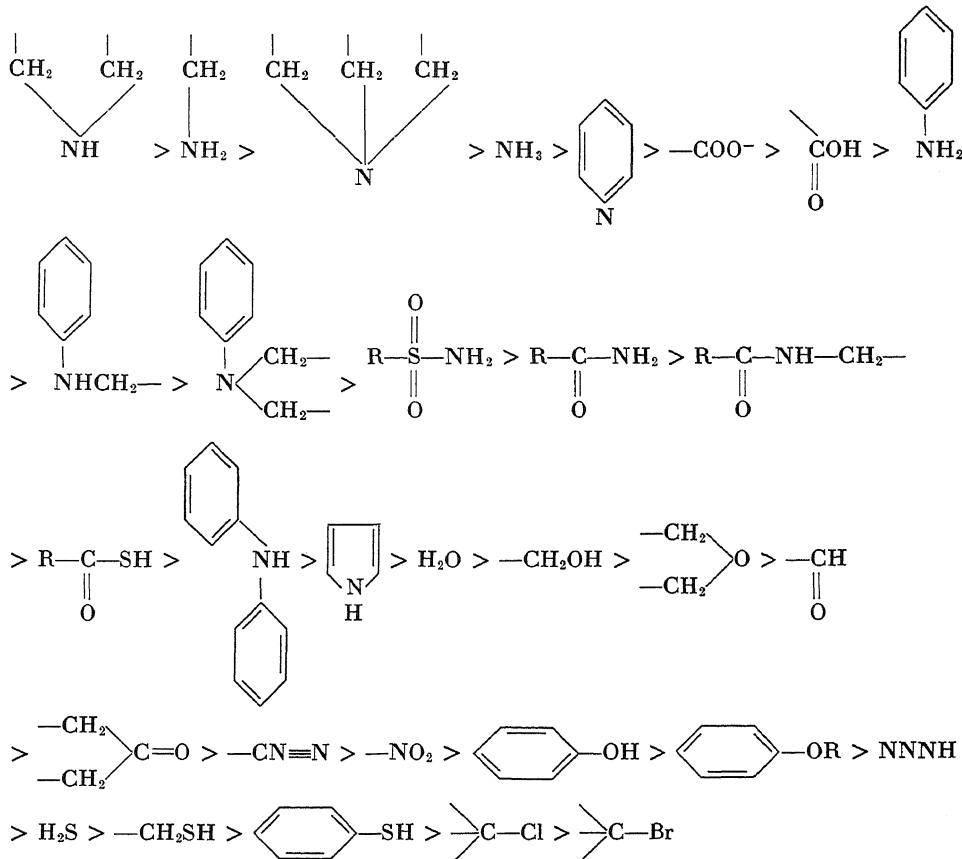
Let us introduce into a length of peptide chain a single cysteine residue flanked by two cationic groups. The inductive effect of the cationic groups on the cysteinyl SH group will be to promote a general rise of its  $\varepsilon^0$  value, such that it will not react to the extent that an independent cysteine SH group would with mild oxidizing agents. In the extreme, it may appear to be totally masked and not react with the SH reagent at all. If we increase the urea concentration, a point will be reached at which an indirect *F*-effect sets in; after this, urea will occupy the peptide H-bonding sites. With its predominant electropositivity, the urea will increase the electron density and hence lower the  $\varepsilon^0$  value of the SH group. This will increase its reactivity with SH reagent and it will appear to be unmasked.

To verify this hypothesis, we cite the work of Benesch *et al.* (1954); they studied the effect of 7*M* urea on the intensity of the nitroprusside color developed with various thiols. The change of molecular extinction  $\varepsilon$  at 550m $\mu$  is expressed as a quotient  $\varepsilon$  in urea/ $\varepsilon$  in water. It is 1 for ethyl mercaptan and thioglycolic acid, 1.14 for homocysteine, 1.43 for cysteine, 1.71 for L-cysteinyl-D-valine, 1.78 for L-glutamyl-L-cysteine, 1.91 for L-cysteinyl-L-valine, 2.34 for glutathione, and 2.51 for phenacylcysteinyl-D-valine. Benesch *et al.* concluded that their results were due to the effect of urea in breaking the H-bonds between the ionized S-groups of the sulphhydryl groups and NH, NH<sub>2</sub>, or OH groups in the vicinity.

Proton-donating power in decreasing order from left to right



Proton-accepting power in decreasing order from left to right



**Table 7.4.** PROTON-DONATING AND PROTON-ACCEPTING POWER OF VARIOUS H-BONDING GROUPS. [Data from Tsuboi (1951, 1952), as quoted by Mizushima (1954).]

They thought that the greater effect of urea on the more complex thiols was due to their greater potentiality for forming such H-bonds before the addition of urea.

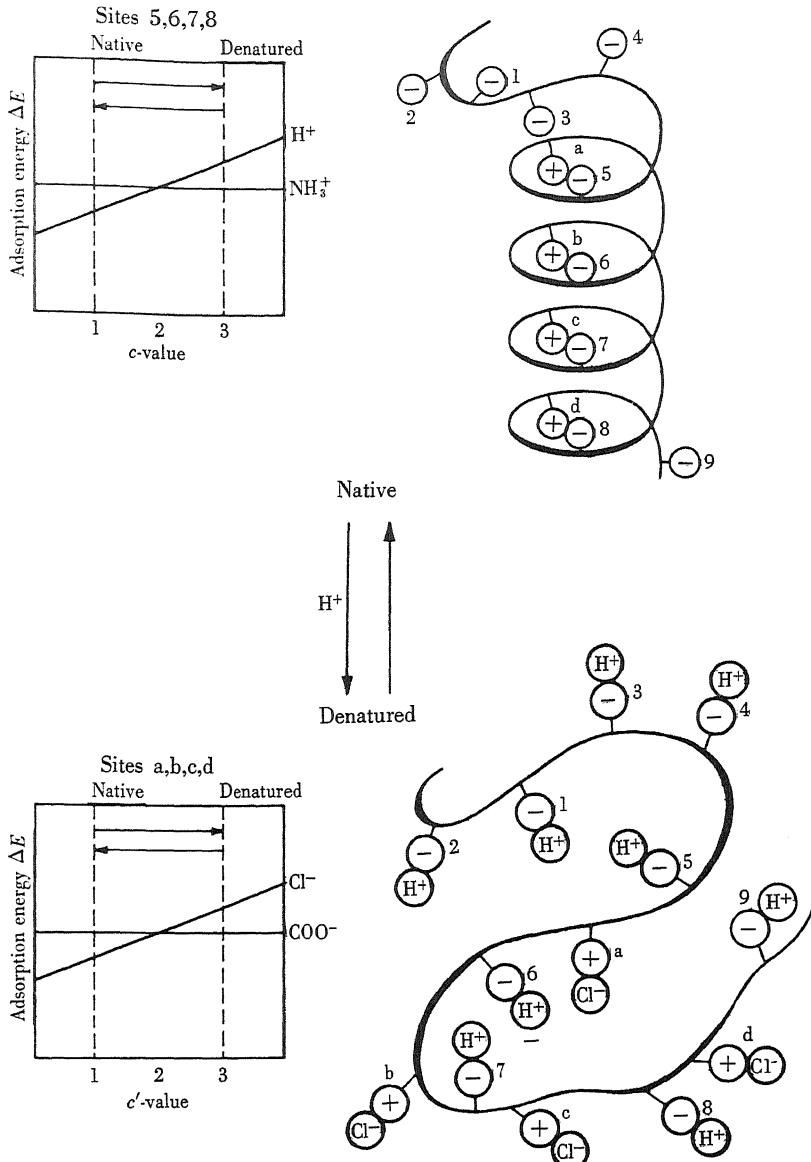
We suggest that this increasing reactivity is due to the increasing number of urea-binding NH and C=O groups. The highly resonating nature of the backbone allows the transmission and accumulation of the inductive effect of urea; due to its electron-enriching effect, this inductive effect decreases the  $\varepsilon^0$  value of the SH groups and thereby increases their reactivity toward the nitroprusside reagent. Consistent with this view, cysteine has a  $\varepsilon$  in urea/ $\varepsilon$  in water quotient of 1.43 but homocysteine, differing from cysteine only by the possession of one more CH<sub>2</sub> group, has a quotient of only 1.14, indicating the relative nonpolarizability of the saturated fatty-acid chain. We see further support for our view in the finding (Ling and Kalis, to be published) that the absolute-extinction coefficient of the nitroprusside color, determined in accordance with the procedure of Benesch *et al.*, increases in the order CH<sub>3</sub>SH < CH<sub>3</sub>CH<sub>2</sub>SH < CH<sub>3</sub>CH<sub>2</sub>CH<sub>2</sub>SH; this agrees with the facts that the CH<sub>2</sub> group is a stronger electron-donating group than an H atom and that the donating power of methyl, ethyl, and isopropyl groups follow the order CH<sub>3</sub>CH<sub>2</sub>CH<sub>2</sub> > CH<sub>3</sub>CH<sub>2</sub> > CH<sub>3</sub>.

Based on our assumption that the values of  $c$ ,  $c'$ , and  $\varepsilon^0$  for a particular side-chain functional group are primarily determined by its immediately neighboring groups, we predict that whenever a preponderance of cationic groups is found in the immediate vicinity of a cysteinyl residue, the  $\varepsilon^0$  value of the SH group will be raised significantly. Thus, a reagent that reacts with, say, simple cysteine will not react with the cysteinyl residue; the SH group thus appears to be masked. When the urea concentration is increased, an all-or-none denaturation eventually occurs, resulting in the occupation of the peptide sites by urea. This enriches the electron density of the SH group and lowers its  $\varepsilon^0$  value. The increased reactivity toward the mild SH reagents is now seen as an unmasking phenomenon.

#### (b) *The liberation of acidic and basic groups*

(i) *The cardinal sites in an indirect F-process.* We have used acid denaturation as a prototype in discussing heterocooperative and autocooperative interaction. To emphasize the crucial importance of the indirect F-effect, we present a diagram (Figure 7.13) that illustrates the following detailed theoretical interpretation of the experiments of Steinhardt and Zaiser (1951, 1953).

Since the  $c$ -value is determined primarily by the charges of immediate neighbors, the  $pK$  values for the various sites, 1, 2, and so on, represented as  $pK$  (1),  $pK$  (2), and so on, should follow the order:  $pK$  (1) >  $pK$  (2) =  $pK$  (3) >  $pK$  (9) =  $pK$  (4) >  $pK$  (8) >  $pK$  (5) =  $pK$  (6) =  $pK$  (7); see Figure 7.13. Thus, at the time of the initial introduction of HCl, only site 1 will be titrated, followed by site 2, site 3, and sites 4 and 9. But the binding of an H<sup>+</sup> ion onto site 1 produces a heterocooperative effect on sites 2 and 3; this makes it possible for sites 4 and 9 to be titrated before sites 2 and 3. In this range of titration, three factors should operate: the heterogeneity factor, the electrostatic factor, and the heterocooper-



**Figure 7.13. A THEORETICAL MODEL OF PROTEIN DENATURATION.** A native protein molecule is represented as a polypeptide chain along which are distributed polar side chains, either isolated (site 4) or in gangs. Anionic sites 1, 2, and 3 represent a heterocooperative gang and alternately cationic and anionic sites 5, a, 6, b, 7, c, 8, d represent an auto-cooperative gang which, in a folded state, forms a helix. Assuming the intrinsic  $c$ -values of the anionic groups to be the same, the charges of the neighboring sites lead to  $pK$  values of the sites such that  $pK(1) > pK(2) = pK(3) > pK(9) = pK(4) > pK(8) > pK(5) = pK(6) = pK(7)$ . As this model protein is titrated with the denaturant HCl, the sites of high  $pK$  value (1, 2, 3, 4, and 9) first bind the  $\text{H}^+$  ion. As the HCl concentration is increased, sites 8 and d will adsorb  $\text{H}^+$  and  $\text{Cl}^-$ , leading to a sequential shift of the  $c$ - and  $c'$ -values of all the sites in the auto-cooperative gang and a sequential binding of HCl to these sites. In this unfolded state, the protein takes on new macroscopic characteristics; if the original state was called the native state, we refer to the new state as denatured. Insets (simplified after Figures 4.9 to 4.11) show the theoretical relations between the  $c$ - and  $c'$ -values and the adsorption energies of the various ions, free and fixed.

ative interaction. All three act to lower the *n*-value below unity, as shown in Figures 7.7 to 7.10. When the five fixed anions, 1, 2, 3, 4, and 9, have been titrated, the gang, 5, a, 6, b, 7, c, 8, d, will be titrated in an all-or-none fashion. Since site 8 is flanked by the least number of cationic sites, it will have a higher *c*-value than sites 5, 6, and 7. The splitting of salt linkage 8d by a pair of H<sup>+</sup> and Cl<sup>-</sup> ions will then, by an indirect *F*-process, lead to the zipperlike autocooperative response, revealing all the fixed anionic and cationic groups in the gang.\*

If the HCl is added in infinitesimal increments, a range of HCl concentrations will be found in which site 8 will have a key function in determining, in an all-or-none fashion, whether 5a, 6b, and 7c will remain in salt linkages or become dissociated by reacting with H<sup>+</sup> and Cl<sup>-</sup> ions. Since these salt linkages hold this stretch of the protein chain in a helix or a similar folded configuration, site 8 also controls the configuration transformation: folded ⇌ unfolded. Therefore, site 8 is a cardinal site. There is, however, no sharp distinction between cardinal and non-cardinal sites. If we visualize the chain reaction discussed as proceeding from "upstream" to "downstream," then each upstream site is cardinal with respect to the sites downstream from it.

Since the environment of the titrated hemoglobin varies, a site that is cardinal under one set of conditions may not be a cardinal site under other conditions. By varying the nature of the titrant (by making the adsorption energy of the anion greater than that of the cation as in sodium dodecylsulfate, for instance), site a may become the controlling cardinal site; site 8 will then be the last site of the gang and will not be cardinal at all. However, within a living cell, the milieu is fairly constant and the cardinal sites probably have a permanent importance.

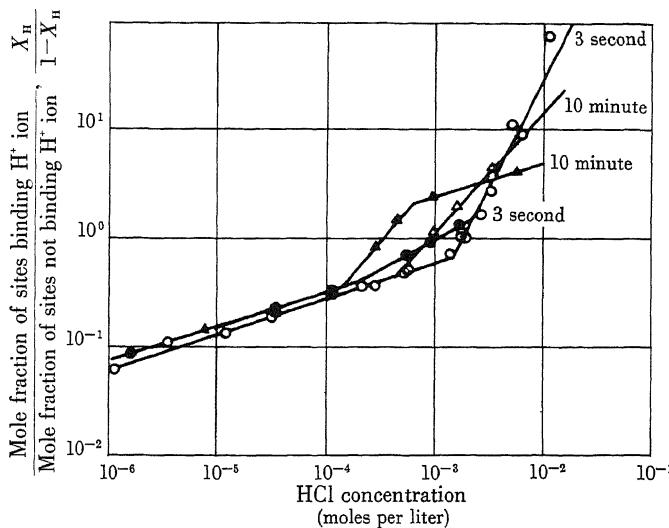
(ii) *The cardinal site in the modulation of all-or-none responses.* In the presence of HCl, the all-or-none response of the gang (taking Figure 7.13 as an example) depends crucially upon whether cardinal site 8 reacts with the H<sup>+</sup> ion or not; the gang of autocooperative sites behaves as if it were a single site. *Thus if site 8 adsorbs the H<sup>+</sup> ion, the whole gang unfolds; if not, the gang remains folded. It is clear that a second adsorbent, say, on site 9, will affect the chain event as a whole precisely as it would affect the adsorption of a particular adsorbent on site 8.* This point, mentioned in Chapter 6, will be discussed again in Chapter 15.

(iii) *The importance of both cation and anion in denaturation.* The theoretical interpretation of the acid denaturation of hemoglobin presented above introduces another factor which is not usually emphasized, the importance of the nature and quantity of the anionic component of the acid used in acid denaturation.

Figure 7.14 presents a log-log plot of the data of Steinhardt and Zaiser on the three-second titration of carbonylhemoglobin in pure water and in the presence of 0.02M NaCl. The usually innocuous 0.02 molar NaCl significantly decreased the

\* In the experiment of Steinhardt and Zaiser the gang appears to have more than the four pairs of sites shown in our diagram.

HCl concentration at which the  $n$ -value bends sharply. In the words of the original authors, "hemoglobin in salt-free solutions is more stable to acid." This result points out the significant fact that the effective occupation of the cationic site by the counteranion ( $\text{Cl}^-$  ion) in the indirect  $F$ -process may be assisted by an increase of its concentration, particularly if the counteranion's free energy of



**Figure 7.14.** HYDROCHLORIC-ACID BINDING BY CARBOXYLHEMOGLOBIN IN THE PRESENCE AND ABSENCE OF SODIUM CHLORIDE (25°C). The facilitative effect of  $\text{NaCl}$  (0.02M) on denaturation is shown by the shift toward a lower acid concentration of the point marking the abrupt change of slope of the titration curve. This is true for both the three-second and the ten-minute titrations. Curves with empty circles and triangles represent titration with no salt; solid circles and triangles represent titration in the presence of 0.02M  $\text{NaCl}$ . [Data calculated from experimental values of Steinhardt and Zaiser (1951), Figures 5 and 6.]

adsorption is not sufficiently great for it to compete with the fixed anion at low concentrations.

The experiment of Steinhardt and Zaiser substantiates the relation of the quantity of the acid anion to the onset of the denaturation process. Our expectation that the nature, and hence the relative  $\Delta E$ -versus- $c'$ -value plot of the anionic component also influences the onset of denaturation is supported by another group of experiments published by Steinhardt *et al.* (1941). Replotted on a log-log scale in Figure 7.4, these data show that an  $n$ -value shift did not occur in the HCl titration of egg albumin (as was observed by Cannan *et al.*, 1941, in their classical experiment). It did occur in titration with  $\text{HNO}_3$  and picric acid, and the

rapidity of onset of the *n*-value shift follows the order,  $\text{HNO}_3 >$  picric; this order is the same as the order of binding energies of these anions on wool (Steinhardt *et al.*, 1941).

(iv) *The reversible change of selective preference for counterions.* The net result of the indirect *F*-process as seen in the acid titration of hemoglobin by Steinhardt and Zaiser is that a large number of fixed anions adsorb the  $\text{H}^+$  ion; before the indirect *F*-process took place, such combinations were not favorable.

According to equation (5-12), the intercept on the abscissa of a log-log plot at

$$\frac{X_{\text{H}^+}^{f^-}}{X_{\text{RNH}_3^+}^{f^-}} = \frac{X_{\text{H}^+}^{f^-}}{1 - X_{\text{H}^+}^{f^-}} = 1$$

gives the reciprocal of the apparent equilibrium constant  $K'$ . In Figure 7.10 for ferrihemoglobin this is  $1.66 \times 10^{-3}$ , giving an apparent equilibrium constant  $K' = 6.02 \times 10^2$ , before the indirect *F*-effect took place. The three-second back titration represents an evaluation of the acid affinity after the denaturation process is completed. It yields  $K' = 5.89 \times 10^3$  which indicates that a considerable increase of HCl affinity has occurred.\* If the denatured protein is brought back to a nearly neutral *pH* and permitted to equilibrate for two hours, the value of  $K'$  reverts to  $3.60 \times 10^2$ . This demonstrates a cycle of events involving a reversible change of ionic affinity, and is in agreement with the concept of the indirect *F*-process which is essentially a reversible process.†

A system might start out with a high preference for salt linkages between its fixed cations and anions and a low preference of  $\text{Cl}^-$  and  $\text{H}^+$  ions. Reaction with a small amount of HCl may trip the equilibrium into another state through the indirect *F*-process. The result is an increase of preference for  $\text{H}^+$  and for  $\text{Cl}^-$  ions. When these triggering  $\text{H}^+$  and  $\text{Cl}^-$  ions are removed (as by back titration with KOH), the fixed anions and cations revert to their original states of lower HCl preference. According to the present theory, the experiments of Steinhardt and Zaiser are an *in vitro* demonstration of the indirect *F*-process which occurs during the nerve impulse, muscular contraction, and a host of other important physiological phenomena involving reversible changes.

### (3) Solubility changes

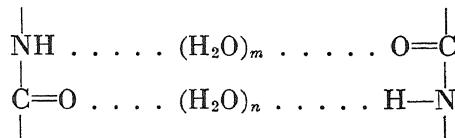
The important sites of a protein which interact with water and thereby contribute to its solubility are the NHCO groups of the peptide chain and polar groups like carboxyl, amino, and guanidyl groups.

The role of the peptide linkages as sites of interaction with water molecules has

\* Although we have considered only the increase of  $\text{H}^+$ -ion affinity of sites downstream from the 8d pair, such an increase of  $\text{H}^+$ -ion affinity is not limited to downstream. Counterion exchange downstream may have a reciprocating effect on the *c*-values of the upstream sites. This effect will be discussed in Section 17.2.

† One may expect that, if the protein contains SH groups, change in the *c*-value ensemble may lead to their oxidation, rendering the reaction irreversible.

long been recognized (Wu, 1931). The demonstration by Mellon and his co-workers (1948) of significant water sorption on polyglycine and particularly on benzoyltetraglycine, which contains neither polar side-chains nor a terminal amino group (see also Sponsler *et al.*, 1940; Katchalski, 1951; Gustavson, 1956, p. 150), strongly suggests that the backbone itself offers important sites for water binding. Peptide linkages and water molecules are both proton donors and proton acceptors. Since each H-bond involves only one donor and one acceptor, in a system containing a large number of peptide linkages and water molecules, we may have the following combinations:



where  $n$  and  $m$  are zero or positive integers.

Solubility is determined by the free energy of solution. The solubility of polar molecules in water depends largely on the free energy of hydration of the ionic components. The free energy of hydration is determined by (1) the magnitude of  $\Delta F$  per water molecule coordinated, and (2) the number of water molecules coordinated per polar group. If  $n$  and  $m$  are equal to zero or one, there could be at most one "valence" left to the unit  $\text{NH} \dots \text{O}-\text{H} \dots \text{O}=\text{C}$  which was



free to interact with other water molecules. Since this complex must displace water molecules with more than one free "valence," there might be a loss of solubility due to its presence. When  $n, m > 1$ , the peptide chain would begin to contribute to protein solubility. The values of  $n$  and  $m$  are analogous to configurations in ionic group interaction ( $n, m = 0, 1, 2, 3$  would roughly correspond to configurations 0, I, II, III\*) and, like the configurations, will vary with the  $c$ -value ensemble. The backbone contribution to solubility will thus also vary with the  $c$ -value ensemble.

It has long been recognized that ionic groups ( $\beta$ - and  $\gamma$ -carboxyl,  $\epsilon$ -amino, histidyl, guanidyl groups, for instance) may contribute to the solubility of proteins (Pauli, 1899). It is further known that the formation of salt linkages leads to dehydration (Pauli, 1947; Haurowitz, 1950, p. 97). We interpret this as a consequence of both a reduction of the number of valences that are free to form H-bonds with the general water lattice and a decrease in the free energy of hydration of each such residual valence. The mechanism for the reduction of the number of free valences is obvious; the decrease in free energy of hydration is due to the overlapping and cancellation of the opposing electric fields of opposite charges and the concomitant decrease of electrostatic energy. We have presented

\* It is entirely possible that what corresponds to  $m, n > 1$  is, by definition, the dissociation of the two H-bonding peptide groups since the effective limit of dielectric saturation cannot extend as far for H-bonding groups as for ionic groups.

the statistical interpretation of the large free energy of association for the formation of salt linkages by amino and carboxyl groups (Section 7.3A). The abundance of salt linkages in some proteins could account for the low solubility of such proteins despite the large number of ionic groups they possess. Other proteins with ionic groups are readily soluble; but their solubility decreases on heat denaturation.

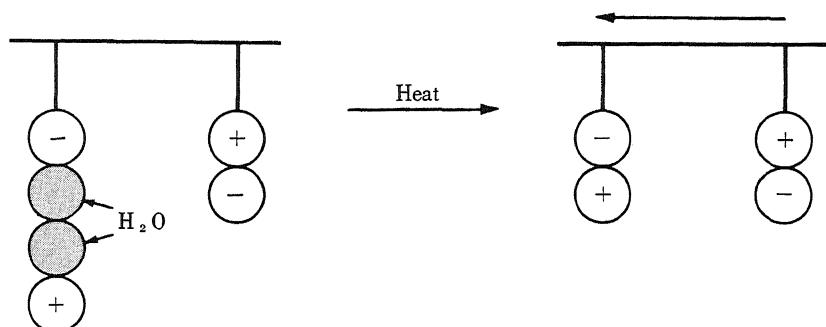
Wu (1931), defining denaturation as "a change in the natural protein whereby it becomes insoluble in solvents in which it was previously soluble," stressed the importance of solubility changes for denaturation. We use two types of denaturation phenomena to illustrate the interaction of agents with the two important types of sites that determine the normal solubility of a protein.

(a) *The phenomena of increased solubility of proteins in urea.* Urea-denatured protein, when brought back into an isoelectric salt solution, does not recover its original solubility; but the solubility of native, as well as heat-denatured proteins, often increases when they are placed in urea solution (Putnam, 1953). It is commonly held that urea reacts with the peptide linkage (R. B. Simpson and Kauzmann, 1953; Bresler, 1949). This is consistent with our knowledge of urea as a strong hydrogen-bonding agent (Gordy, 1936; Gordy and Stanford, 1941). At high concentrations, peptide-water H-bonds and peptide-peptide H-bonds should be replaced by H-bonds to urea. Each urea molecule has five potential H-bonding sites, more sites than either the water molecule or the peptide linkage possesses. Adsorption of urea onto the peptide chain would leave a number of free valences available for interaction with water molecules in the medium. Displacement of the peptide-peptide and peptide-water linkages by urea should thus be expected to increase protein solubility.

(b) *Heat denaturation and solubility.* A rise of temperature from 25°C to 100°C causes a rise of  $RT$  from 0.6 kcal/mole to 0.76 kcal/mole. The rise in average kinetic energy is not very large. Its first-order effect on a large molecule is likely to be small. But it will have a relatively great effect on the large number of adsorbed water molecules because they are held by weak bonds (weak energy of association) and are small discrete particles (high entropy gain on dissociation). The desorption of such particles results in a large entropy gain which a dislocation of, say, a backbone-to-backbone H-bond with the same dissociation energy does not enjoy. This is because the remainder of the backbone peptide chain is anchored and consequently the configurational- and rotational-entropy gain for the dissociated group is restricted (see Section 2.4).\*

\* That a general dehydration at ionic groups and at peptide linkages results from a small rise of temperature is consistent with the following facts: (1) When organic acids are heated there is first an increase and then a decrease in their  $pK$  values (Branch and Calvin, 1941). I suggest that the increase is due to dehydration of the acid group with an increase in the statistical weight of the lower configurations. The decrease at higher temperatures is attributed to the decrease of  $\Delta F/RT$  due to increased  $T$  where  $\Delta F$  is the dissociation energy of  $H^+$  at the zero or other low configuration. (2) In heating the sulfonic-acid exchange resin, this fixed-charge system changes the selectivity from the  $K^+ > Li^+ > Li^+ > K^+$ ; from  $K^+ > H^+ > H^+ > K^+$  (Gregor and Bregman, 1951; Cosgrove and Strickland, 1950); this has been interpreted by these authors as a consequence of dehydration.

In terms of the present hypothesis, raising the temperature increases the statistical weight of the lower configurations. In the case of proteins, increasing the statistical weight of lower configurations affects water sorption, not water solubility.\* Nevertheless, a primary effect on water sorption may secondarily affect solubility. We argued that a protein may exist in more than one metastable state, and that heterogeneity of  $c$ - and  $c'$ -values in a protein is the rule rather than the exception. Consequently, different fixed ion-counterion pairs are coupled



**Figure 7.15. MODEL OF A SEGMENT OF PROTEIN UNDERGOING HEAT DENATURATION.** A moderate increase of temperature causes the loss of interspersed water molecules; the shorter distance of separation between the fixed anion and its counterion leads to an inductive change of the  $c$ -value of the adjacent fixed ion.

with a different number of intervening water molecules. Among these fixed ion-counterion pairs, those assuming configuration III contain more easily displaceable water molecules than those assuming configuration II. The water of configuration II can be displaced more easily than that of configuration I. Thus a rise of temperature from 25°C to 100°C is likely to affect configuration III, but will not affect configuration 0. If a microscopic section of part of a protein molecule resembles that shown in Figure 7.15, then a rise of temperature will effect a loss of interspersed water, allowing the free cation to be drawn closer to the fixed anion  $f^-$ . Electrons will then be drawn toward  $f^-$  and create an imbalance with respect to the original state of equilibrium. Removal of more water will result in a  $c$ -value shift sufficient to precipitate an indirect  $F$ -effect, creating a new  $c$ -value ensemble more compatible with the higher temperature.

Salmine sulfate, whose side chains are virtually all positively charged guanidyl groups (see Figure 0.2), may be boiled at 100°C without loss of solubility. However, if the simple sulfate ion is replaced with sodium dodecylsulfate or polystyrene sulfonate, the protein immediately forms an insoluble coagulate

\* As pointed out by Haurowitz (1950), water solubility does not necessarily parallel water sorption.

(Ling and Ochsenfeld, unpublished). This is interpreted to mean that the long chains of dodecyl sulfate and polystyrene sulfonate decrease the entropy gain that occurs with dissociation of the sulfate groups from the guanidyl groups of the protein; the long chains thus favor association. We can also achieve this effect by increasing the adsorption energy (to increase the magnitude of  $\Delta E$ ) of the anion with no change in the entropy contribution. Thus, solubility decreases when salmine is treated with  $\text{SCN}^-$ ,  $\text{Br}^-$ , but not when it is treated with  $\text{SO}_4^-$ . The adsorption energies of these anions follow the order  $\text{SCN}^- > \text{Br}^- > \text{SO}_4^-$ , as shown in Table 7.5. Hofmeister (1888) described a similar anion order for the precipitation of egg globulin from solution.

The results of these experiments lead to an important conclusion: Only freely dissociating fixed ionic groups significantly increase the solubility of a protein molecule. Formation of an ion pair, even of the highest configuration, reduces the solubility effect of the fixed site. A number of native proteins bearing both anionic and cationic side chains are quite soluble in pure water. The  $c$ - and  $c'$ -value ensembles of these native proteins in pure water must be unfavorable to the formation of unionized polar groups and salt linkages; thus a number of free, dissociated ionic groups must exist.

The  $c$ -value ensemble is stabilized largely by cardinal sites. A dehydration-activated indirect  $F$ -process could overthrow stabilizing adsorptions at cardinal sites and alter the  $c$ -value ensemble to favor the formation of more salt linkages (or associated ion pairs, if the protein is in a salt solution); this would yield a loss of solubility. This type of heat denaturation is basically reversible (Kunitz, 1949; Eisenberg and Schwert, 1951). However, the  $c$ -value ensemble change may lead, for instance, to an  $\varepsilon^0$  value change of SH groups and to their oxidation. This might result in irreversibility.

#### D. SEMIQUANTITATIVE EXPERIMENTAL VERIFICATION OF THE BASIC EQUATIONS

We shall present a number of experimental findings concerning the phenomenon of protein denaturation in an attempt to verify several basic relations which we have presented only on theoretical foundations.

- (1) The verification of the relation between the adsorption energy and the change in the  $c$ -value of neighboring sites

From our discussion of protein denaturation, it follows that, to be a denaturant, an agent must be successfully adsorbed. Adsorption depends on the free energy of adsorption  $\Delta F_p$  given by:

$$\Delta F_p = RT \ln \left[ \frac{(\text{p.f.})_p^{\text{dissoc}}}{(\text{p.f.})_p^{\text{assoc}}} \right] + \Delta E_p. \quad (7-22)$$

The partition-function ratio should be more or less constant for small ions, particularly monovalent, monoatomic ions. To be an effective denaturant, the adsorbed ion must be able to produce a significant *c*-value change. In Section 5.3A we introduced the relation,

$$\Delta_{0j} = \frac{\tau_{0j}}{\beta_{0j}} I_{0j}(p).$$

If the adsorbent *p* is attached at only one point,  $I_{0j}(p)$  should be a direct function of the energy of adsorption of *p* on site  $f_j$ , and we have the equation,

$$\Delta_{0j}(p) = \tau_{0j}(\Delta E_p)_j.$$

One therefore expects a strongly adsorbed denaturant with a high energy of adsorption  $\Delta E_p$  to be more effective than a more weakly bound entity. In the case of denaturants such as acids, bases, and salts, if one of the ions is unvaried (all as Na salts), the effectiveness of the denaturant should be proportional to the adsorption energy of the other ion, see equation (5-6).

The cationic groups in proteins are the  $\epsilon$ -amino group of lysine and the guanidyl group of arginine. It is likely that their ion-binding characteristics are simulated by anion exchange resins such as Dowex 1 and 2 which bear similar amine functional groups. Column one of Table 7.5 is taken from Peterson's (1953) review of the work of Wheaton and Bauman (1951) and of Belle (1951) where

$$K_{Cl} = \frac{[A^-]_r [Cl^-]_s}{[A^-]_s [Cl^-]_r}. \quad (7-23)$$

The concentration of the studied anion in the resin phase is represented by  $[A^-]_r$ ;  $[Cl^-]_s$  represents the concentration of the  $Cl^-$  ion in the solution phase;  $K_{Cl}$  is thus the affinity of the anion groups of Dowex 1 and 2 for various anions relative to their affinity for chloride. By assuming that the least preferred acetate ion has a  $\Delta F$  equal to zero, we obtain the relative free energy of adsorption  $\Delta F_{rel}$  for each ion from the value of  $K_{Cl}$  for that ion. The  $\Delta F_{rel}$  values so calculated are presented in column two of Table 7.5. Columns three and four present the data available to the author on the protein binding of anions. The order of preference here is the same as the order of preference for binding on Dowex 1 and 2 resins.\*

Column five quotes data related to the effect of anions on the rate of change of optical rotation (R. B. Simpson and Kauzmann, 1953). Even though different cations are involved, the order of effectiveness is the same as the order of binding energies of anions to proteins and to Dowex 1 and 2. Similarly, the excellent data of Burk (1943) on the interaction of urea and salts in the revelation of SH groups (columns six and seven) can be interpreted within the framework of the present theory and so can the well-known data of Greenstein (1939) which shows the

\* All anions with  $K_A$  greater than one are known to be protein denaturants.

Exchange resins, anion adsorption on Dowex 1 and 2		Proteins					
		Anion adsorption on proteins			Anion effect on protein denaturation		
Anion	$K_{\text{Cl}}$	$\Delta F_{\text{rel}}$ , kcal/mole	On wool, $-\log K'_A$	On bovine serum albumin, moles of ions per mole of protein	Effect on rate of change of optical rotation of egg albumin in urea, $d \log t_{1/2}/dc$	Inhibitory effect on urea liberation of SH groups, $dx/dy$	Minimum concen- tration of guani- dine salt to liberate SH of egg albumin, $M$
Perchlorate	32	-3.43					
Salicylate	30.1	-3.38				-2.77	-6.31
Thiocyanate	18.5	-3.09		24	-4.8 <sup>b</sup>		1.0
Trichloracetate	18.2	-3.08	1.16				
Iodide	13.2	-2.89			-3.7 <sup>b</sup>	-0.81	-1.99
Nitrate	3.5	-2.11	0.89	19	-1.8 <sup>c</sup>	-0.10	-0.33
Bromide	2.8	-1.97	0.69	15		-0.10	-0.44
Dichloroacetate	2.3	-1.87					
Chloride	1.0	-1.36	0.43	8	0.2 <sup>c</sup>	+0.67	+0.64
Bicarbonate	0.43	-0.86					
Formate	0.22	-0.47					

Monochloracetate	0.21	-0.44				
Acetate	0.10	-0.00	0.7 <sup>c</sup>	+3.50	+0.94	6.0
SO <sub>4</sub> <sup>-</sup>		1.78 <sup>a</sup>	4.0 <sup>c</sup>	+5.62	+6.0	6.0
CO <sub>3</sub> <sup>-</sup>						6.0

**Table 7.5. COMPARISON OF THE RELATIVE AFFINITY OF VARIOUS ANIONS FOR THE FIXED AMINO GROUP IN THE MODEL DOWEX 1 AND 2 SYSTEMS AND FOR THE FIXED CATIONIC GROUPS OF SEVERAL PROTEINS.** Adsorption on Dowex 1 and 2 is expressed as an arbitrary selectivity ratio ( $K_{\text{Cl}}$ ) with respect to chloride-ion adsorption, where  $K_{\text{Cl}} = [\text{A}^-][\text{Cl}^-]_s/[\text{A}^-]_s[\text{Cl}^-]$ , values in tables represent the average of three sets of values given by Peterson (1953, Table 1). The relative free energy of adsorption  $\Delta F_{\text{rel}}$  is calculated from  $K_{\text{Cl}}$ , assuming the free energy of adsorption for acetate ion to be zero. Wool-binding data has been taken from Steinhardt *et al.* (1942);  $K'_A$  is the dissociation constant of the protein-anion complex. Data on anion binding by bovine serum albumin are taken from Carr (1952). The effects of anion on the rate of change of optical rotation in urea at 30°C are expressed in terms of  $d \log t_{1/2}/dc$ ; here,  $t_{1/2}$  is the half time for change in optical rotation in minutes and  $c$  is the salt concentration in moles per liter. These data are taken from R. B. Simpson and Kauzmann (1953). The "inhibitory" effects on urea liberation of masked SH groups (Burk, 1943) are expressed as the slope at 0.5M salt concentration against salt concentration at the points at which these combine to give a minimal liberation of SH groups (see Figures 7.16, 7.17, 7.18). The last column, showing the minimum concentrations of guanidine salts necessary to liberate SH of egg albumin, was obtained from Greenstein (1939).

<sup>a</sup> Value given as if the sulfate ion were monovalent.

<sup>b</sup> As potassium salts.

<sup>c</sup> As sodium salts.

same order of effectiveness of anions of guanidine salts on denaturation (column eight).

The conclusion that emerges from a consideration of Table 7.5 is this: *In a variety of proteins and model fixed-charge systems bearing amino groups, the order of affinity for different anions is preserved.* This constancy of anionic affinity is found in protein interaction *in vitro*, in enzyme reactivity (see Section 14.2A), and in selective anionic accumulation (Section 9.3D). We contrast it with the high degree of flexibility found in the order of cationic selectivity. A reasonable explanation can be deduced from equation (5-5) which relates the transmissivity factor  $\tau$  to  $\omega$  and  $\chi$ :

$$\tau_{ij} = \pi_i \chi^{n_i + n_j} \omega^{m_{ij}} \beta_{ij}. \quad (7-24)$$

Since saturated hydrocarbon chains are less polarizable than the polypeptide backbone (in other words,  $\chi \ll \omega$ ) the more  $\text{CH}_2$  or similar saturated groups intervene between the effector and target groups, the smaller the inductive effect brought about by a change in the effector group will be. The  $\epsilon$ -amino group of the lysine residues has four  $\text{CH}_2$  groups interspersed between the peptide chain and the charge-bearing  $\text{NH}_3^+$  group; the guanidyl group of the arginine residue has three  $\text{CH}_2$ , one C, and one NH group. On the other hand, there is only one  $\text{CH}_2$  group between the peptide chain and the SH in a cysteine residue; and only one and two  $\text{CH}_2$  groups between the carboxyl group and the peptide in aspartic and glutamic residues, respectively. We thus expect a greater variability of  $c$ -values and  $\varepsilon^0$  values than of  $c'$ -values. This generalization is valid only for the average affinity of a large number of  $\epsilon$ -amino groups and guanidyl groups of a protein, cell or tissue. It does not imply that a particular group cannot have a  $c'$ -value corresponding to a different order of preference. In our discussion of enzyme activity, we shall show that an individual site can have an anion-preference order that deviates from the one above.

## (2) The experimental verification of the principle of additivity

In Section 5.3A we introduced equation (5-4),

$$c_0 = (c_0)_0 + \sum_j \Delta_{0j}(p_j)$$

which states that the  $c$ -value of a site is determined by the sum of its intrinsic  $c$ -value  $(c_0)_0$  and the changes of  $c$ -values produced by its neighbors,  $\Delta_{0j}(p_j)$ . We have just presented experimental evidence which shows that the equation,

$$\Delta_{0j}(p_j) = \tau_{0j}(\Delta E_{p_j})_j \quad (7-25)$$

is valid.

We can predict that there are two classes of denaturing agents; in accordance with our discussion of electronegative and electropositive bioregulants, we desig-

nate them as electronegative and electropositive denaturants. The adsorption of an electronegative reactant leads to an average *c*-value decrease; the adsorption of an electropositive reactant leads to an average *c*-value increase.

In discussing the relation of the oxidation-reduction potential  $\varepsilon^0$  to the *c*-value, we have attempted to establish that  $\varepsilon^0$  varies inversely with the *c*-value of a site. In the case of sulfhydryl groups, we have presented the hypothesis that the masking of an SH group is due to the presence of strongly positive groups sufficiently near it to draw electrons away from the cysteinyl residue; from the data of Tsuboi on H-bonding agents, we deduced that the unmasking of these SH groups by urea is due to the over-all electropositivity of the urea molecule. Because it reacts directly with the peptide chain, the effect of urea is highly additive, according to our interpretation of the data of Benesch *et al.* (1954). It follows that the concentration of urea needed to unmask an SH group is a measure of the *c*-value change needed to lower the  $\varepsilon^0$  value from that corresponding to the masked state,  $\varepsilon_{\text{mask}}^0$ , to that corresponding to the threshold reactive state,  $\varepsilon_{\text{thres}}^0$ , with respect to a particular mild oxidizing agent.

According to the present theory, a strongly adsorbed, relatively electronegative molecule like urea tends to increase the *c*-value and decrease the  $\varepsilon^0$  value of a neighboring site. We thus expect that if the salts of a certain cation (at a definite concentration) are added to a native protein, there will be a direct relation between the urea concentration needed to unmask the SH group and the adsorption energy of the cationic component of the salt. There should be an inverse relation between the minimum urea concentration needed for the unmasking of the SH group and the adsorption energy of the anionic component of the salt (as obtained, for instance, from the Dowex 1 and 2 exchange data, Table 7.5).

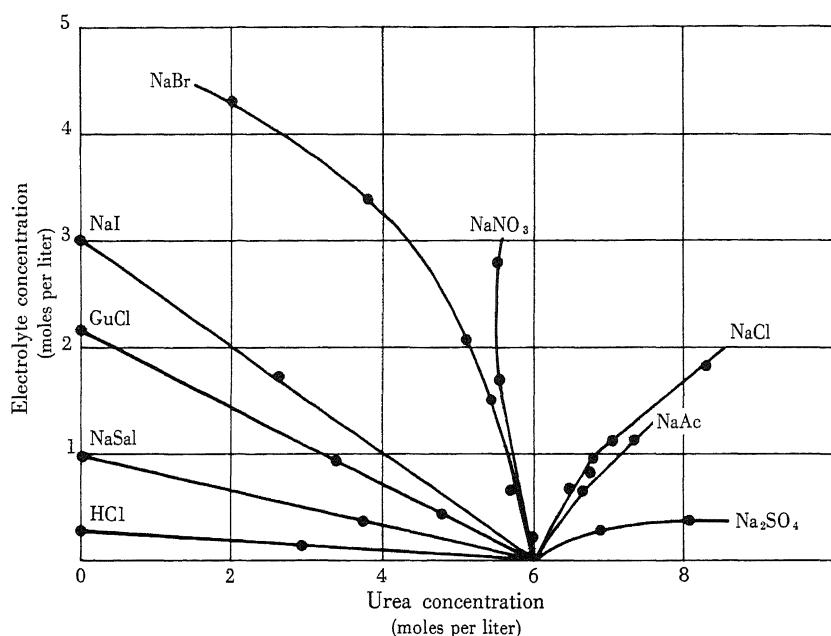
(a) *Egg albumin and edestin.* In Table 7.5 we present data which indicate that the adsorption energies of various anions on proteins follow the sequence: sulfate < acetate < chloride < bromide < nitrate < iodide < salicylate.\* We predict that the minimum urea concentration needed for a positive nitroprusside test should always decrease in this order when a protein is treated with varying concentrations of electrolytes bearing the same cationic component but these various anions. Burk, following the observations of Wu and Yang (1931) on the effect of salts on the urea denaturation of proteins, performed these experiments in 1943. His data on egg albumin and edestin are partially reproduced in Figures 7.16 and 7.17; they confirm our prediction.

(b)  *$\beta$ -lactoglobulin.*† Experiments with egg albumin and edestin demonstrate the additivity of anionic adsorbents and urea in changing the  $\varepsilon^0$  value;  $\beta$ -lactoglobulin provides another demonstration of the same relationship. In Figure 7.18

\* The relative positions of bromide and nitrate are the only ones that differ in various columns of Table 7.5. However, the difference between the effect of the two is never great and appears to be related to concentration (see Figures 7.16 and 7.17).

† Burk's "lactalbumin" was later named  $\beta$ -lactoglobulin.

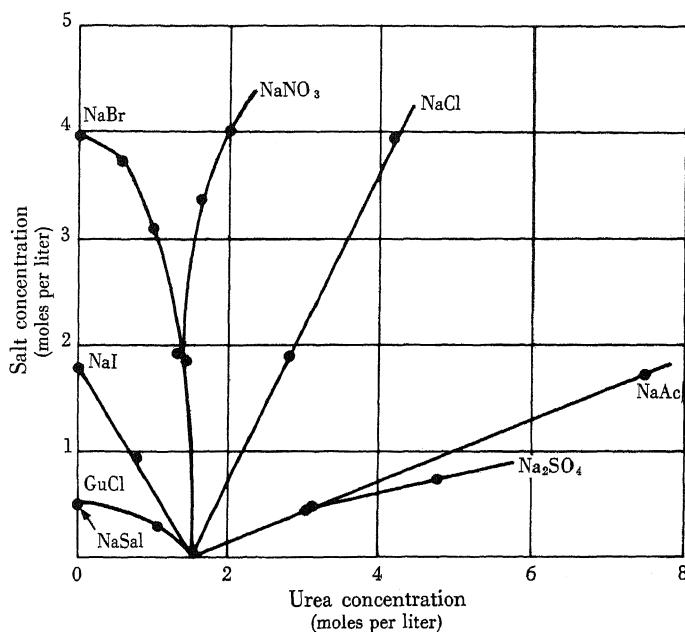
we have presented more of Burk's data. Using various chloride salts, he found that the critical urea concentration needed to reveal the SH group varies with the cation. This effect is also seen for egg albumin in Figure 7.16. However, we note an important difference between the effects of HCl on the two proteins. Concentrated HCl denatures both egg albumin and  $\beta$ -lactoglobulin. In egg albumin, it



**Figure 7.16.** CONCENTRATION OF ELECTROLYTES REQUIRED FOR A MINIMAL LIBERATION OF SULFHYDRYL GROUPS IN EGG ALBUMIN AS A FUNCTION OF UREA CONCENTRATION. Urea and electrolyte concentrations represented in the abscissa and ordinate, respectively. GuCl represents guanidine hydrochloride; NaSal represents sodium salicylate. Nitroprusside was used as the indicator for the reactive sulfhydryl group. (Redrawn from Burk, 1943.)

reduces the urea concentration needed to unmask the SH groups and eliminates the need for urea altogether. In  $\beta$ -lactoglobulin, it increases the need for urea. The present theory offers the interpretation that although the basis of denaturation is the indirect  $F$ -process, an all-or-none process, the net  $\varepsilon^0$ -value change of an SH group depends only on the altered counterions of polar groups in its immediate vicinity. Visualizing the SH group, flanked by both cationic and anionic groups, we expect the critical urea concentration to depend on the opposing inductive power of the cationic and anionic components. If, in egg albumin, the anionic and cationic neighbors of the SH groups were arrayed in a manner similar to that shown in Figure 7.19A, adsorption of the Cl<sup>-</sup> ion onto the cationic site

would thus increase the reactivity of the sulphydryl group, decreasing the amount of urea necessary to unmask it. The adsorption of the  $H^+$  ion onto the next-neighboring group would be less effective because of the greater intervening distance. If the arrangement of the polar groups were similar to that shown in Figure 7.19B, the hydrogen ion would be the more effective adsorbent and the amount of urea



**Figure 7.17.** CONCENTRATION OF ELECTROLYTES REQUIRED FOR A MINIMAL LIBERATION OF SULPHYDRYL GROUPS IN EDESTIN AS A FUNCTION OF UREA CONCENTRATION. GuCl represents guanidine hydrochloride; NaSal represents sodium salicylate. (Redrawn from Burk, 1943.)

necessary to unmask the SH group would be increased. We suggest that the arrangement of polar groups in  $\beta$ -lactoglobulin resembles this latter case. In either case, if we kept the cationic component constant and varied the anionic component, we would expect that the urea necessary to unmask the SH groups would vary inversely with the adsorption energy of the anion. Figure 7.20 shows that if the cation is held constant the critical urea concentration decreases in the order: acetate > monochloroacetate > dichloroacetate > trichloroacetate for egg albumin; this is exactly the order one would expect from the relative adsorption energies (Table 7.5).

The critical concentrations of urea necessary to unmask the SH groups of egg albumin and  $\beta$ -lactoglobulin in the absence of salt are also in agreement with the

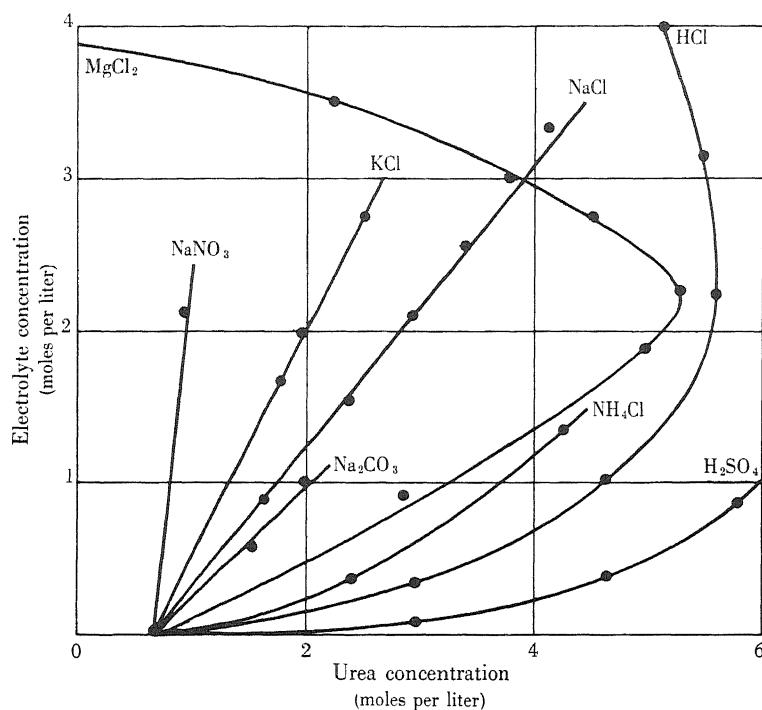


Figure 7.18. CONCENTRATIONS OF ELECTROLYTES REQUIRED FOR A MINIMAL LIBERATION OF SULPHYDRYL GROUPS IN  $\beta$ -LACTOGLOBULIN AS A FUNCTION OF UREA CONCENTRATION. Burk referred to  $\beta$ -lactoglobulin as lactalbumin in his paper. (Redrawn from Burk, 1943.)

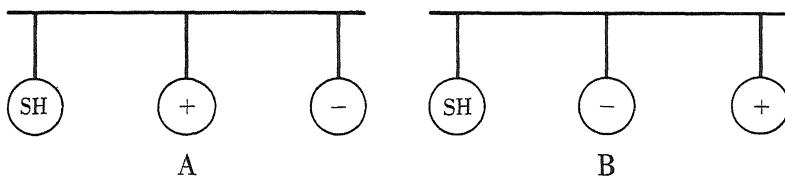
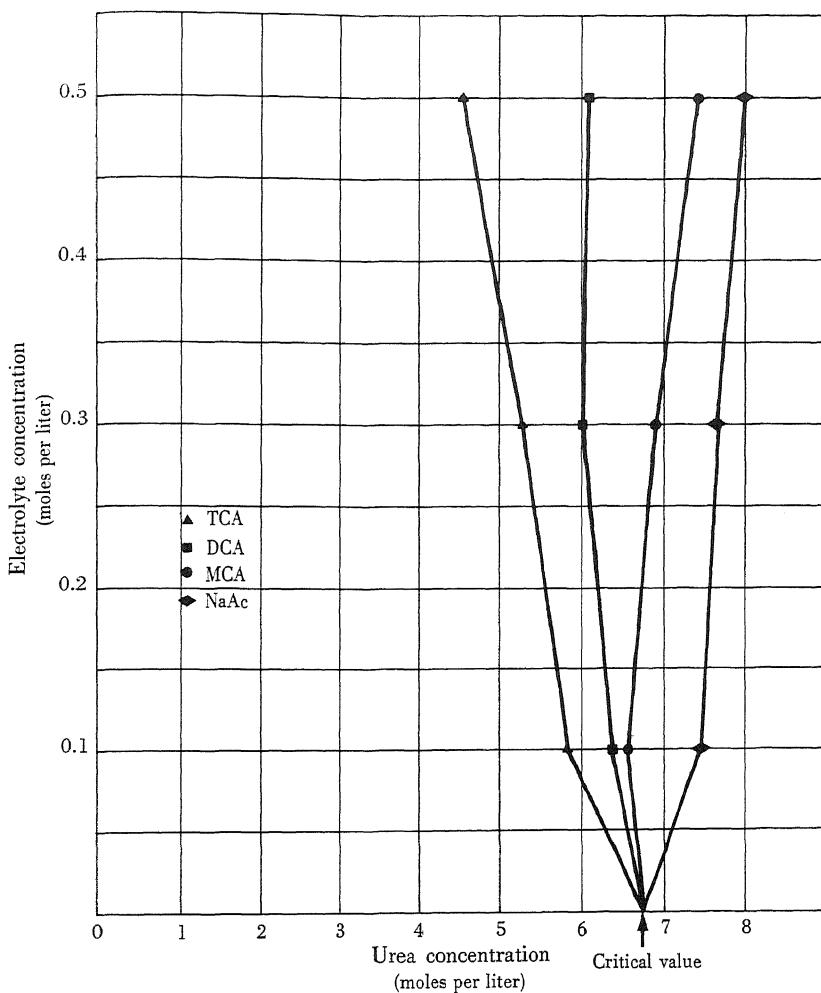


Figure 7.19. MODEL OF A SEGMENT OF A POLYPEPTIDE CHAIN BEARING A SULPHYDRYL GROUP. The reactivity ( $\varepsilon^0$  value) of the sulfhydryl group is a function of the adsorbents on the neighboring cationic and anionic sites. In A, the anionic adsorbent will have a greater influence on the reactivity of the SH group than a cationic adsorbent with equal or even greater adsorption energy due to the closer proximity of the fixed cationic site to the sulfhydryl group. In B, the cationic adsorbent will have more influence on the SH group.



**Figure 7.20.** CONCENTRATION OF SUBSTITUTED ACETATE SALTS REQUIRED FOR LIBERATION OF SULFHYDRYL GROUPS IN EGG ALBUMIN AS A FUNCTION OF UREA CONCENTRATION. Experimental procedures were similar to those of Burk (see Figure 7.16) except that end point was measured on a spectrophotometer using a wave length of  $550\text{m}\mu$ . Concentrations of salt and urea necessary to give a uniform reading of 300 at pH 7.0 are plotted. NaAc represents sodium acetate; MCA, sodium monochloroacetate; DCA, sodium dichloroacetate; TCA, sodium trichloroacetate. (Data of Ling and Kalis, unpublished.)

arrangements of polar groups shown in Figure 7.19. Thus, if the SH group is flanked by a cationic site, the concentration of urea necessary to unmask it should be higher than the concentration needed to unmask an SH group flanked by an anionic site. Figures 7.16 and 7.18 show that the critical urea concentration in the absence of salt is six molar for egg albumin but only one molar for  $\beta$ -lactoglobulin.

## E. CONCLUDING REMARKS ABOUT THE PHENOMENON OF PROTEIN DENATURATION

Our investigation of denaturation illustrates the unique behavior of protein molecules—a behavior arising from the long resonating polypeptide chain which permits the ready transmission of electronic changes. To be specific, denaturation is directly linked to an alteration of the number of salt linkages and H-bonds. Since the indirect *F*-process usually involves a large number of these bonds, variations in viscosity, light scattering, optical rotation, and solubility, factors influenced by the gross molecular properties of the molecule, should generally accompany the indirect *F*-effect. On the other hand, changes in the reactivity of individual groups do not necessarily proceed in a given direction. Thus, sulfhydryl reactivity may vary only in accord with variations in the near-neighboring polar groups and H-bonding sites; whether all the rest of the protein molecule changes its shape or not is, to the SH group, a matter of secondary importance. The results of Benesch *et al.* demonstrate that the extent of the reaction of simple cysteine and cysteinyl peptides with urea is proportional to the urea concentration; these results show that the adsorption at  $550m\mu$  increases linearly with urea concentration (Section 7.4C). In proteins, the interaction with urea is different; here it assumes the properties of an all-or-none response, suggesting that urea adsorption follows an indirect *F*-process. Thus, as soon as the near-neighbor sites of the SH group have adsorbed counterions compatible with their new equilibrium state, a new  $\varepsilon^0$  value is entirely determined. This change is not always an increase of the SH-group reactivity. The fact that it usually does increase concurrently with changes in viscosity, light scattering, and so on, shows that attention has been focused on changes in the masked type of SH group, whose masking is due to the presence of adjacent positively charged polar groups. The result of the indirect *F*-process is usually an exchange of the counterions on these cationic polar groups for more strongly adsorbed anions (Section 7.4D); this lowers the  $\varepsilon^0$  value of the SH groups, thus unmasking them.

It is conceivable that SH groups can be flanked by anionic groups. In this case, the SH group must be more reactive than simple cysteine toward a particular mild oxidizing agent. The indirect *F*-process may lead to a change of counterions on all neighboring sites, cationic and anionic, such that the balance of the  $\varepsilon^0$ -value changes is an increase rather than a decrease. If so, one should expect a greater masking of the SH groups to accompany denaturation (see Figure 7.12B). In support, we cite three pieces of experimental evidence: (1) Timasheff and Nord (1951) found that the cationic detergent, decylamine hydrochloride, denatures egg albumin, producing viscosity, optical rotation, and titration-curve changes; yet it unmasks no SH groups whatsoever. This becomes explicable if one assumes that, in the immediate vicinity of the SH group, the change of *c*-value brought about by the cationic component decylamine is actually greater than the change of *c'*-value due to the chloride ion, a reasonable conjecture. (2) Similarly, few

would doubt that 4*M* HCl is an effective denaturing agent. Yet we have shown that it acts on  $\beta$ -lactoglobulin to *enhance* the masking of the SH groups so that a much higher urea concentration must be used to unmask them (Figure 7.18). Twenty per cent TCA is a much higher concentration than is usually employed

Reagent	Concentration of reagent, mM per gm H <sub>2</sub> O	Cysteine, %
0.5 <i>M</i> KCl in H <sub>2</sub> O		0.40
Urea	16.7	0.64
N-Methylurea	10.0- 16.7	0
	1.7	0.40
O-Methylisourea HCl	16.7	0
Guanidine HCl	16.7	1.16
Guanidine H <sub>2</sub> SO <sub>4</sub>	1.1	0.40
Methylguanidine HCl	16.7	1.16
Arginine monohydrochloride	0.5	0
Glycine	0.01- 1.0	0
Ammonium chloride	1.7	0
Methylamine HCl	1.7	0
Dimethylamine HCl	1.7	0
Hydrazine monohydrochloride	1.7	0
Acetamide	16.7	0
KI	1.0	0.40
KSCN	1.0	0.40
MgCl <sub>2</sub>	1.0	0.40
CaCl <sub>2</sub>	1.0	0.40
LiCl	1.0	0.40

**Table 7.6. THE EFFECT ON THE SULFHYDRYL GROUPS OF MYOSIN OF VARIOUS AMMONIUM SALTS AND RELATED SUBSTANCES.** The amount of the dye, porphyrindin, sufficient to cause a negative nitroprusside reaction with the protein was taken as a measure of the reactive sulfhydryl groups present. The dye is added in  $1.16 \times 10^{-3} M$  concentration in 0.5*M* KCl to two milliliters of a 0.78 per cent solution of the protein. The amount of dye required is then expressed in terms of equivalents of cysteine per 100 grams of protein. (*From* Greenstein and Edsall, 1940.)

to denature proteins in ordinary biochemical work, yet it does not liberate the SH groups of  $\beta$ -lactoglobulin (Ling and Kalis, unpublished). These facts demonstrate a dissociation of gross changes in the properties of the protein and SH-group alteration. (3) Greenstein and Edsall (1940) observed that the SH group of "myosin," which is normally reactive, *disappears* on the addition of ammonium salts (Table 7.6). We shall present evidence to show that myosin has a strong affinity for the ammonium ion (Section 7.5). The experiment strongly suggests

that in myosin the cysteinyl residue has a nearby anionic residue (among others) which, through the direct *F*-effect, lowers the  $\delta^0$  value of the SH group and makes it normally reactive. When the anionic group preferentially adsorbs an  $\text{NH}_4^+$  ion, the direct *F*-effect then draws electrons from the SH group, decreasing its reactivity and causing it to "disappear" (see Figure 7.12B).

The alteration of counterions which leads to a change of the  $\delta^0$  of a sulfhydryl group is, like the acid titration curve of hemoglobin, time dependent (see Section 7.4B). Only on this basis can we derive a rational understanding of the use of the nitroprusside test for SH groups. Ordinarily, it would be meaningless to study the effect of, say, 0.1*N* HCl on the reactivity of cysteine with the nitroprusside reagent since the nitroprusside reaction must be carried out in a basic medium of about pH 12.0. Unless decomposition occurs, the  $\text{H}^+$  ionic dissociation of the monomeric cysteine molecule is instantaneously reversible; a cysteine molecule, therefore, does not remember its previous history and will react with nitroprusside in exactly the same way whether it has been treated with 0.1*N* HCl or not. But, through the operation of the time-dependent indirect *F*-effect, a protein does remember its previous encounter with HCl; within the time interval necessary to read the nitroprusside color, the neighboring polar groups are still associated with the components of HCl, momentarily unaffected by the medium that is no longer acidic.

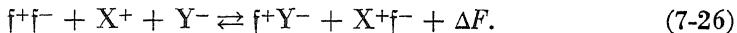
### 7.5. The Classification of Proteins on the Basis of Ammonium-Sulfate Fractionation and the Relation of this Classification to Protein *c*-Value Ensembles

Cells are composites of many different proteins; but when a cell, such as a muscle fiber, is broken and soaked in distilled water, most of these molecules do not diffuse out. They must, therefore, be bound together. The bonds cannot be S—S bonds since the proteins may be readily extracted by various salts of negligible reducing power and only a reducing agent can split the S—S bond. The simplest assumption is that the most significant of these bonds are salt linkages (see Figure 7.11; also Table 6.1).

Not all salts are equally effective in the extraction of all proteins. Myosin can be extracted from muscle with ammonium sulfate at a comparatively low concentration, but neither low nor high concentrations of ammonium sulfate will extract actin (Dubuisson, 1954, p. 31). Yet actin can be easily extracted with KI, particularly in the presence of a small amount of KSCN (Szent-Györgyi, 1951). This indicates that successful extraction is not merely a matter of ionic strength; specific ionic interactions must be involved (see Table 7.5 for the large adsorption energies of  $\text{I}^-$  and  $\text{SCN}^-$  in comparison with  $\text{SO}_4^{2-}$ ).

That different types of salts are needed to extract different proteins from the

same cells suggests a significant heterogeneity from protein to protein in the intermolecular salt linkages. On the basis of the above concept, the rupture of salt linkages preparatory to extraction of a protein may be represented by the simple equation,



Here  $\Delta F$  can be resolved into the algebraic sum of three terms,

$$\Delta F = \Delta F_{\text{f}^+\text{Y}^-} + \Delta F_{\text{f}^-\text{X}^+} - \Delta F_{\text{f}^+\text{f}^-}. \quad (7-27)$$

For the greatest efficiency in salt-linkage rupture,  $-(\Delta F_{\text{f}^+\text{Y}^-} + \Delta F_{\text{f}^-\text{X}^+})$  should be as much greater than  $-\Delta F_{\text{f}^+\text{f}^-}$  as possible. However, another factor must be considered: For proper extraction, salt linkages must be broken and then the protein thus dislocated must remain soluble. If both  $-\Delta F_{\text{f}^+\text{Y}^-}$  and  $\Delta F_{\text{f}^-\text{X}^+}$  are very high, the ionized groups of the dislocated protein will be completely bound to  $\text{X}^+$  and to  $\text{Y}^-$ , leaving no net ionized charge; such a protein will be insoluble (see discussion of the solubility of salmine, Section 7.4C). To solve this problem, we must make one of the factors,  $-\Delta F_{\text{f}^+\text{Y}^-}$ , very high and the other,  $-\Delta F_{\text{f}^-\text{X}^+}$ , very low while keeping  $-(\Delta F_{\text{f}^+\text{Y}^-} + \Delta F_{\text{f}^-\text{X}^+})$  as much larger than  $-\Delta F_{\text{f}^+\text{f}^-}$  as possible. We must choose a highly soluble salt, one of whose ions is very strongly adsorbed while the other ion is very weakly adsorbed. These characteristics are found in solutions of ammonium sulfate; this is probably the reason for the success of ammonium sulfate as a protein extractant; sulfate is among the anions most weakly bound to proteins (see Table 7.5). Thus, in the case of ammonium-sulfate extraction,  $\Delta F_{\text{f}^+\text{Y}^-}$ , the free energy for anion ( $\text{SO}_4^{2-}$ ) binding, is negligible. The chemical equilibrium,

$$\frac{[\text{f}^-\text{X}^+][\text{f}^+\text{Y}^-]}{[\text{f}^-\text{f}^+][\text{X}^+][\text{Y}^-]} = \exp \left[ \frac{-(\Delta F_{\text{f}^+\text{Y}^-} + \Delta F_{\text{f}^-\text{X}^+} - \Delta F_{\text{f}^+\text{f}^-})}{RT} \right] \quad (7-28)$$

reduces to

$$\frac{[\text{f}^-\text{X}^+][\text{f}^+\text{Y}^-]}{[\text{f}^-\text{f}^+][\text{X}^+][\text{Y}^-]} = \exp \left[ \frac{-(\Delta F_{\text{f}^-\text{X}^+} - \Delta F_{\text{f}^+\text{f}^-})}{RT} \right]. \quad (7-29)$$

The thoroughness of extraction depends on the quotient

$$\frac{[\text{f}^-\text{X}^+]}{[\text{f}^-\text{f}^+]} = \frac{[\text{X}^+][\text{Y}^-]}{[\text{f}^+\text{Y}^-]} \exp \left[ \frac{-(\Delta F_{\text{f}^-\text{X}^+} - \Delta F_{\text{f}^+\text{f}^-})}{RT} \right]. \quad (7-30)$$

Thus, for proteins with a similar number\* of salt linkages  $\text{f}^-\text{f}^+$  joining them to other structural proteins in the cell, the extent of extraction will depend on

\* We have neglected the variability in the number of intermolecular salt linkages in protein; this will undoubtedly vary. Figure 0.2, however, shows a general homogeneity in the frequency of charge-bearing amino-acid residues in enzyme proteins (those quoted in Table 7.7). Furthermore, no relationship appears to exist between these numbers and the concentration range of  $(\text{NH}_4)_2\text{SO}_4$  needed for extraction. For these reasons, the density of fixed ionic groups cannot account for the differences in salt concentration necessary to extract different proteins.

$\Delta F_{f-x^+} - \Delta F_{f+f^-}$  and on the concentration of extractant  $[X^+][Y^-]$ . If a given protein needs an extractant concentration of 30% saturation to accomplish a 90% extraction and another requires 80% of saturation to achieve the same extraction, a large difference in the value  $\Delta F_{f-x^+} - \Delta F_{f+f^-}$  for the two proteins should be expected. In other words, a large difference will exist between the relative affinities of the fixed anionic groups of the two proteins for free ammonium ions and for fixed amino

Enzymes	Ammonium sulfate salting-out limits, % of saturation
Globulins	Actomyosin
	Myosin
	Phosphorylase <i>a</i>
	Phosphorylase <i>b</i>
	Phosphofructokinase
	Tropomyosin
	Pyruvodehydrogenase
	Hexosephosphate isomerase
	Aldolase
	ATP-ADP creatine phosphopherase
Albumins	Myokinase
	Triosephosphate isomerase
	<i>d</i> -3 phosphoglyceraldehyde dehydrogenase
	72-80

**Table 7.7. SALTING-OUT LIMITS OF VARIOUS MUSCLE PROTEINS IN AMMONIUM SULFATE.** Concentrations expressed in terms of percentage saturation of ammonium sulfate necessary to precipitate the particular fraction. [Data from A. Distèche as quoted in Dubuisson (1954).]

groups. The difference between the proteins must be due to differences in the relative affinities of the fixed carboxyl groups for the fixed amino groups and for the ammonium ion. We can explain this in terms of differences between the *c*- and *c'*-values of the respective groups.

The *c*- and *c'*-value difference occurs widely among proteins. A. Distèche (Dubuisson, 1954) tabulated the ranges of ammonium sulfate concentrations required for the extraction of the various muscle proteins (Table 7.7). Each of these proteins has a relatively narrow and characteristic range. Myosin from fresh muscle is extracted at a low ammonium concentration. It must have a greater affinity for the ammonium ion than do tropomyosin or actin, for example. Since these proteins are almost indistinguishable on the basis of amino-acid composition (the composition of six of these proteins is given in Figure 0.2), the *c*-value and the *c'*- and  $\varepsilon^0$ -value ensembles must be of primary importance for the characterization

of proteins. We reached this conclusion from a consideration of the heats of ionization in Section 7.1.

## B I B L I O G R A P H Y

### BOOKS AND MONOGRAPHS

- Haurowitz, F., "Chemistry and Biology of Proteins." Academic Press Inc., New York, 1950. One of the few books on proteins written by a single author; it offers welcome readability and continuity.
- Cohn, E. J., and Edsall, J. T., eds., "Proteins, Amino Acids, and Peptides as Ions and Dipolar Ions." Reinhold, New York, 1943. Although nearly twenty years old, this classical book remains an important source of information on the physical and chemical properties of isolated proteins. Most of the chapters were written by Drs. Edsall and Cohn; a few chapters were contributed by other prominent authors. This book provides advanced discussion of individual topics rather than information about proteins in general. It does not deal with the more biological aspects of proteins, such as those discussed in some detail in Haurowitz's book, nor does it specifically treat the phenomenon of protein denaturation.
- Neurath, H., and Bailey, K., eds., "The Proteins: Chemistry, Biological Activity, and Methods," 2 volumes, 4 parts. Academic Press, New York, 1953-1954. These four volumes comprise the most up-to-date and comprehensive book available on proteins; they contain articles by many contributors with emphasis on authoritative treatment. This book provides details of the information available up to 1954 on practically all aspects of protein chemistry. As background for the present theory, Chapter 9 of Volume I, Part B, written by F. W. Putnam on Protein Denaturation, would be very valuable.
- Greenberg, D. M., ed., "Amino Acids and Proteins: Theory, Methods, Application." Charles C Thomas, Springfield, Illinois, 1951. Written cooperatively by 18 contributors, each working on a specific topic, this book is extremely valuable for its rich information about methods used in the study of proteins.
- Edsall, J. T., and Wyman, J., "Biophysical Chemistry," Vol. I: *Thermodynamics, Electrostatics, and the Biological Significance of the Properties of Matter*. Academic Press, New York, 1958. A general book "dealing with a number of limited topics with some depth" in the realm of biophysical chemistry. This first volume contains four chapters (3, 8, 9, and 11) particularly informative about proteins from the viewpoint of the most up-to-date current concepts.

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# PART IV

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ASSOCIATION AND INDUCTION

*IN VIVO*

In 1838–1839, both Schleiden and Schwann—following the work of Leeuwenhoek, Malpighi, Grew, and Hooke—enunciated a “cell” theory; the essence of it was that all complex organisms are built from small, basically similar units. It is unfortunate that Hooke (1665) had casually referred to these units as “cells,” thereby coining the name. Wilson, in his treatise “The Cell” (1928, p. 4), has pointed out that cells “do not, in general, have the form of hollow chambers as the name suggests but are typically solid bodies.”\* These solid bodies are composed of protoplasm, the material that Thomas Huxley called the “physical basis of life” (1853). *Pursuant to this philosophy, we submit that all cells and cytological structures share certain properties that arise from the attributes of protoplasm as a true fixed-charge system of protein, salt, and water. These attributes are reflected in the unique properties of the living state.* Protoplasm can assume varied characteristics that permit the distinction of cytological structures such as microsomes, nuclei, mitochondria, and cytoplasm; these are all proteinaceous fixed-charge systems and, as such, must possess the common attributes shared by all such systems.

In Part III, we showed that a protein molecule represents more than a simple summation of the attributes of its individual components. The interaction between these components when they are fixed into a single large molecule forces the protein to assume an entirely different set of attributes, unique to this level of complexity. As an illustration we refer to the autocooperative interaction of the polar groups on hemoglobin (Figures 7.9 and 7.10); such interaction can occur only when individual groups exist together as a close-knit unit. We shall suggest that the complex protoplasmic structure we call the cell, like the protein molecule, is not merely a simple summation of its constituent parts: membranes, contractile proteins, enzymes, and so on. It represents a functional continuum at a still higher level.

We regret that the complexity of the subject matter necessitates the imposition of arbitrary divisions on our discussion. Thus, the division of Part IV into chapters, should be recognized as an organizational device for transmitting information imposed by the limitations of human capability.

\* In this connection, we quote further from Wilson’s treatise, “The researches of Dujardin, De Bary, Cohn, Max Schultze, and many others long since showed . . . that most living cells are not hollow, but solid bodies . . . . The term “cell” thus became a biological misnomer. In the older cells of plants, it is true, the cytosome [cytoplasmic mass in contradistinction to the nucleus] itself often becomes sac-like through the appearance of watery vacuoles which enlarge and finally fuse to form a single large central vacuole, surrounded by a thin peripheral layer . . . . In such cases the living protoplasm does indeed assume the form of a hollow chamber, but this is of secondary origin and significance. In their young and less differentiated condition, these same cells are solid, like those of animals generally.” [From Wilson, 1928, p. 22. The phrase in brackets was inserted by the present author.]

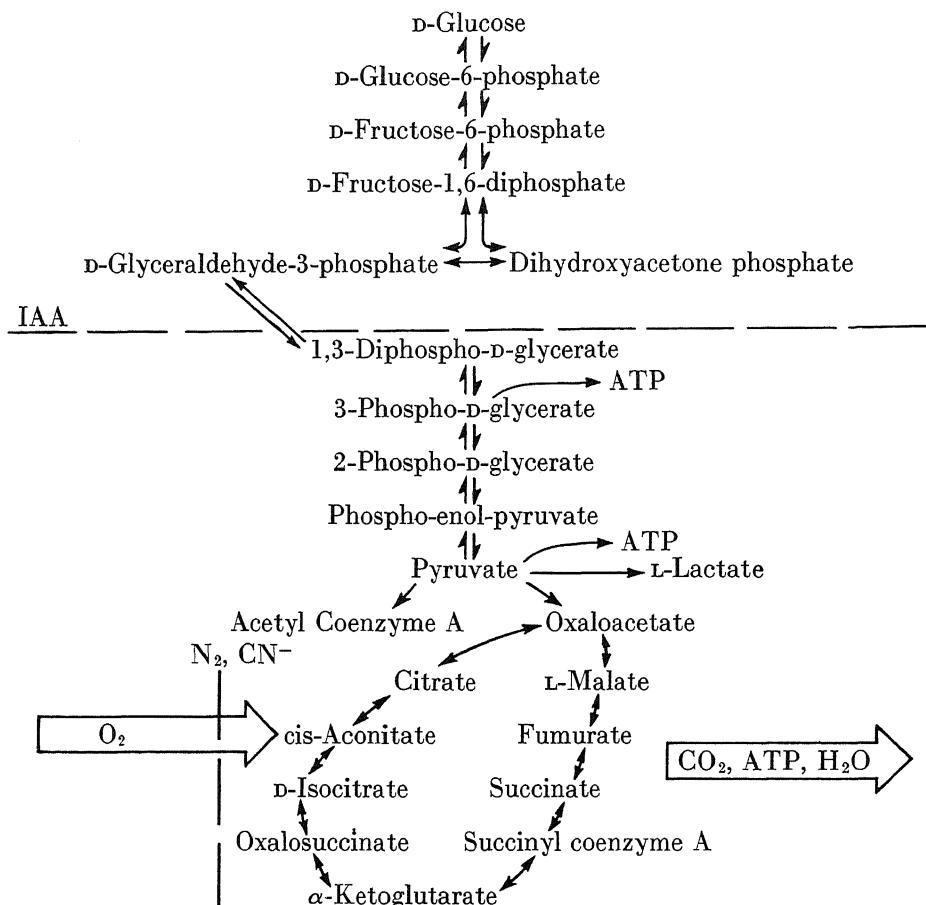
# 8

## THE ROLE OF METABOLISM IN BIOLOGICAL FUNCTIONS

- 8.1 The Role of High-Energy Phosphate-Bond Compounds in the Maintenance of Biological Functions 191
- 8.2 Evidence that the Maintenance of Selective Ionic Accumulation in the Resting Cell Represents a Metastable Equilibrium State Rather than a Steady State 195

Although isolated nonliving systems approach lower states of energy and higher degrees of randomness with the passage of time, living systems appear to counter this universal trend in two ways. They perform work reversibly, returning to their initial state after each cyclic event. They also maintain—for a considerable length of time—a labile but constant state, which invariably disintegrates after death. To reconcile these phenomena with physical laws, biologists have contended that reversible work performance, as illustrated by mechanical work in muscular contraction and electrical work in nerve action potentials, is coupled with energy-yielding reactions. Many biologists maintain that the sustenance of the labile living system, even in the resting condition, requires a continuous supply of energy; for example, maintenance of the high level of  $K^+$ -ion selectively accumulated in most living cells is thought to require a machinelike pump operation. This type of work is referred to as osmotic work and is conceived as the continuous use of metabolic energy to pump out undesirable elements (the  $Na^+$  ion, for instance) and to maintain a steady living state.

While they were developing these ideas, physiologists and biochemists added a comprehensive store of exact information about the complex sequence of chemical processes that constitute metabolism. Of central interest is the oxidative and

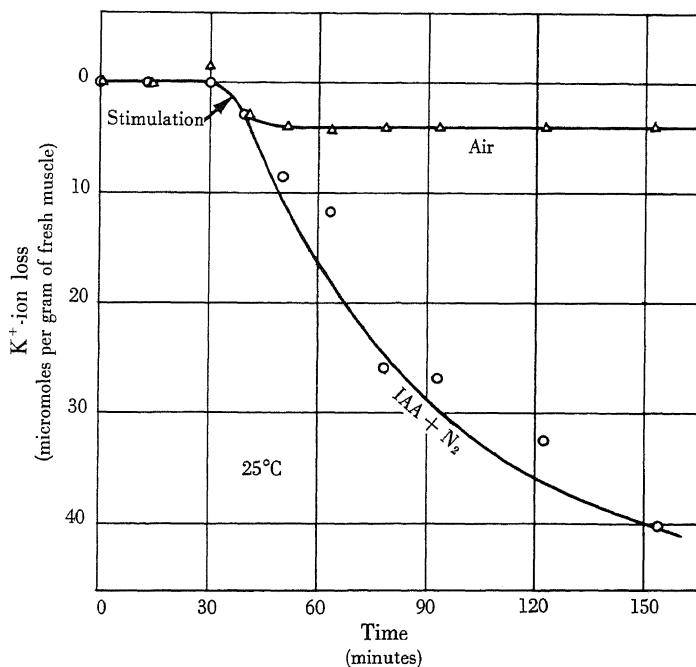


**Figure 8.1. THE MAIN PATHWAYS OF GLYCOLYTIC AND OXIDATIVE METABOLISM.** The main products of metabolism are ATP, lactate, CO<sub>2</sub>, and H<sub>2</sub>O. The points of action of the inhibitors, iodoacetate (IAA), cyanide (CN<sup>-</sup>), and nitrogen (N<sub>2</sub>), are indicated by broken lines.

glycolytic conversion of food matter into those usable packets of metabolic energy, which we now call high-energy phosphate bonds, residing in compounds such as phosphoenolpyruvate, acetyl phosphate, creatine phosphate (CrP), and particularly ATP. This process is outlined in Figure 8.1 (see Baldwin, 1957).

### 8.1. The Role of High-Energy Phosphate-Bond Compounds in the Maintenance of Biological Functions

To point out the significance of the high-energy phosphate bond in the present theory, first, we shall show that oxidation, glycolytic reactions, and the stores of high-energy phosphate-bond compounds such as CrP and ATP are the only significant sources of metabolic energy that maintain biological function; then we shall demonstrate that normal functions are maintained when both oxidative and glycolytic activities are entirely blocked, leaving high-energy phosphate bonds in the form of CrP and ATP as the only source of energy. Finally we shall ask: If



**Figure 8.2. THE LOSS OF K<sup>+</sup> ION FROM MUSCLE STIMULATED IN THE PRESENCE AND IN THE ABSENCE OF IODOACETATE (IAA) PLUS NITROGEN (25°C).** Paired muscles from the same frog were placed in tandem in separate watch glasses electrically connected through a moist cotton wick. At the time indicated by the arrow, tetanic stimulation from an inducitorium was applied directly to both muscles for two minutes. Successive aliquots of the bathing solution were then analyzed for K<sup>+</sup> ion. Points marked with triangles indicate the control muscle in normal Ringer's solution and air; points marked with circles indicate the experimental muscle in Ringer's solution containing 0.005M IAA and N<sub>2</sub>. Other experiments showed that the loss of K<sup>+</sup> ions from the experimental muscles continued until external and internal K<sup>+</sup>-ion concentrations were equal—about four and a half hours.

ATP and CrP can maintain functions, do they accomplish this by continually hydrolyzing and directly delivering their high-energy phosphate-bond energy or do they act through another mechanism?

Date	Frog No.	Environment	Muscle types	Muscle wt, mg	Temp., °C	Duration, hr	K, $\mu\text{M/g}$
3-3-55	1	Air	1, 2, 3	287.6	25	1	77.6
	1	N <sub>2</sub>		293.4		1	81.7
	2	Air		280.6		2	72.6
	2	N <sub>2</sub>		281.0		2	77.0
	3	Air		325.6		3	70.0
	3	N <sub>2</sub>		318.2		3	74.9
	4	Air		265.2		4	65.9
	4	N <sub>2</sub>		240.0		4	68.5
	5	Air		221.4		5	65.1
	5	N <sub>2</sub>		222.4		5	65.4
2-2-55	6	Air	1, 2, 3, 4	368.2	20	4	87.4
	6	N <sub>2</sub>		376.6		4	92.8
	7	Air		318.6		4	87.4
	7	N <sub>2</sub>		317.0		4	83.5
	8	Air		270.6		4	91.7
	8	N <sub>2</sub>		260.8		4	96.1
	9	Air		210.6		4	95.4
	9	N <sub>2</sub>		201.2		4	81.2
	10	Air		392.8		4	95.2
	10	N <sub>2</sub>		408.6		4	83.8
	11	Air		316.6		4	95.6
	11	N <sub>2</sub>		314.8		4	82.7

**Table 8.1. THE EFFECT OF ANOXIA UPON THE K<sup>+</sup>-ION CONTENT OF FROG MUSCLES.** The K<sup>+</sup>-ion content of paired *sartorius* (1), *semitendinosus* (2), *tibialis anticus longus* (3), and *iliofibularis* (4) muscles from frogs (selected by criteria discussed in Appendix G) was determined by means of flame photometry after the muscles had been soaked for one to five hours in Ringer's solution bubbled with either air or N<sub>2</sub> purified by passage through a column of activated copper. The data indicate no significant loss of K<sup>+</sup> ions after complete blockage of oxidative metabolism for up to five hours at room temperature. For the composition of the Ringer's solution employed in this and all subsequent experiments from our laboratory, see Appendix H.

Figure 8.2 presents the results of an experiment in which a frog *sartorius* muscle, poisoned with iodoacetate (which completely blocks glycolysis) and nitrogen (which completely inhibits respiration), was stimulated electrically. Exhaustive stimulation under such conditions removed the ATP and CrP content of the muscle. As the figure shows, following such stimulation, the muscle promptly began to lose its selectively accumulated K<sup>+</sup> ions. On the other hand, any one of the

factors, pure nitrogen (Table 8.1), glycolytic blockage (Table 8.4), or *sciatic* stimulation (Figure 8.2, control muscle), by itself, produced little effect (pure nitrogen, electrical stimuli), or a very slow one (0.005M iodoacetate alone, Ling,

Muscle condition	Potential <sup>b</sup>	
	Average, mv	Maximum fiber
In 0.005M IAA for 1 hr at 0°C, then at 22.5°C	85.2 ± 2.9	
After 100 shocks to muscle, 2/sec <sup>a</sup>	82.1 ± 3.8	
After 100 more shocks to muscle	76.0 ± 3.8	
After 100 more shocks to muscle	80.3 ± 3.0	
After 100 more shocks to muscle	84.9 ± 5.1	
20 min after stimulation	84.1 ± 2.0	
40 min after stimulation	64.4 irreg.	
60 min after stimulation	15.0 irreg.	
In 0.01M NaCN for 3 hr at 20°C	80.0 ± 2.2	
After 90 sec tetanization of muscle, 100/sec	47.7 ± 5.5	
45 min after tetanization	62.8 ± 9.5	
120 min after tetanization	75.4 ± 4.8	
In 0.005M IAA for 15 min at 22.5°C	77.6	
After 3 min tetanization of nerve, 100/sec	55.7	56.0
30 min after tetanization	55.7	56.0
60 min after tetanization	13.0	47.5
90 min after tetanization	12.0	45.6
120 min after tetanization	8.5	43.0

**Table 8.2. RESTING POTENTIALS OF SINGLE MUSCLE FIBERS IN IODOACETATE OR CYANIDE BEFORE AND AFTER STIMULATION.** These experiments demonstrate that the normal resting potential ( $84.5 \pm 3.2$ mv *in vivo*, Ling and Gerard, 1949a) can be maintained in the absence of active glycolytic and oxidative metabolism. The potentials of such poisoned muscle cells degenerate after electrical stimulation which removes CrP and ATP from them, see Table 8.3, also Ling, unpublished. (Data from Ling and Gerard, 1949b.)

<sup>a</sup> The interval between successive periods of stimulation is 3 to 5 minutes during which readings are made.

<sup>b</sup> Each figure is obtained on 4 to 12 fibers. The probable error of most figures given is between 2 and 5mv.

unpublished; see Dean, 1940). Table 8.2 shows the analogous behavior of the resting potential of muscle fibers; Figure 8.3, Table 8.3, Figure 8.4, and Figure 8.5 illustrate the companion observations on contractile function, action potentials, and the rate of  $\text{Na}^+$ -ion exchange in frog muscle. All of these functions can be performed normally in the absence of active oxidative and glycolytic activity as long as the normal CrP and ATP contents are present. But, as soon as these compounds are removed from the poisoned cells, all functions cease. We must conclude that

respiration, glycolysis, and high-energy phosphate-bond compounds in the form of CrP and ATP are the only energy sources which effectively maintain cellular excitability, contractility, and ability to accumulate ions selectively. Other sources of energy, if they exist, are so trivial in effect that they can be ignored.

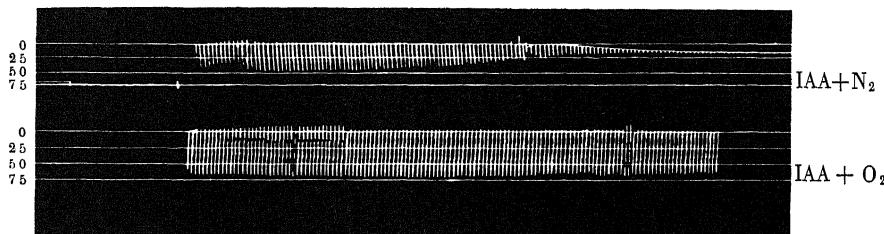
Date	Environment	Creatine phosphate in milligrams $P_2O_5$ per gram of <i>sartorius</i> muscle
5-17-30	Muscle in $N_2$	0.20
	Muscle in $O_2$	1.32
5-19-30	Muscle in $N_2$	0.15
	Muscle in $O_2$	1.23
5-19-30	Muscle in $N_2$	0.17
	Muscle in $O_2$	0.89
5-20-30	Muscle in $N_2$	0.09
	Muscle in $O_2$	0.73
5-23-30	Muscle in $N_2$	0.11
	Muscle in $O_2$	1.25
5-23-30	Muscle in $N_2$	0.27
	Muscle in $O_2$	0.97
5-25-30	Muscle in $N_2$	0.16
	Muscle in $O_2$	0.93
Average	Muscles in $N_2$	0.164
	Muscles in $O_2$	1.046

**Table 8.3. CREATINE PHOSPHATE CONTENT OF IODOACETATE-POISONED *SARTORIUS* MUSCLES AFTER STIMULATION IN OXYGEN AND NITROGEN.** Paired muscles were used as for Figure 8.3. The early exhaustion of the ability to perform mechanical work in IAA plus nitrogen was accompanied by an early exhaustion of CrP content as compared with the paired muscle contracting in IAA plus oxygen. (Data from Lundsgaard, 1930.)

Next, experiments were designed to assure complete suppression of respiration (oxidative metabolism) and glycolysis in frog muscle. Under these conditions and at a temperature of 0°C, the tissues maintained their excitability, contractility, and normal selective ionic accumulation pattern for at least eight hours (Tables 8.4 and 8.5). We concluded that, in adequate quantities, the high-energy phosphate-bond compounds, ATP and CrP, are capable, by themselves, of maintaining the tissue's capacity for normal performance of mechanical and electrical work as well as the normal asymmetric ionic distribution. This conclusion serves as the foundation for our discussion on the mechanisms of functions such as contraction and nerve activity.

### 8.2. Evidence that the Maintenance of Selective Ionic Accumulation in the Resting Cell Represents a Metastable Equilibrium State Rather than a Steady State

We have shown that respiration, glycolysis, and the store of high-energy phosphate-bond compounds are the only significant sources of energy in the cell. Yet a normal ionic accumulation pattern is maintained when both respiration and glycolysis are blocked. Thus, *if the maintenance of the ionic accumulation pattern*

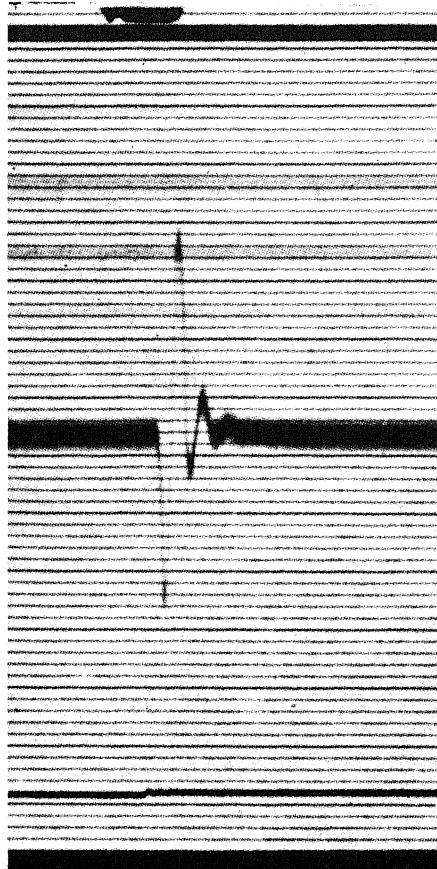


**Figure 8.3.** SUCCESSIVE MUSCLE TWITCHES FROM PAIRED FROG *SARTORIUS* MUSCLES POISONED WITH IODOACETATE. Stimulation was carried out in nitrogen (upper record) and in oxygen (lower record). These experiments establish that normal contractile function can be totally independent of active glycolytic and active respiratory metabolism. The ability to perform mechanical work ceases with the exhaustion of the CrP in the muscles, see Table 8.3. (Figure from Lundsgaard, 1930.)

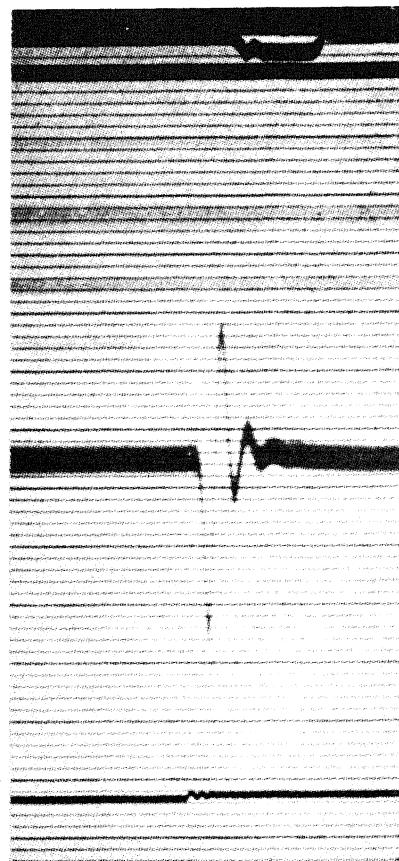
*depends upon a continuous energy expenditure, this energy must be that liberated in the degradation of the ATP and CrP originally present in the system.* Thus we can estimate the *maximum energy-delivery rate* by observing the difference between the amount of ATP and CrP in a muscle at the beginning of an experiment and the amount present at the end (in paired muscles).

We can also estimate the *minimum energy-delivery rate* required by the membrane-pump theory to maintain the normal asymmetric ionic distribution through the operation of a metabolic pump. If  $\text{Na}^+$  ions are to be pumped out of a cell, work must be done against both a concentration gradient (the intracellular concentration  $[\text{Na}]_{\text{in}}$  is much lower than the extracellular concentration  $[\text{Na}]_{\text{ex}}$ ) and an electrical potential gradient (the resting potential is positive outside the cell). The work done against the concentration gradient, per mole of  $\text{Na}^+$  transported, is given by

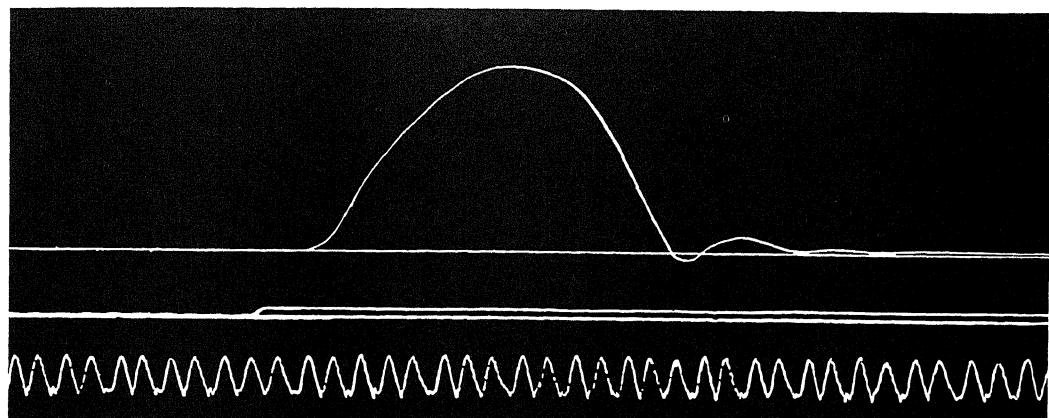
$$E_{\text{Na}}(t) = RT \ln \left( \frac{[\text{Na}]_{\text{ex}}}{[\text{Na}]_{\text{in}}} \right)_t \quad (8-1)$$



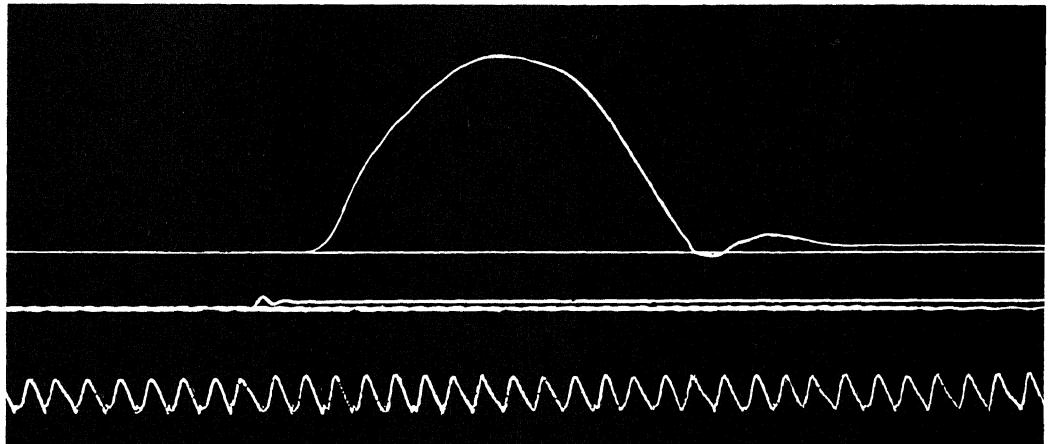
A



B

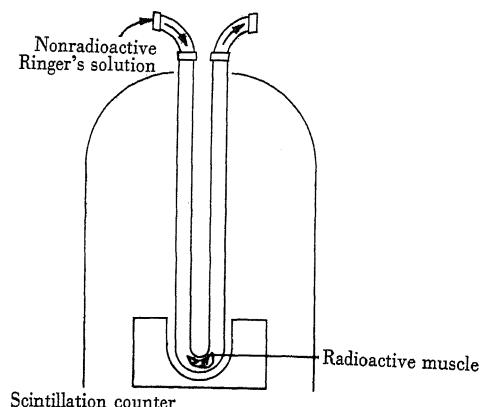
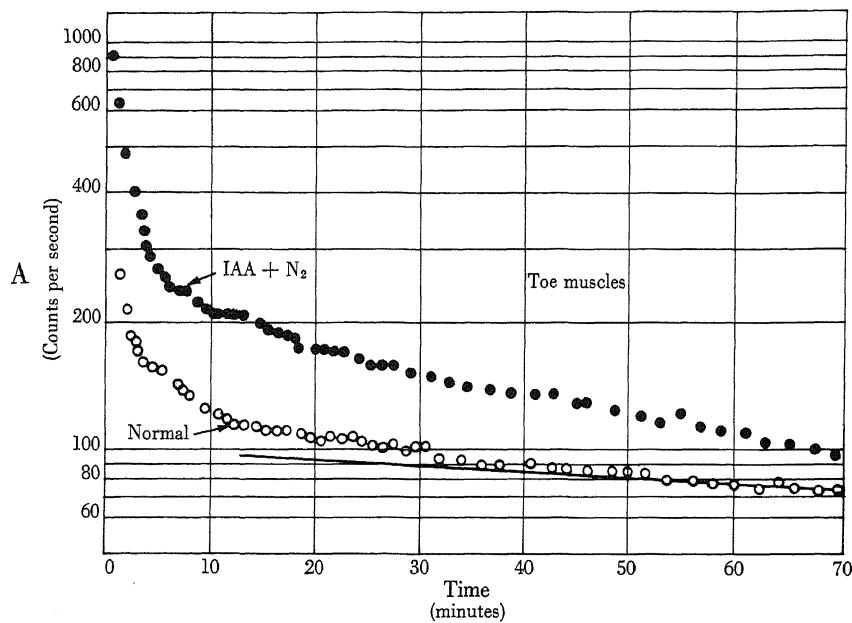


C

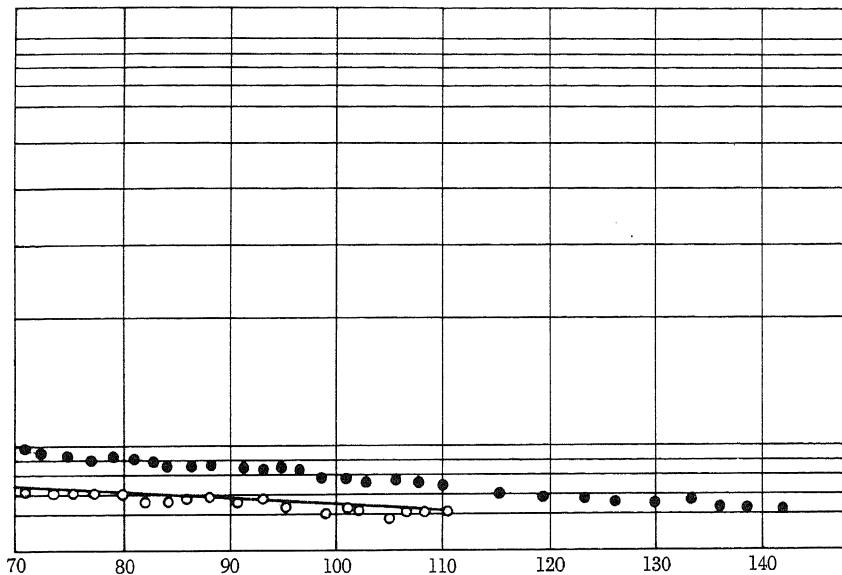


D

**Figure 8.4. ELECTRICAL AND MECHANICAL ACTIVITY OF IODOACETATE-POISONED FROG GASTROCNEMIUS MUSCLES.** (Room temperature.) A and B are electromyograms from indirectly stimulated *gastrocnemii*; C and D are isometric contraction tracings. Muscles A and D have been poisoned with IAA; muscles B and C are normal controls. The figure shows that normal electrical and mechanical behavior can be maintained in muscles whose glycolytic metabolism is blocked. (Figures from Henriques and Lundsgaard, 1931.)



B



**Figure 8.5. WASHOUT OF  $\text{Na}^{22}$  FROM FROG TOE MUSCLE.** A, paired *extensor digitorum* IV muscles were equilibrated overnight at  $0^\circ\text{C}$  in Ringer's solution containing  $\text{Na}^{22}$  and washed in Ringer's solution at  $0^\circ\text{C}$ . The washing solution used to produce the upper curve contained  $0.001M$  IAA and was bubbled with nitrogen; the lower curve was obtained with normal Ringer's solution bubbled with oxygen. These curves are similar in nature; the differences in their absolute magnitudes are largely results of the initial quantities of tracer present in and weight difference among the muscles. B, the washing-out apparatus used in this experiment and others described in Chapter 11 consists of a U-shaped glass tube that can be placed in the well of a scintillation counter. A radioactive muscle is held at the bottom of the tube by pieces of thread tied to either end of the muscle and affixed to the inlet and outlet of the glass tube. A continuous stream of nonradioactive Ringer's solution is drawn through the tube by negative pressure. Since small changes of position of the specimen do not affect the radioactive count (because of the  $4\pi$  geometry of the well), several samples can be studied simultaneously if the U-tubes containing them are placed in the well alternately. To assure a constant temperature for the experiments the entire apparatus was kept in a constant-temperature room.

Frog No.	Tissue	Control or experiment	Tissue wt, mg	K, $\mu\text{M/g}$	Na, $\mu\text{M/g}$
1	muscle 2 <sup>a</sup>	control	96.8	78.6	25.8
	muscle 2	experiment	96.8	78.6	28.5
	muscle 3 <sup>a</sup>	control	94.6	82.8	23.0
	muscle 3	experiment	93.6	81.1	24.6
2	muscle 2	control	109.6	70.2	18.0
	muscle 2	experiment	109.6	73.0	18.0
	muscle 3	control	105.6	67.6	20.8
	muscle 3	experiment	103.6	70.5	20.5
3	muscle 2	control	85.4	74.8	25.2
	muscle 2	experiment	84.6	74.4	30.9
	muscle 3	control	100.0	64.6	30.5
	muscle 3	experiment	101.4	75.6	23.2
4	muscle 2	control	86.4	55.5 <sup>b</sup>	41.6 <sup>b</sup>
	muscle 2	experiment	86.4	79.5	18.5
	muscle 3	control	88.8	45.1 <sup>b</sup>	57.7 <sup>b</sup>
	muscle 3	experiment	91.3	77.5	24.9
	nerve	control	33.0	34.8	73.0
	nerve	experiment	31.4	35.0	69.8
5	muscle 2	control	100.2	71.8	29.9
	muscle 2	experiment	101.0	71.0	29.9
	muscle 3	control	83.2	71.0	24.5
	muscle 3	experiment	84.3	62.0	34.4
	testis	control	26.8	56.0	35.8
	testis	experiment	23.0	56.7	41.3
	kidney	control	66.4	30.0	51.4
	kidney	experiment	64.0	35.2	51.9
	nerve	control	32.8	38.7	62.8
	nerve	experiment	28.0	35.8	51.1
6	muscle 2	control	100.0	70.5	36.7
	muscle 2	experiment	102.6	75.5	26.3
	muscle 3	control	97.0	71.4	31.0
	muscle 3	experiment	97.0	65.6	52.0
	testis	control	26.4	43.5	52.3
	testis	experiment	17.4	39.1	47.1
	kidney	control	74.2	40.4	60.7
	kidney	experiment	69.4	45.9	54.8
	nerve	control	30.2	37.1	79.5
	nerve	experiment	28.6	40.6	84.0
7	muscle 2	control	102.2	75.2	38.6
	muscle 2	experiment	103.6	86.7	27.5
	muscle 3	control	97.0	83.8	29.5
	muscle 3	experiment	97.0	82.6	28.1

Table 8.4 (Continued on facing page)

Frog No.	Tissue	Control or experiment	Tissue wt, mg	K, $\mu\text{M/g}$	Na, $\mu\text{M/g}$
8	muscle 2	control	88.0	83.0	27.6
	muscle 2	experiment	82.0	86.2	31.7
	muscle 3	control	80.4	78.4	33.7
	muscle 3	experiment	83.4	84.4	27.6
9	muscle 2	control	98.2	80.0	28.6
	muscle 2	experiment	94.8	84.3	27.4
	muscle 3	control	92.6	78.9	30.4
	muscle 3	experiment	92.8	77.4	35.9
10	muscle 2	control	90.8	71.5	29.6
	muscle 2	experiment	90.8	72.7	32.8
	muscle 3	control	98.2	73.5	28.1
	muscle 3	experiment	100.3	72.8	26.1
9	heart	experiment	97.6	29.8	52.5
5	heart	experiment	104.0	21.4	38.7
6	heart	experiment	88.6	33.9	48.6
7	heart	experiment	90.4	22.2	63.0
8	heart	control	83.4	36.3	56.3
4	heart	control	80.0	45.0	49.0
10	heart	control	83.4	40.2	47.2

**Table 8.4. THE EFFECT OF IODOACETATE AND PURE NITROGEN ON THE K<sup>+</sup>- AND Na<sup>+</sup>-ION CONTENTS OF FROG TISSUES.** Isolated frog tissues were placed in a Ringer's solution (0°C) containing 0.005M sodium iodoacetate (Eastman). The solution had been equilibrated with 99.99% pure nitrogen (Linde Air Corp.). At the end of the experiment (after 7 hours and 45 minutes), the individual tissues were boiled in 3ml of distilled water to extract their K<sup>+</sup>- and Na<sup>+</sup>-ion contents. (For experiments after 1953, a different procedure was adopted; extraction was accomplished by overnight emersion of the tissues, in 3ml of 0.1N HCl without heating.) The aliquot for the K<sup>+</sup>-ion determination was diluted to contain a final concentration of 0.1M NaCl; aliquots for the Na<sup>+</sup>-ion determination contained 0.1M KCl. These radiation buffers in the samples, as well as in all standards, eliminate both self-interference and radiation interference which can be considerable for direct flame photometry. A Beckman DU spectrophotometer with a flame attachment was employed. The results presented are those of an experiment performed on February 27, 1953; this experiment is one of more than 20 similar series performed since 1950; all yielded similar results. (For example see Table 8.5; here the control of respiration and glycolysis was more rigorous.)

<sup>a</sup> Muscles 2 and 3 represent the *semitendinosus* and *tibialis anticus longus*, respectively; "nerve" refers to *sciatic* nerve.

<sup>b</sup> Low K<sup>+</sup>, high Na<sup>+</sup> found in these control muscles were probably from the same abnormal leg.

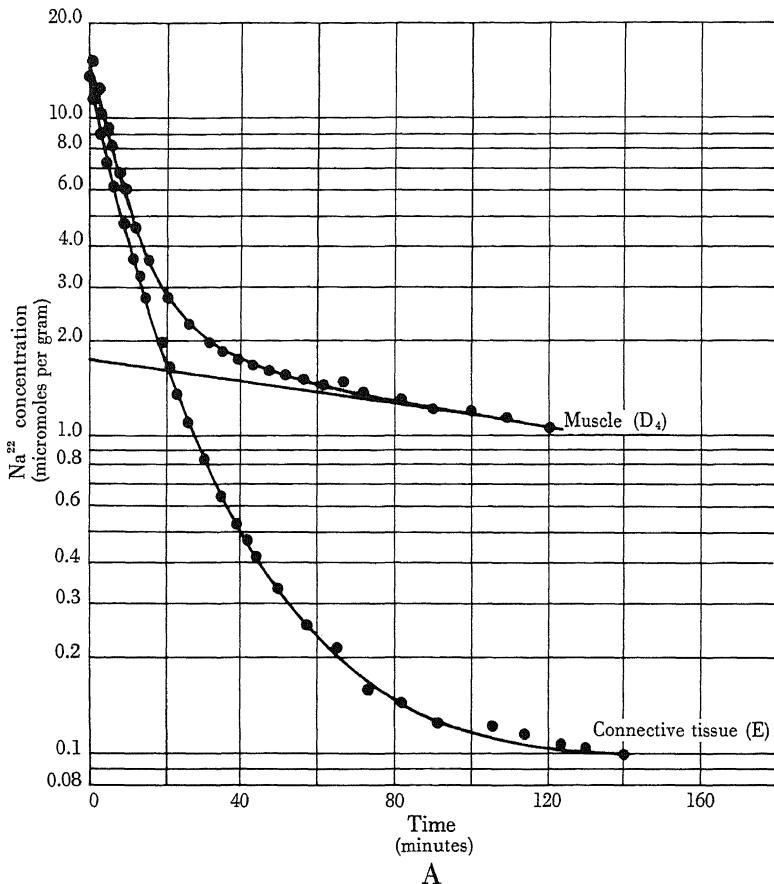
Frog No. and sex	Control or experiment	Muscle wt, mg	Duration of soaking in IAA-CN-N <sub>2</sub> , hr						
				K, $\mu\text{M/g}$	CrP, $\mu\text{M/g}$	$\Delta\text{CrP}$ , $\mu\text{M/g}$	$\Delta F$ (CrP), cal/kg/hr	ATP, $\mu\text{M/g}$	$\Delta\text{ATP}$ , $\mu\text{M/g}$
1 ♀	Control	418.1	0	72.5	10.5	-3.7	-11.8	7.31	-0.83
	Experiment	421.5	4	61.7	6.8			6.48	
2 ♀	Control	421.3	0	72.5	17.7	-7.4	-23.7	7.00	-0.34
	Experiment	441.1	4	67.8	10.3			6.66	
3 ♀	Control	373.3	0	77.7	11.3	-5.8	-18.6	6.78	+0.27
	Experiment	350.5	4	68.3	5.5			7.05	
4 ♂	Control	367.9	0	73.5	17.2	-8.5	-27.2	7.46	-0.65
	Experiment	374.9	4	72.4	8.7			6.81	
5 ♂	Control	407.3	0	69.5	24.2	-7.1	-22.8	6.49	+0.53
	Experiment	412.7	4	76.3	17.1			7.02	
6 ♂	Control	307.1	0	66.4	19.2	-7.9	-25.3	6.15	+0.63
	Experiment	313.8	4	74.7	11.3			6.78	
Average							-21.57		

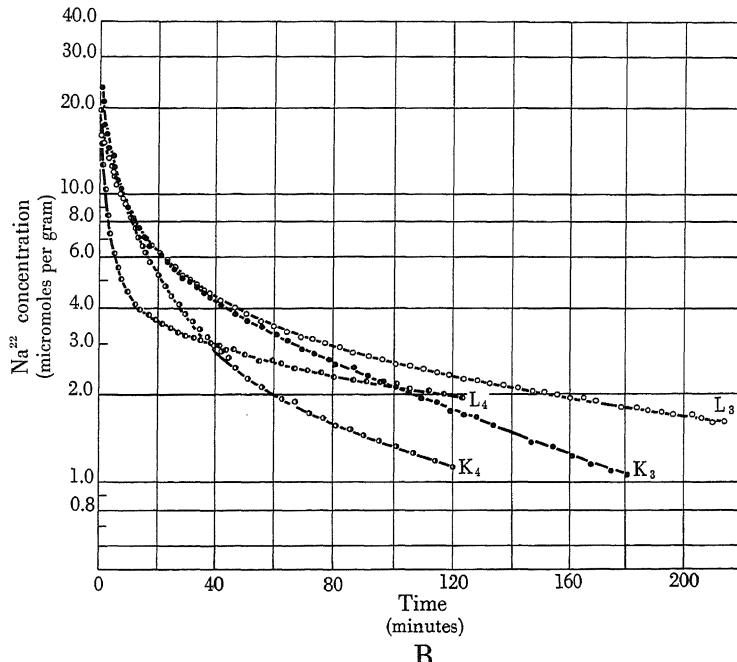
Frog No. and sex	$\Delta F$						$\Delta F$ (Lactate), <sup>a</sup> cal/kg/hr	$\Delta F$ (Total), cal/kg/hr
	$\Delta F$ (ATP), cal/kg/hr	ADP, $\mu\text{M/g}$	$\Delta\text{ADP}$ , $\mu\text{M/g}$	$\Delta F$ (ADP), cal/kg/hr	Lactate, $\mu\text{M/g}$	$\Delta$ Lactate, $\mu\text{M}^a$		
1 ♀	-6.10	0.09 0.45	+0.36	+1.35	0.972 0.415	-0.235		-17.20
2 ♀	-2.49	0.0 0.42	+0.42	+1.58	0.848 0.352	-0.219		-25.18
3 ♀	+1.00	0.10 0.21	+0.11	+0.45	0.719 0.387	-0.117		-17.71
4 ♂	-4.75	0.93 0.61	-0.32	-1.20	0.623 0.478	-0.055		-33.71
5 ♂	+3.87	0.38 0.0	-0.38	-1.35	0.511 0.364	-0.065		-20.84
6 ♂	+4.63	0.50 0.0	-0.50	-1.95	0.540 0.369	-0.053		-23.18
Average	-0.64			-0.18			-0.56	-22.97

Total loss from muscles,  $0.74 \mu\text{M}$ . Bathing solution contained  $0.0196 \mu\text{M}/\text{ml}$  before experiment;  $0.0298 \mu\text{M}/\text{ml}$  after experiment. Total gain in solution,  $0.929 \mu\text{M}$ . Net production,  $0.185 \mu\text{M}$ .

**Table 8.5.** MAXIMUM RATE OF ENERGY DELIVERY IN MUSCLES POISONED WITH IODOACETATE, CYANIDE, AND PURE NITROGEN. *Sartorius*, *semitendinosus*, *tibialis anticus longus*, and *iliofibularis* muscles from one frog were used in each experimental group; the paired muscles served as controls. First, both control and experimental muscle groups were equilibrated with 0.001M IAA at 0°C for one hour; this ensures complete penetration of IAA and inhibition of glycolysis. The control muscles were then homogenized in 10 per cent perchloric acid and the extract was immediately neutralized. The experimental muscles were transferred to a special flask with 100ml of Ringer's solution (pH 7.4) containing 0.001M IAA and 0.001M NaCN (also at 0°C); the solution had been equilibrated with a 5% CO<sub>2</sub> + 95% N<sub>2</sub> mixture. The oxygen tension of this mixture was no higher than  $4 \times 10^{-5}$  per cent; to assure this, it was passed first through heated copper turnings and then through a tower of activated copper. After the solution containing the experimental muscles had been bubbled for two hours with the CO<sub>2</sub>-N<sub>2</sub> mixture, both inlet and outlet tubes were closed and the entire flask was immersed in the ice water bathing the flask. At the end of four hours in IAA-CN-N<sub>2</sub>, the experimental muscles were also homogenized. Aliquots of the perchloric acid extract from both controls and experimentals were analyzed for K<sup>+</sup> ion, CrP, ATP, ADP, and lactate. The lactate contents of the bathing solution were determined at the beginning and end of the experiments. K<sup>+</sup>-ion concentrations were determined by flame photometry, CrP by Gomori's modification (1942) of the method of Fiske and Subbarow (1929), ATP and ADP by a modification of the method of Kalckar (1947) using 5-adenylic-acid deaminase, myokinase, and potato apyrase. (Appendix D outlines an easy method for preparation of a large quantity of stable adenylic-acid deaminase that can be used for the accurate and simultaneous determination of the ATP, ADP, and AMP contents of a large number of samples.) Average lactate production (under IAA) was determined by the method of Barker and Summerson (1941), taking into account the changes both in the muscles and in the bathing solution. The calculation is shown in the column under  $\Delta F$  (lactate); the average shown is the total change in free energy, taking both the muscle and the bathing-medium lactate into account. For calculation of the maximum rate of free energy delivery, we used the relatively high values of Burton and Krebs (1953; see also Levintow and Meister, 1954; Vladimirov *et al.*, 1957; George and Rutman, 1960):  $\Delta F$  (ATP → ADP + P) between -12.5 and -16.0, averaging -14.3 kcal/mole;  $\Delta F$  (ADP → AMP + P) -15.0 kcal/mole;  $\Delta F$  (CrP → Cr + P) -12.8 kcal/mole and  $\Delta F$  (glucose → 2 lactate) -28 kcal/mole of lactate. These  $\Delta F$  values are free energy changes for the conditions presumed to exist in living cells and are not standard free energy changes.

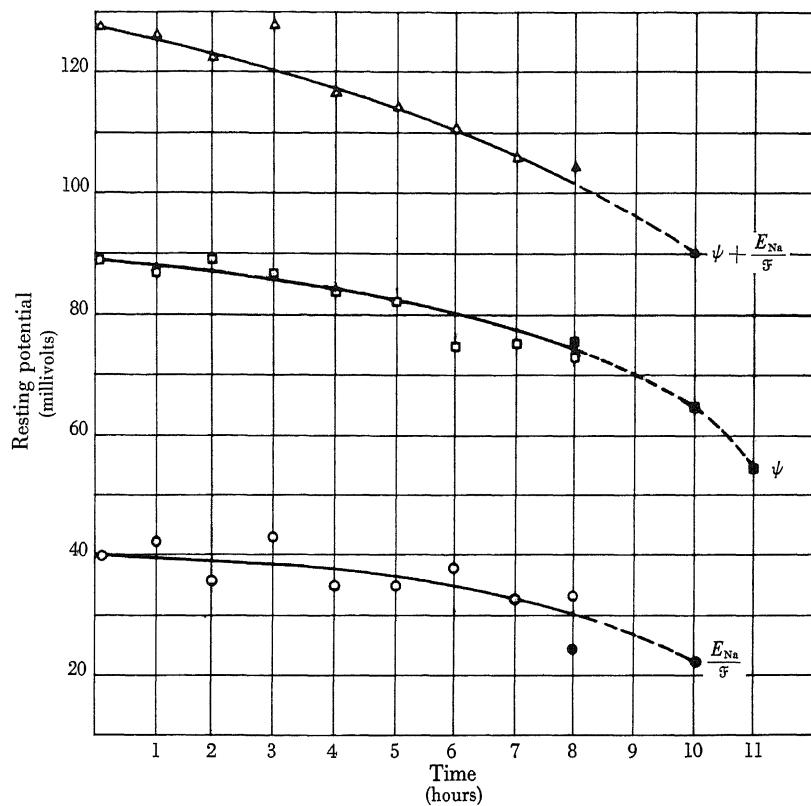
\* Total amount of lactate change by muscle was obtained by multiplying the difference between experimental and control lactate concentrations by the weight of the experimental muscle.





**Figure 8.6. THE RATE OF  $\text{Na}^+$ -ION EXCHANGE IN NORMAL CONNECTIVE TISSUES AND MUSCLE-FIBER BUNDLES POISONED WITH IAA-CN-N<sub>2</sub>.** A, connective-tissue elements weighing 5.35 mg were soaked in  $\text{Na}^{22}$  Ringer's solution for 4 minutes and 24 seconds. For data on normal muscle-fiber bundle D<sub>4</sub>, see Table 8.7. For details of the experimental procedure, see legends of Table 8.7 and Figure 8.5. The straight line through the last points on curve D<sub>4</sub> gives the extrapolated initial concentration of  $\text{Na}^{22}$  ion. B, similar to A except that all muscle-fiber bundles were treated with IAA-CN-N<sub>2</sub>. Details are given in Table 8.7. From these curves, the extrapolated initial concentrations of  $\text{Na}^{22}$  ion were obtained.

From microelectrode measurements, we obtained the electrical potential gradient  $\psi(t)$  at various times during the experiment (Table 8.6). The work per mole done against the potential gradient is equal to  $\mathfrak{F}\psi(t)$ , where  $\mathfrak{F}$  is the Faraday constant.



**Figure 8.7. TIME COURSE OF CHANGE OF THE RESTING POTENTIAL  $\psi$  AND THE INTRACELLULAR  $\text{Na}^+$ -ION CONCENTRATION IN IODOACETATE-, CYANIDE-, AND NITROGEN-POISONED FROG *SARTORIUS* MUSCLES ( $0^\circ\text{C}$ ).** The  $\text{Na}^+$ -ion concentration is expressed as  $E_{\text{Na}}/\mathfrak{F}$ , a potential gradient derived from the Nernst equation,  $E_{\text{Na}} = RT \ln ([\text{Na}]_{\text{ex}}/[\text{Na}]_{\text{in}})$  for  $0^\circ\text{C}$ . Empty triangles, squares, and circles are taken from a single experiment (Table 8.5); solid points are averages of other experimental values.

The minimum energy required by the  $\text{Na}$  pump to sustain selective ionic accumulation for a length of time  $t_f - t_0$  is given by the total work  $\Delta W$  done in this time:

$$\Delta W = \int_{t_0}^{t_f} [\mathfrak{F}\psi(t) + E_{\text{Na}}(t)] J_{\text{Na}}^{\text{in} \rightarrow \text{ex}}(t) dt \quad (8-2)$$

where  $J_{\text{Na}}^{\text{in} \rightarrow \text{ex}}(t)$  is the rate of pumping in moles of  $\text{Na}^+$  ion exchanged per kilogram

Frog No.	Muscle No.	Muscle wt, mg	Duration in hr	CN-N <sub>2</sub> -IAA, fibers measured	Resting potential $\psi$ , mv		$E_{Na}/\mathfrak{F}^a$ , mv	$\psi + E_{Na}/\mathfrak{F}$ , mv
					No.	Mean $\pm$ standard deviation		
1	1	58.0	0	8	99.5 $\pm$ 4.0	97.0	40.5	39.1 128.0
	2	62.5	0	8	94.5 $\pm$ 2.9		37.7	
2	1	84.0	1	8	91.5 $\pm$ 5.5	93.3	37.0	41.4 126.8
	2	86.6	1	8	94.8 $\pm$ 2.9		45.8	
3	1	62.4	2	8	97.5 $\pm$ 4.9	96.7	31.7	33.8 122.3
	2	62.3	2	8	96.0 $\pm$ 1.1		35.8	
4	1	62.2	3	10	94.8 $\pm$ 3.5	94.0	43.2	42.6 128.6
	2	60.2	3	10	93.0 $\pm$ 3.7		41.9	
5	1	41.3	4	8	90.4 $\pm$ 3.1	89.0	34.6	34.0 115.5
	2	38.6	4	8	97.5 $\pm$ 6.6		33.3	
6	1	44.9	5	8	84.9 $\pm$ 2.5	88.0	34.3	33.6 114.1
	2	42.6	5	8	90.8 $\pm$ 2.0		32.8	
7	1	63.7	6	8	79.4 $\pm$ 4.6	80.0	33.3	37.7 111.0
	2	60.5	6	8	80.6 $\pm$ 4.8		42.0	
8	1	61.5	7	8	79.4 $\pm$ 4.9	80.5	39.0	32.5 106.2
	2	62.9	7	8	81.7 $\pm$ 3.8		26.0	
9	1	57.2	8	10	74.2 $\pm$ 7.8	78.5	36.6	34.6 106.5
	2	51.6	8	10	82.6 $\pm$ 5.0		32.6	

**Table 8.6. THE AVERAGE RESTING POTENTIAL AND INTRACELLULAR  $Na^+$ -ION CONCENTRATION OF FROG *SARTORIUS* MUSCLES POISONED WITH NITROGEN, CYANIDE, AND IODOACETATE ( $0^\circ C$ ).** We determined the intracellular  $Na^+$  ion by flame photometry and measured resting potentials of individual fibers with Gerard-Graham-Ling microelectrodes. The intracellular-extracellular  $Na^+$ -ion concentration gradient is expressed as a potential gradient derived with the Nernst equation for  $0^\circ C$ . The last column gives the minimum energy, on the basis of a membrane-pump hypothesis, for the extrusion of one mole of  $Na^+$  ions against the measured electrical and concentration gradients. Experimental conditions were similar to those described under Table 8.4 except that only *sartorius* muscles were used.

\*  $E_{Na}/\mathfrak{F}$  values are given by the relation  $E_{Na}/\mathfrak{F} = 54.3 \log \frac{[Na]_0}{[Na]_i}$ , where  $[Na]_0 = 107.6$  millimoles per liter.

of fresh muscle per hour; this measurement was also made from time to time on the experimental muscles using the radioactive tracer  $Na^{22}$  (Table 8.7);\* these measurements enable us to estimate the minimum energy necessary for the effec-

\* For accurate measurements of the rate of  $Na^+$ -ion exchange and for criticism of other methods usually employed, see Section 11.1.

	Muscle No.	Muscle wt, mg	Muscle-fiber length, cm	Total No. of muscle fibers in bundle	Fiber diameter		$\frac{V}{A}$ , $\mu$	$\frac{A}{W}$ , $\text{cm}^2/\text{g}$	Duration in poison, hr
					No. of fibers counted	Average, $\mu$			
A	1	6.82	1.8	59	9	71	18	530	0.33
	2	2.07	1.8	17	9	73	20	477	0.33
	3	2.26	1.2	24	10	63	16	596	0.83
	4	5.68	1.3	70	11	63	16	596	1.50
	5	5.18	1.5	53	9	63	16	596	1.50
	6	8.60	1.4	80	10	82	20	477	2.33
	7	2.76	1.5	20	10	71	18	530	2.33
	8	7.45	1.4	73	10	75	19	500	4.33
	9	3.49	1.5	32	11	67	17	560	4.33
	10	9.84	1.7	67	10	69	17	569	8.00
	11	4.99	1.5	39	10	65	16	596	8.00
Average								550	
B	D <sub>4</sub>	17.03							0.00
	L <sub>4</sub>	6.00		76					1.00
	L <sub>3</sub>	17.45		204					2.50
	K <sub>3</sub>	3.50		45					4.50
	K <sub>4</sub>	8.60		101					8.00

	Muscle No.	Duration in Na <sup>22</sup> Ringer's solution, min	Rate of Na <sup>+</sup> flux				
			M/kg/hr			M/cm <sup>2</sup> /sec	
			Uncorrected	Cor-rected	Aver-age	Uncorrected	Corrected
A	1	3.0	0.133	0.121	0.118	$6.97 \times 10^{-11}$	$6.33 \times 10^{-11}$
	2	3.0	0.125	0.114		$7.28 \times 10^{-11}$	$6.62 \times 10^{-11}$
	3	3.0	0.073	0.066		$3.40 \times 10^{-11}$	$3.09 \times 10^{-11}$
	4	3.0	0.114	0.104		$5.31 \times 10^{-11}$	$4.83 \times 10^{-11}$
	5	3.0	0.121	0.110		$5.64 \times 10^{-11}$	$5.13 \times 10^{-11}$
	6	3.0	0.071	0.064	0.081	$4.13 \times 10^{-11}$	$3.75 \times 10^{-11}$
	7	3.0	0.108	0.098		$5.66 \times 10^{-11}$	$5.14 \times 10^{-11}$
	8	3.0	0.173	0.157		$9.61 \times 10^{-11}$	$8.73 \times 10^{-11}$
	9	3.0	0.109	0.099	0.128	$5.41 \times 10^{-11}$	$4.92 \times 10^{-11}$
	10	3.0	0.169	0.154		$8.25 \times 10^{-11}$	$7.50 \times 10^{-11}$
	11	3.0	0.165	0.150		$7.69 \times 10^{-11}$	$6.99 \times 10^{-11}$
B	D <sub>4</sub>	3.1	0.035	0.035			$1.76 \times 10^{-11}$
	L <sub>4</sub>	2.8	0.074	0.074			$3.74 \times 10^{-11}$
	L <sub>3</sub>	2.7	0.162	0.162			$8.18 \times 10^{-11}$
	K <sub>3</sub>	2.5	0.197	0.197			$9.95 \times 10^{-11}$
	K <sub>4</sub>	2.3	0.113	0.113			$5.71 \times 10^{-11}$

**Table 8.7. THE RATE OF  $\text{Na}^+$ -ION EXCHANGE IN MUSCLE-FIBER BUNDLES POISONED WITH IAA AND PURE NITROGEN.** Muscle-fiber bundles were isolated from the parts of frog *semitendinosus* muscles that are practically free of small-fiber-innervated muscle fibers (Kuffler and Vaughan-Williams, 1953). Series-A bundles were soaked in Ringer's solution containing IAA (0.001M) and bubbled with pure  $\text{N}_2$  for up to eight hours at 0°C. The container of Ringer's solution was in a large covered glass jar containing ice water and continuously flushed with pure nitrogen; the glass jar was kept in a constant-temperature room maintained at 0° to 2°C. After the specified time, the muscle bundle was transferred by means of a remote-handling device to a smaller vessel of Ringer-IAA mixture containing  $\text{Na}^{22}$ . After soaking for 3 minutes in this solution, the muscle bundle was placed in a large beaker and washed there for 1.5 minutes in a nonradioactive Ringer's solution vigorously bubbled with nitrogen. The radioactivity of the tissue was then assayed in the well of a scintillation counter. Diffusion through the extracellular space can be determined from the following equation (Jost, 1952):

$$\frac{\bar{C}(t) - C(\infty)}{C(0) - C(\infty)} = \frac{8}{\pi^2} \sum_{\nu=0}^{\infty} \frac{1}{(2\nu+1)^2} \exp \left[ -\pi^2 \left( \frac{2\nu+1}{h} \right)^2 Dt \right].$$

Here  $\bar{C}(t)$  is the concentration at time  $t$ ;  $C(\infty)$  is the final concentration, and  $C(0)$  is the initial concentration;  $\nu$  is an integer equal to 0, 1, 2 . . . ;  $h$  is the height of the capillaries, and  $D$  is the self-diffusion constant which, for the  $\text{Na}^+$  ion, is equal to  $1.30 \times 10^{-5} \text{ cm}^2/\text{sec}$  (Mills, 1955). From this calculation, we find that washing for 1.5 minutes removes virtually all  $\text{Na}^{22}$  contained in the extracellular space. A more significant error arises from the connective-tissue elements which, in whole *sartorius* muscle, constitute about 9.1 per cent of the wet weight (see Table 8.8). Such connective-tissue elements take up less  $\text{Na}^{22}$  than whole muscle does (see Figure 8.6) and release it more rapidly. A deliberately overestimated correction for connective tissue was made by the subtraction of 9.1 per cent of the assayed  $\text{Na}^{22}$  content from this latter figure. For series B, a totally different method of estimating the  $\text{Na}^+$  ion exchange rate was adopted. The muscle-fiber bundles were kept in IAA-CN- $\text{N}_2$  Ringer's solution for the specified length of time. Then they were soaked in a Ringer's solution containing  $\text{Na}^{22}$  for a few minutes, mounted in the washing apparatus (see Figure 8.5), and washed continuously with nonradioactive Ringer's solution containing IAA and cyanide. In this case, the muscle-fiber bundle was tied to a small lucite rod to prevent tearing of the tissue. A straight line was fitted to the last part of the plot of  $\text{Na}^{22}$  content against time (see Figure 8.6). Extrapolation of this line to zero time gives another deliberately underestimated initial  $\text{Na}^{22}$  content for the muscle fibers. Dividing this by the time the muscle was actually soaked in the  $\text{Na}^{22}$  Ringer's solution, we obtained a minimum value of the rate of  $\text{Na}^{22}$  exchange in such poisoned muscles. The last column of values for series B gives the rate of flux in moles per square centimeter per second; we obtained this by using the average value for area/weight of  $550 \text{ cm}^2/\text{g}$  obtained for series A.

tive operation of a hypothetical Na pump for the duration of the experiment; we made the unlikely assumption that all energy transfers and utilization processes are 100 per cent efficient.

Representative data accumulated from more than five years of work (1950–1956) are presented in Tables 8.5, 8.6, 8.7, 8.8, and 8.9, and in Figures 8.6 and 8.7.

Source	No.	Tissue wt, mg	Collagen wt, mg	Collagen content, %	Average percentage of connective tissue in muscle, fresh wt/fresh wt
Muscle	1	496.9	1.2	0.24	
	2	550.4	1.3	0.23	
	3	546.6	1.6	0.29	
	4	547.3	1.5	0.27	
Average				0.26	9.09
Connective tissue	1	51.9	1.7	3.28	
	2	49.1	1.5	3.05	
	3	56.4	1.3	2.30	
	4	42.6	1.2	2.81	
Average				2.86	

**Table 8.8. THE PERCENTAGE IN WEIGHT OF CONNECTIVE TISSUES IN FROG SARTORIUS MUSCLES.** To estimate this, we used the fact that pure collagen can be isolated from tissues by alkaline digestion (Lowry *et al.*, 1941) and the reasonable assumption that the collagen content of the connective-tissue elements is closely approximated by the similar connective-tissue elements immediately surrounding the muscle. From the percentage of pure collagen found in these “pure” connective tissues and the content of pure collagen found in intact muscles, the average percentage by weight of fresh connective tissues in fresh muscle was calculated.

We were forced to conclude that the mean maximum energy liberated per kilogram of muscle per hour cannot exceed 25 calories (Table 8.9); the mean minimum energy needed to perform the postulated pumping operation is more than ten times greater (343 cal).

Experiments of this type necessitate the conclusion that the energy available to the cell is not sufficient to operate the Na pump—even if the cell performs no energy-requiring function but the maintenance of its asymmetrical  $\text{Na}^+$ -ion distribution.\* Thus the phenomenon of selective ionic accumulation, *per se*, does not belong in the same category as the performance of mechanical work (in contraction) or electrical work (in the action potential). It cannot depend upon a con-

\* We have discussed other facts of cellular physiology equally difficult to reconcile with the concept of a Na pump in previous publications (Ling, 1952, 1955a).

Date	Duration, hr	Rate of Na exchange, integrated average, M/kg/hr	$\psi + E_{Na}/\bar{F}$ integrated average, mv	Minimum rate of energy required for Na pump, cal/kg/hr	Maximum rate of energy delivery, cal/kg/hr	Minimum required energy	
						Maximum available energy	
9-12-56	10	0.138	111	353	11.57 (highest value, 22.19)	3060%	
9-20-56	4	0.121	123	343	22.25 (highest value, 33.71)	1542%	
9-26-56	4.5	0.131	122	368	20.47 (highest value, 26.10)	1800%	

**Table 8.9. ENERGY BALANCE SHEET FOR THE Na PUMP IN FROG *SARTORIUS* MUSCLES (0°C).** The minimum rate of energy delivery required to operate a Na pump according to the membrane-pump theory was calculated from integrated values of the measured rates of  $\text{Na}^+$ -ion exchange (Table 8.7) and the energy needed to pump each mole of  $\text{Na}^+$  ion out against the measured electrical and concentration gradients (Table 8.5; Figure 8.7). The maximum energy-delivery rate was calculated from the measured hydrolysis of CrP, ATP, and ADP, the only effective energy sources available to the muscles which were poisoned with IAA and  $\text{N}_2$ . Total inhibition of respiration and of glycolysis was assured by the simultaneous presence of 0.001M NaCN (in addition to  $\text{N}_2$ ) and verified by the actual measurement of residual lactate production (in addition to IAA). The ratios between the required and available energy are underestimations. There is no significant difference if the data on flux rate for series A in Table 8.7 are used rather than those for series B. Details of one of the three complete sets of data obtained in September of 1956 are given in Table 8.4. It should be pointed out that six more series of similar experiments were completed (3-20-53, 4-12-54, 1-13-55, 5-20-55, 5-30-55, and 8-9-55); the duration of soaking in the poison gave a mean maximum rate of energy delivery even lower than the data from the three series used in these calculations. Since the development of the procedure for ATP-ADP determinations (Appendix D) was not completed until the end of 1955, the earlier data have not been included.

tinuous expenditure of energy, and, therefore, cannot be said to involve the performance of osmotic work.

Although the above experiments indicate that selective ionic accumulation\* cannot depend on a continuous expenditure of energy, they do not suggest that metabolism is not essential. Metabolism is essential; even though muscle deprived of its ability to carry on either oxidative or glycolytic metabolism can maintain its functions at 0°C for eight hours or longer, the tissue eventually does degenerate.

\* The importance of selective ionic accumulation in the mechanisms of other biological functions is discussed separately in following chapters.

It loses its excitability and contractility, and liberates all of its selectively accumulated K<sup>+</sup> ions. It has been noted that there is a simultaneous exhaustion of the CrP and ATP contents (see Section 9.5 and Figure 9.18).

We conclude that the maintenance of physiological function depends crucially upon the presence of ATP and other metabolic products within the protein fixed-charge system. This dependence rests on the role of ATP and CrP as cardinal adsorbents (Section 6.1) onto the fixed-charge system rather than on the continuous liberation of energy from these compounds through their hydrolytic degradation. This conclusion follows naturally from our hypothesis which implies that the major properties of normal resting cells can be explained best if the cell is described, not as a system in an energy-consuming steady state, but rather, if it is described as a system in a metastable equilibrium state. Like any equilibrium state, this equilibrium state requires no expenditure of energy for its maintenance. How ATP functions as a cardinal adsorbent to maintain this metastable equilibrium state and the role of ATP in the establishment of new metastable states form a central theme of later chapters.

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# 9

## IONIC ACCUMULATION AND OSMOTIC PROPERTIES

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Two aspects of the physiological behavior of living cells, selective ionic accumulation, the subject matter of this chapter, and cellular potentials, discussed in Chapter 10, have traditionally been explained in terms of the differential permeability of postulated cell membranes to various ionic substances.\* Selective ionic accumulation was understood as a consequence of either permeability of the cell membrane toward the substance involved, or an absolute impermeability (Mond and Amson, 1928).  $\text{Na}^+$ -ion impermeability would lead to its exclusion from cells;  $\text{K}^+$ -ion permeability would lead to its selective accumulation in cells. Neuschloss (1923, 1924, 1925, 1926) proposed an alternative view of the mechanism of selective  $\text{K}^+$ -ion accumulation; he postulated that most of the intracellular  $\text{K}^+$  ions are *bound* and thus *nondiffusible*. Ernst† and co-workers expressed a similar view of bound potassium in cells (Ernst and Fricker, 1934; Ernst and Morocz, 1940–1941). The concept of nondiffusible, nonexchangeable  $\text{K}^+$  ions was soon disproved experimentally. (See Raab, 1927; Sager and Roth, 1930; Höber, 1929; Callison, 1931; see also Ling, 1955a.)

Within the framework of the then limited methods available for the study of permeability, the membrane concept seemed to be consistent with a considerable number of observed facts (see Ling, 1955a). With respect to ions, this view culminated in the sieve theory of Boyle and Conway (1941) who proposed a physico-chemical mechanism relating permeability to pores of a critical size in the cell membrane and to the diameter of the hydrated ion. Thus the hydrated  $\text{K}^+$  ions, which are smaller than hydrated  $\text{Na}^+$  ions (see Table 1 in Appendix C), would be able to pass through the small pores and could thus be accumulated in cells, while the larger hydrated  $\text{Na}^+$  ions would be excluded.

\* It may serve to avoid confusion if we mention here that a special case of the membrane theory, proposed in various forms by Teorell (1953), Meyer and Sievers (1936), and Sollner (1949), is known, coincidentally with the earlier form of the present theory, as the "fixed-charge theory." These authors followed Michaelis (1929) in accepting the notion that the hypothetical cell membrane gains certain of its attributes from the presence within it of localized ionic charges, the "fixed charges." In none of the versions of this theme is the question of the effect of immobilizing the charged site on the degree of ionic association considered; following current views of electrolyte solutions, these authors considered ions in the membrane to be completely dissociated (see Teorell, 1953, Figure 5; Sollner, 1949, Figure 1, for diagrams).

The central importance of charge fixation in increasing ionic association was the reason for my original use of the term "fixed-charge hypothesis." For reasons presented in the Introduction we have abandoned this term as well as the later term, "fixed-charge-induction hypothesis," for the present title, "the association-induction hypothesis." It is hoped that this title indicates the broad nature of the present hypothesis and helps to forestall further confusion.

† For the latest view of Ernst, see Ernst and Hajnal (1959).

Though this theory maintains its historic importance, its basic assumptions were disproved with radioactive-tracer techniques. These techniques soon made it obvious that intracellular  $\text{Na}^+$ , as well as  $\text{K}^+$ , ions are constantly exchanging with similar ions in the external medium (Heppel, 1940; Abelson and Duryee, 1949; and Steinbach, 1951; see also Table 9.1 and Figures 11.26 and 11.27 of this book).

Rat No.	Weight, g	Days on $\text{K}^+$ -ion-free diet	Time, min	$\text{Na}^{24}$ , counts/g/min		$\text{Na}^{24}$ ratio (muscle/serum)	$\text{Na}^+$ ion, mM/kg fresh tissue		$\text{Na}^+$ -ion ratio (muscle/serum)
				Muscle	Serum		Muscle	Serum	
5	117	40	5	640	4700	14	53.0	135 <sup>b</sup>	39.2
4	101	36	10	770	4500	17	32.4	138	23.5
8	90	34	10	1000	6600	15	35.4	132	26.8
1	102	40	20	960	4200	23	51.0	140	36.4
3	117	37	31	350	1390	25	48.1	140	34.4
9	80	37	60	430	1400	31	43.8	134	32.7
14	98	44	60	140	430	33	46.0	131	35.0
10	108	34	182	1500	5300	28	39.8	128	31.1
7	110	44	187	90	240	38	56.0	136	41.2
1 <sup>a</sup>	101	42	215	730	2300	32	43.2	143	30.2
2	99	34	260	1320	4000	33	44.7	137	32.6

**Table 9.1. THE PENETRATION OF  $\text{Na}^{24}$  INTO THE MUSCLES OF POTASSIUM-DEPRIVED RATS.** The fourth column gives the time between the intraperitoneal injection of radioactive solution and the sacrifice of the animal. The ratio of tracer  $\text{Na}^{24}$  in muscle to  $\text{Na}^{24}$  in serum approached the value of the ratio of total  $\text{Na}^+$  ion in muscle to that in serum after about 3.5 hr. This indicates nearly complete renewal of the intracellular  $\text{Na}^+$  ion in that time and disproves the concept of the absolute impermeability of the cells to the  $\text{Na}^+$  ion. The  $\text{Na}^+$ -ion concentration within the muscle is not the true intracellular  $\text{Na}^+$ -ion concentration, but includes some extracellular fluid. (Data from Heppel, 1940.)

<sup>a</sup>  $\text{Na}^{24}$  was introduced by means of a stomach tube.

<sup>b</sup> The sample was lost and the average value for serum sodium has been substituted.

Thus, the low concentration of  $\text{Na}^+$  ion found in cells cannot be due to impermeability of the cell membrane to this ion.

The failure of the sieve concept led to the gradual emergence of the pump concept (Dean, 1941; Krogh, 1946) which hypothesizes that a metabolically energized pumping mechanism maintains a low intracellular  $\text{Na}^+$ -ion concentration in spite of constant inward diffusion. The gross disparity between the energy requirements of such a hypothetical pump and the measured rate of delivery of metabolic energy (see Chapter 8) offers unequivocal evidence against this type of mechanism. Although these energetic considerations with respect to the  $\text{Na}^+$  ion alone are suffi-

cient cause to discard the pump idea, the application of this type of analysis as a test of the membrane hypothesis is much more general.

A fundamental corollary of the membrane concept (Boyle and Conway, 1941) is that, given the existence of a semipermeable membrane and, say, impermeant anions within the cells (creatine phosphate, ATP, hexosephosphates, for example) the permeant ions will distribute themselves at equilibrium according to a Donnan ratio  $r$ . Thus, if  $[p_i^+]_{\text{in}}$  and  $[p_i^+]_{\text{ex}}$  represent the intracellular and extracellular concentrations of the  $i$ th cation, and  $[p_j^-]_{\text{in}}$  and  $[p_j^-]_{\text{ex}}$  the intracellular and extracellular concentrations of the  $j$ th anion, and  $n$  and  $m$  are their respective valences,

$$r = \left[ \frac{[p_i^+]_{\text{in}}}{[p_i^+]_{\text{ex}}} \right]^{1/n} = \left[ \frac{[p_j^-]_{\text{ex}}}{[p_j^-]_{\text{in}}} \right]^{1/m}.$$

Sodium, an ion previously supposed to be impermeant, as well as other ions such as  $\text{Cl}^-$  (Boyle and Conway, 1941; Tasaki *et al.*, 1961),  $\text{Ca}^{++}$  (Campbell and Greenberg, 1940; Rothenberg and Field, 1948; Tasaki *et al.*, 1961),\*  $\text{Mg}^{++}$  (Fenn and Haage, 1942; Conway and Cruess-Callaghan, 1937), lactate (Eggleton *et al.*, 1928; Ghaffar, 1935), orthophosphates (M. G. Eggleton, 1933; Tasaki *et al.*, 1961), and inorganic sulfate (Tasaki *et al.*, 1961; see also Figure 11.31 of this book), have all been demonstrated to be permeant. Furthermore, the rates of exchange of these ions are not necessarily low. Thus the  $\text{Ca}^{++}$  ion injected into squid axon was found to have a time constant several times higher than that of the  $\text{Na}^+$  ion (Tasaki *et al.*, 1961). However, the Donnan ratio calculated on the basis of the actual distribution of these ions, contrary to the prediction demanded by the membrane concept, differs markedly from ion to ion (Table 9.2). It became necessary to propose the  $\text{Na}^+$  pump because the distribution ratio of the  $\text{Na}^+$  ion differs from that of the  $\text{K}^+$  ion, although  $\text{Na}^+$  ions are permeant. These criteria (asymmetrical distribution and permeability) are also satisfied by ions other than the  $\text{Na}^+$  ion. If the membrane-pump concept is to be a consistent view describing ionic distribution in general, there must also be a pump for each of these ions. We have shown that there is insufficient energy available to the cell to operate the  $\text{Na}^+$  pump; thus the energy to maintain a non-Donnan distribution for other ions is clearly not available. We must conclude that the membrane-pump concept, as a mechanism for selective ionic accumulation in typical cells, is erroneous. In Chapter 10, we shall present evidence to show that the membrane-pump concept is untenable as a mechanism for cellular electric potentials. The failure of the membrane-pump hypothesis to explain the two phenomena to which it owes its only justification leads to no alternative but to discard it as the basic mechanism of cell physiology.

Both the membrane hypothesis and the bound-potassium concept ascribe the ability of cells to accumulate ions to special properties of components of living

\* It is frequently argued that  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  cannot be treated like  $\text{Na}^+$  because these divalent ions are bound. We should, however, point out that if this is the case, the Donnan ratio for these ions should be higher, not lower, than that for the  $\text{K}^+$  ion.

Ion	Intracellular		Extracellular		Ratio, (column 3 column 5)	Donnan ratio, <i>r</i>
	mM/kg fresh muscle	Milliequivalents/l of intracellular water	mM/l of plasma	Milliequivalents/l of extracellular water		
<b>Cations</b>						
K	85.8 (TBF'E')	128.0	2.5 (F)	2.53	50.59	50.59
Na	24.9 (F'B)	16.9	103.8 (F)	105.0	(6.2) <sup>-1</sup>	
Ca	4.08 (KMUB'F)	11.3	2.0 (F)	4.04	2.8	1.67
Mg	10.8 (KMUB'FC)	31.6	1.2 (B)	2.46	12.8	3.58
Amino acids	6.8 (E)	10.0	8.5 (F)	8.60	1.16	
Carnosine	11.0 (E)	10.2	0	0		
Creatine	7.2 (L)	10.7	0	0		
<b>Anions</b>						
Cl	10.7 (BF)	1.04	74.3 (F)	76.8	(73.9) <sup>-1</sup>	73.9
HCO <sub>3</sub>	10.7 (F)	9.2	25.4 (F)	26.4	(28.7) <sup>-1</sup>	28.7
Lactate	2.8 (F)	3.5	3.3 (F)	3.42	1.02	1.02
Inorganic phosphate	7.2 (L)	16.2	3.1 (F)	3.21	5.05	1.85
Creatine	7.2 (L)	10.7	0	0(?)		
Amino acids	6.8 (E)	10.0	8.5 (S)	8.60	1.16	
CrP	21.8 (L)	71.0	0	0		
ATP	5.0 (E)	27.4	0	0		
Hexose- <i>m</i> - phosphate	1.4 (E)	4.0	0	0		
Other P	4.2 (F)	10.5	0	0		
Carnosine	11.0 (E)	6.2	0	0		

**Table 9.2. THE IONIC COMPOSITION OF FROG MUSCLES.** The most accurate data known to the author were collected in 1946 and averaged, weighing the data from each source according to the number of determinations. Keys to the symbols are: B (Bialaszewicz and Kupfer, 1936); B' (Boyle and Conway, 1941); C (Conway and Cruess-Callaghan, 1937); E (Eggleton, 1935); E' (Ernst and Morocz, 1940–1941); F (Fenn, 1936); F' (Fenn and Cobb, 1936); K (J. A. Katz, 1896); L (Ling, unpublished); M (Meigs and Ryan, 1912); T (Torda, 1941); U (Urano, 1908a,b). The values for Na<sup>+</sup> and K<sup>+</sup> ions were taken from published values where there are at least five individual determinations and the standard error does not exceed 5 per cent of the mean. (Parts of this table were published earlier; see Ling, 1955a.)

cells—a cell “membrane” and cell proteins and/or other entities. But it has been known for some time that nonliving systems can also selectively accumulate K<sup>+</sup> ions over Na<sup>+</sup> ions. Thus, Fenn, in his 1940 review on the role of potassium in physiological processes, opened with the sentence, “It is well-known potassium is of the soil and not the sea; it is of the cell but not the sap.” In 1952, the present author proposed the earlier version of the present theory, offering a unified *quantitative* theory for selective ionic accumulation in soil, in other inanimate ion exchanges, and in living cells\* (Ling, 1952; also Appendix C of this book).† Recently, a number of other investigators have presented additional experimental evidence against the membrane-pump concept (Troschin, 1958; Shaw and Simon, 1955; Menozzi *et al.*, 1959; Lester and Hechter, 1958; Frater *et al.*, 1959; Ernst and Hajnal, 1959; Bozler *et al.*, 1958). In the meanwhile, the present author’s original “fixed-charge hypothesis” has undergone an evolution which has led to the present “association-induction hypothesis,” no longer a hypothesis dealing with selective ionic accumulation and cellular potentials alone, but a general theory of the living state.

We have demonstrated that both the energy and entropy of dissociation within living protoplasm, as a proteinaceous fixed-charge system, favor the association of counterions with fixed ionic sites. A high degree of ionic association, in turn, makes selection of the adsorbed ion possible; this is a function of differences in the adsorption energies of the various ions available.

The protein of a normal resting cell, like native isolated protein, exists in a metastable equilibrium state in which a certain fraction of the fixed ions prefers to adsorb certain free ions (K<sup>+</sup> ions, for example). This metastable state can be readily and reversibly transformed by the indirect *F*-process into another cooperative state which we represent as the active state of the cell in question (for example, the contracted muscle or the conducting nerve). In this chapter, the metastable resting state will be considered in some detail. The resting state serves as the foundation upon which the physiological activity of the cell is based. From the investigator’s point of view, the behavior of the resting state with respect to ionic selectivity and osmotic behavior is the most direct manifestation of the workings of the fixed-site induction system of the cell.

To demonstrate that the common attributes of all fixed-charge systems give rise to basically similar patterns of ion adsorption, we shall discuss the properties of such diverse materials as synthetic exchange resins, wool, isolated cell proteins such as actomyosin, subcellular particles, and cellular fragments. We shall then show that the principles discussed in earlier chapters can be used to interpret not

\* In this volume we shall not explicitly consider the type of selective ionic accumulation found in plant vacuoles. This type of accumulation is intimately related to the presence of an asymmetrical membrane and is found widely as a transcellular (frog skin, renal tubules, for instance) phenomenon. It will be discussed in a separate publication.

† For earlier qualitative theories on selective ionic accumulation in inanimate systems stressing only some of the factors that make the quantitative theory possible, see Chapter 4.

only the intricate patterns of accumulation of cations, anions, and dipolar ions in living cells, but also the relation of ion-accumulation properties to osmotic equilibrium. Then the problem of the metabolic control of ionic accumulation, introduced in Chapter 8, will be subjected to a final examination.

## 9.1. Selective Ionic Accumulation in Nonliving Fixed-Charge Systems

### A. EXCHANGE RESINS

#### (1) The order of preference

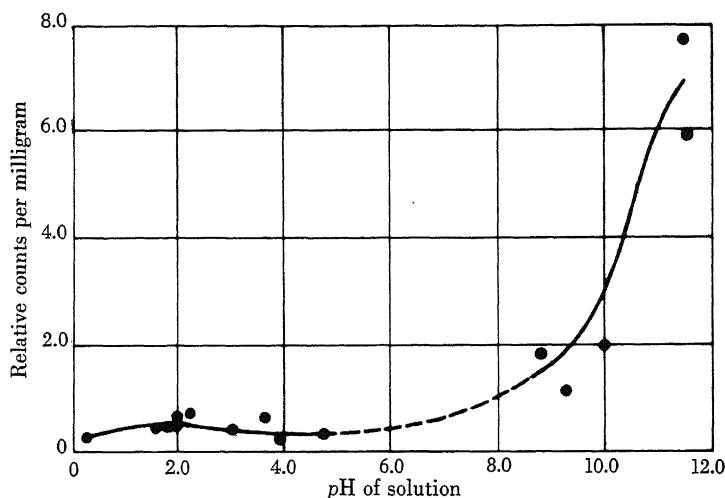
In 1952, using natural and synthetic ion exchangers such as Permutit, soils, and various ion exchange resins as models, the present author postulated that carboxyl groups, carried by the glutamic- and aspartic-acid residues of proteins, function as fixed anionic sites for selective  $K^+$ -ion accumulation. Later, however, experiments with carboxylic exchange resins, showed that these resins actually prefer the  $Na^+$  ion over the  $K^+$  ion (Bregman, 1953, p. 135; see also Conway, 1957). In the intervening years, the significance of variations in the effective charge of the anion (see Chapter 4) has been better understood, and the introduction of the concept of the *c*-value of the fixed anionic group has allowed the resolution of the problem of varying selectivity orders. Thus, differences in the *c*-value spectra must underlie differences in ionic preference between two fixed-charge systems. Evidence supporting this view follows.

If a carboxylic exchange resin selecting the  $Na^+$  ion over the  $K^+$  ion has a high *c*-value, then the theoretical calculations (Figure 4.9) show that it should have a higher *pK* value than a resin like the sulfonic resin, which selects the  $K^+$  ion over the  $Na^+$  ion. Actually, the *pK* value of carboxylic resin is about 6.0; that of sulfonic exchange resin, about 1.5 (estimated from the data of Bregman, 1953, p. 127, Figure 2). The correlation between *pK* and  $Na^+$ -versus- $K^+$  selectivity was first noted by Jenny in clays (1932) and then by Bregman in exchange resins (1953). Our model offers a physical mechanism for this correlation on the basis of theoretically calculated relations (see Figure 4.9). The *pK* values usually given for the  $\beta$ - and  $\gamma$ -carboxyl groups of aspartic- and glutamic-acid residues are 3.0 to 4.7 (Cohn and Edsall, 1943, p. 445), suggesting that the carboxyl group of the living cell has a modal *c*-value between that of a carboxylic exchange resin and that of a sulfonic exchange resin. Support for this view is found in the different orders of selectivity observed in these systems. The carboxyl exchange resin selects ions in the order:  $Li^+ > Na^+ > K^+$  (Bregman, 1953; Gregor *et al.*, 1956), whereas sulfonic exchange resin shows the selectivity order:  $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$  (Samuelson, 1953, p. 35); living cells exhibit selectivity orders intermediate between these extremes (compare Table 9.5 and Figure 9.8 with Figure 4.9). The

variation in order of selectivity among alkali-metal ions thus agrees with that predicted on the basis of the theoretical results and from the  $pK$  values known in each of these instances.

### (2) The magnitude of the selectivity ratio

The above considerations show a general agreement between the qualitative aspects of selective ionic accumulation in living cells and in the man-made fixed-charge system, the exchange resin. The present theory also offers an interpretation

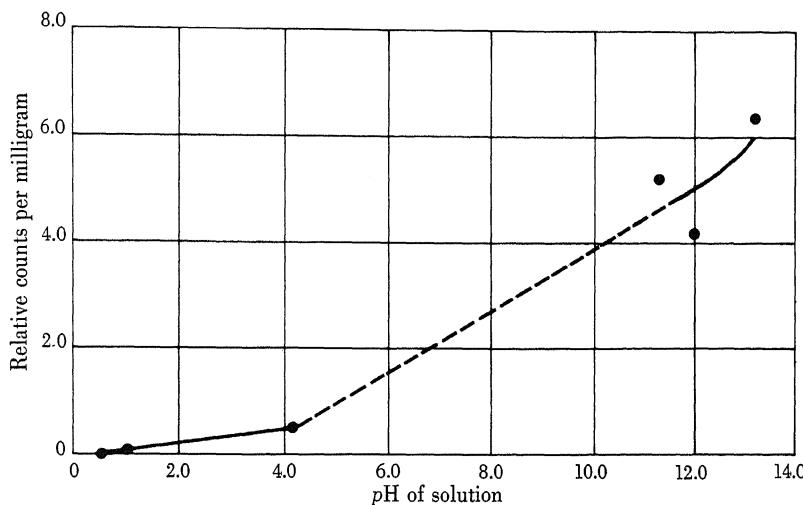


**Figure 9.1. THE ADSORPTION OF THE  $\text{Rb}^+$  ION BY WOOL PROTEIN AS A FUNCTION OF pH.** Defatted virgin wool (sheep) was equilibrated with  $\text{Rb}^+$  ion tagged with  $\text{Rb}^{86}$ . After shaking overnight in a water bath set at  $38^\circ\text{C}$ , the wool samples were drained and washed in  $0.1\text{N NaCl}$  at  $0^\circ\text{C}$  for five minutes to remove adhering fluids. The samples were then dissolved in concentrated  $\text{NaOH}$  and their  $\text{Rb}^{86}$  content determined. The  $\text{Rb}^+$ -ion content is expressed as a ratio of the  $\text{Rb}^+$ -ion concentration of the wool to that of the bathing solution. No points were taken between pH 5 and pH 9 so the position of the curve between these pH values must be taken as tentative.

of a distinct difference between these two systems. In living cells, selective accumulation of the  $\text{K}^+$  ion over the  $\text{Na}^+$  ion is usually accompanied by a high apparent selectivity ratio, defined by equation (7-23) in Section 7.4D. Thus, for frog muscles, this ratio is of the order of magnitude of 300 (Table 9.2; see also Table 9.5). Although selective accumulation of the  $\text{K}^+$  ion over the  $\text{Na}^+$  ion has long been recognized in inanimate systems like Dowex 50 exchange resin, there is no known

instance in which the ratio exceeds 10 (see Table 2.3). Three possible explanations for this difference follow from the present theory.

First, it is clear that the sulfonate exchange resins possess a very low *c*-value at which the dissociation-energy differences between the cations are relatively small compared with the range of differences that occur in the *c*-values



**Figure 9.2. THE REVERSIBILITY OF  $\text{Rb}^+$ -ION ADSORPTION BY WOOL PROTEIN AS A FUNCTION OF pH.** To confirm that the increase of ion adsorption and reversal of preference recorded in Figure 9.1 was the result of the pH change on fixed-ion dissociation and not the result of, say, hydrolysis of wool protein, samples that had been equilibrated at high pH were neutralized with HCl. The complete reversibility of the effect of pH may be seen from a comparison of Figure 9.1 with the curve shown here. The position of this curve between pH 5 and pH 11 is tentative as no values were measured in this range.

usually found in cells. Since the selectivity between ion  $p_i$  and ion  $p_j$  is related to  $\exp(\Delta E_i - \Delta E_j)/RT$ , a high selectivity ratio for muscle and a low selectivity ratio for a sulfonate resin is to be expected (see Section 4.4G on optimum *c*-value).

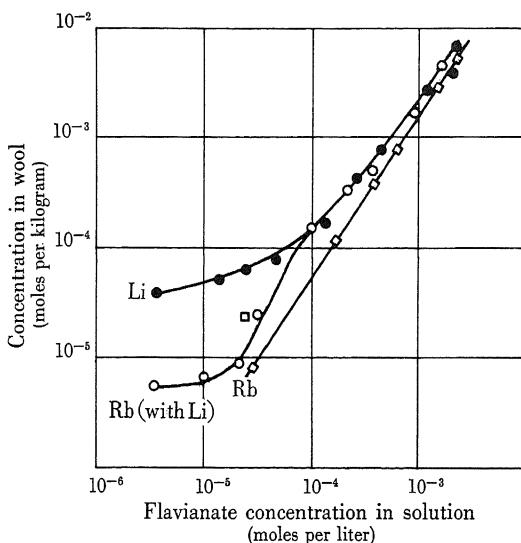
Second, the high density of fixed ionic sites (about 5M) in exchange resins and the relatively small number of water molecules per site in the system do not allow the assumption by the  $\text{Na}^+$  ion of the highest configurations (II, III) on which high  $\text{K}^+/\text{Na}^+$  ratios depend (see Section 4.4F on optimum microcell size).

Third, a heterogeneity of *c*-value also tends to diminish the selectivity ratio. Thus, in the extreme case, if half the sites had a selectivity ratio of infinity for  $\text{K}^+/\text{Na}^+$ , and the other half had a selectivity ratio of infinity for  $\text{Na}^+/\text{K}^+$ , the over-all selectivity ratio would nevertheless be one.

### B. SELECTIVE IONIC ACCUMULATION IN A TRUE FIXED-CHARGE SYSTEM—WOOL PROTEIN

Wool manifests two attributes of proteins that are central to an understanding of physiological behavior according to the present theory.

First, the association-induction hypothesis predicts that many of the fixed cationic groups on wool protein will be joined in salt linkages with fixed anionic



**Figure 9.3. THE CONCENTRATION DEPENDENCE OF Li-FLAVIANATE AND Rb-FLAVIANATE ADSORPTION IN WOOL.** The open circles show Rb-flavianate adsorption in the presence of an equal amount of Li-flavianate; note the enhanced adsorption of Rb-flavianate in the presence of Li-flavianate.

groups. An increase in *pH* from 6 to 12 leads to the partial deionization of the  $\epsilon$ -amino groups of lysine residues and the guanidyl groups of arginine residues, leaving a portion of formerly salt-linked fixed anions with no partners; the free cations in the medium will have to adsorb onto these anionic groups to preserve macroscopic electroneutrality. Thus a sharp increase in free alkali-metal ion accumulation should occur when the *pH* is increased in the critical range; such a phenomenon is demonstrated in Figure 9.1 which shows a tenfold increase in the alkali-metal accumulation of wool as the *pH* is increased from 6 to 12. That this ion accumulation is reversible (Figure 9.2) shows that it results from the breaking of salt linkages and not of peptide bonds.

In Chapter 7, we discussed the ionization of wool protein. Figure 7.1 shows that the  $\epsilon$ -amino and perhaps the guanidyl groups of this protein begin to deionize

at pH 10. A comparison of Figure 9.1 with this figure demonstrates that, concomitant with this deionization, there is an increased uptake of free cations.

Second, Figure 9.3 shows the adsorption of Rb-flavianate by wool. A second curve shows the adsorption of Rb-flavianate when present with an equimolar concentration of Li-flavianate. The amount of Rb-flavianate adsorbed is *increased* rather than *decreased* by the presence of Li-flavianate. This establishes that this proteinaceous fixed-charge system is capable of the type of autocooperative behavior demonstrated in Section 7.4B by the greater-than-unity slope of a log-log plot. That the *F*-effect underlies such an autocooperative phenomenon is a major theme of the present theory.

## 9.2. Selective Ionic Accumulation in Degraded Components of Living Cells

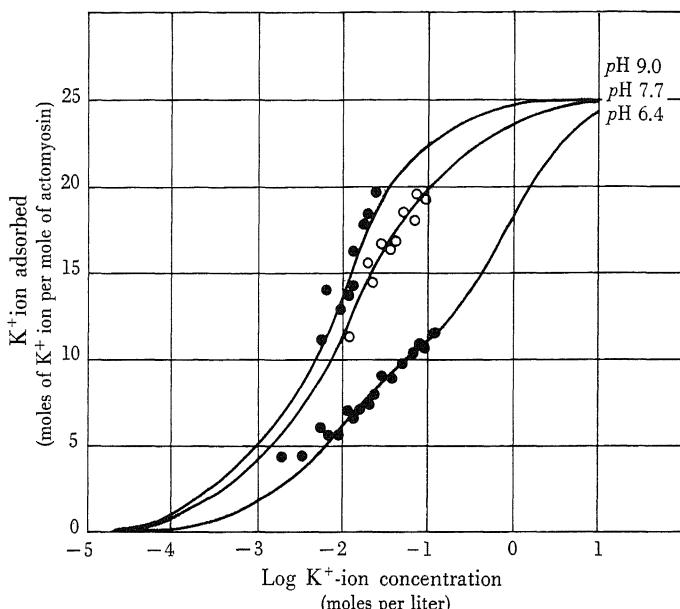
### A. SELECTIVE IONIC ADSORPTION IN SEMIFIXED-CHARGE SYSTEMS—PROTEIN SOLUTIONS

Recently, using permselective-membrane electrodes, M. S. Lewis and Saroff (1957; see also Szent-Györgyi, 1947) demonstrated that the major muscle protein, actomyosin, binds a substantial number of  $K^+$  or  $Na^+$  ions (Figure 9.4). A consideration of these results is profitable for understanding the changes in the adsorption of ions produced by the degradation of cellular fixed-charge systems. These changes are of two types: a substantial decline in the degree of ion-protein association, and a profoundly modified *c*-value ensemble in the isolated protein.

The protein solution employed by Lewis and Saroff contained 1.5 to 2.5 per cent myosin B (actomyosin); the concentration of fixed anionic and cationic charges in such a solution is about 0.05M. If the solution also contains 0.1M KCl or NaCl, the total ionic strength is about 0.15M. At this strength, a monomeric salt solution is completely dissociated (Section 2.1). Lewis and Saroff, nevertheless, found that at pH values near neutrality, 20 moles of  $K^+$  and  $Na^+$  were adsorbed on each mole of myosin B. This adsorption is due to the semifixed nature of the ionic groups on the actomyosin molecule (Chapter 2). Live muscle, on the other hand, adsorbs about ten times as much cation as the preparation of Lewis and Saroff. This disparity has its origin in the degradation of the cellular true fixed-charge system into a semifixed-charge system. Such degradation increases the entropy gain on dissociation of free ions such as  $K^+$  and  $Na^+$ , but does little to increase the entropy gain of the fixed ions (Section 7.3A). An increased probability of the formation of salt linkages and a decrease in the adsorption of free ions result.

The selectivity ratio of  $K^+$  to  $Na^+$  in live muscle may approximate 300:1; yet in isolated actomyosin, the dominant protein in this tissue, there is actually a slight preference for the adsorption of  $Na^+$  over  $K^+$ . We have repeatedly emphasized that the *c*-value of a protein is highly labile and varies with the interaction;

variations in selectivity ratio can be produced experimentally in protein systems *in vitro* as well as *in vivo* and occur following much milder treatment than is usually employed in isolating proteins such as actomyosin. Lability of selective ionic adsorption is the essence of the contractile function (Chapter 15) which actomyosin subserves and which is effected *in vivo* by the subtlest of environmental changes. The ion-adsorption patterns of actomyosin, therefore, must be



**Figure 9.4.** THE CONCENTRATION DEPENDENCE OF THE ADSORPTION OF THE K<sup>+</sup> ION ON MYOSIN B (27°C). The average number of moles of K<sup>+</sup> ion adsorbed per mole of myosin is plotted against the log of the potassium concentration. (Figure after M. S. Lewis and Saroff, 1957.)

considered to be among the most susceptible to environmental modification, and pronounced change of the *c*- and *c'*-value ensembles must be expected when this protein (or any other) is extracted from its resting condition in a true fixed-charge system by exposure to concentrated salt solutions.

#### B. SELECTIVE IONIC ACCUMULATION IN SUBCELLULAR PARTICLES

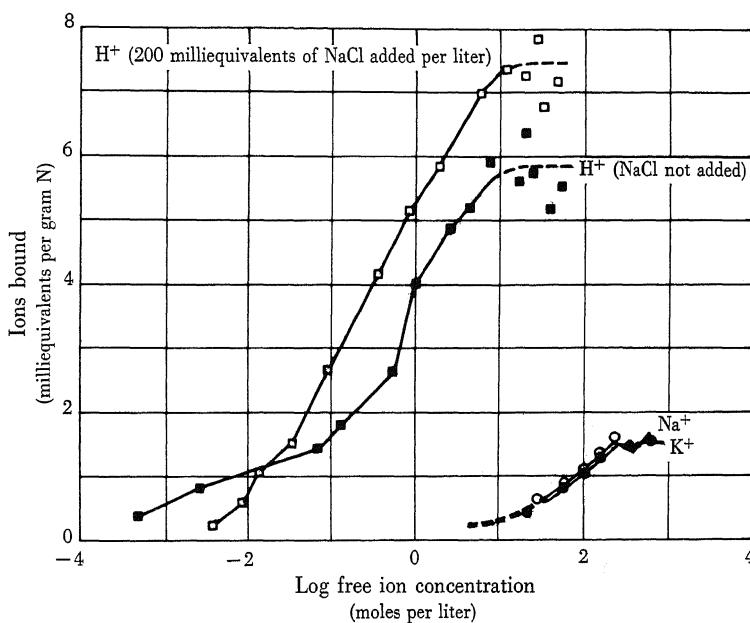
According to the present hypothesis, the cellular protoplasm is a proteinaceous fixed-charge system.\* However, unlike most nonliving fixed-charge systems such

\* Of course, nonprotein material such as lipids and carbohydrates can be anchored onto this protein fixed-charge framework.

as Dowex 50 or even wool, the living state is poised in a delicate metastable balance and it is only in this metastable state that it demonstrates, qualitatively as well as quantitatively, the type of ionic selectivity manifested under normal conditions. Nevertheless, in recent years there has been growing interest in the isolation and study of cellular fragments with some work done on the ion binding of these fragments. We shall consider a number of such cases.

### (1) Microsomes

Sanui and Pace (1959) studied the accumulation of  $\text{Na}^+$  and  $\text{K}^+$  ions by rat liver microsomes.\* They concluded that the retention of cations "is typical of adsorp-



**Figure 9.5. SEMILOGARITHMIC PLOT OF THE CONCENTRATION DEPENDENCE OF  $\text{Na}^+$ -,  $\text{K}^+$ -, AND  $\text{H}^+$ -ION ADSORPTION BY RAT LIVER MICROSOLES.**  $\text{Na}^+$ - and  $\text{K}^+$ -ion adsorption was determined by flame photometry of microsome extracts;  $\text{H}^+$  binding was determined from acid-titration experiments. Note that, at higher ion concentrations, the  $\text{Na}^+$  ion seems to enhance the binding of the  $\text{H}^+$  ion but, at lower concentrations, these ions compete for binding sites. See text for explanation of this phenomenon. (Figure after Sanui and Pace, 1959.)

tion rather than of bathing-medium entrapment," that is, with increasing external ionic concentration, the retained  $\text{K}^+$ - or  $\text{Na}^+$ -ion concentration reached a finite

\* The fraction of whole homogenate suspended in 0.25M sucrose, spun down between  $9,214 \times g$ , 10 min, and  $132,050 \times g$ , 2 hr, at 0 to 5°C.

plateau value rather than increasing linearly as would be the case if the microsomes behaved as Donnan-type osmometers.

These authors also observed that, above 0.01*M* concentration, added NaCl increases H<sup>+</sup>-ion binding (compare Figure 9.5, which is a reproduction of the figure of Sanui and Pace, with Figure 9.3). We suggest that this facilitation is due to the Cl<sup>-</sup> ion added rather than to the Na<sup>+</sup> ion. This implies that the effect is analogous to that seen in the titration of carbonylhemoglobin by Steinhardt and Zaisser where the addition of NaCl moved the titration curve to the left (Figure 7.14).

## (2) Mitochondria

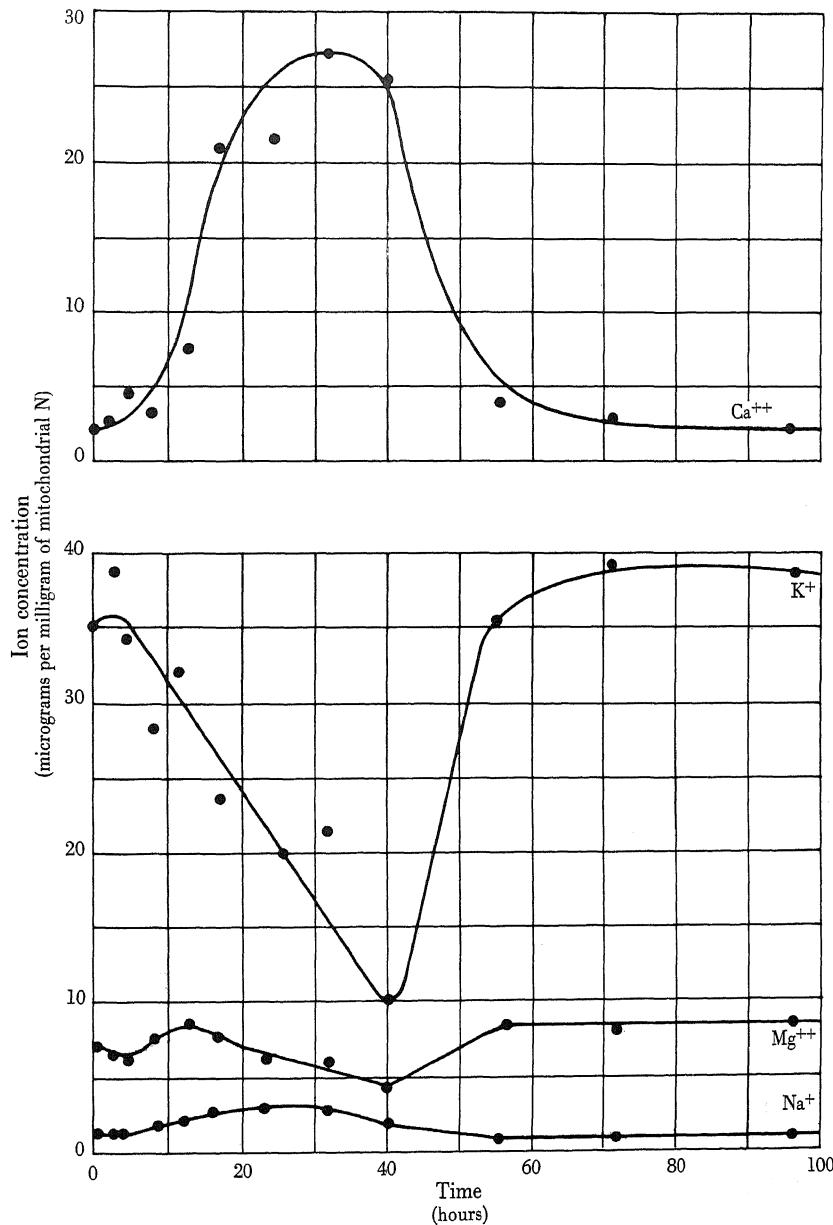
Stanbury and Mudge (1953) and Gamble (1957) found that a "significant quantity of mitochondrial potassium is present in a tightly bound form that is not

Particle	K:N	Na:N
Intact mitochondria.....	0.7-1.0	0.02-0.04
Mitochondrial fragments (digitonin method).....	0.08-0.15	0.01-0.02
Mitochondrial fragments (mechanical method).....	0.005	0.006
Microsomes.....	0.04	0.03

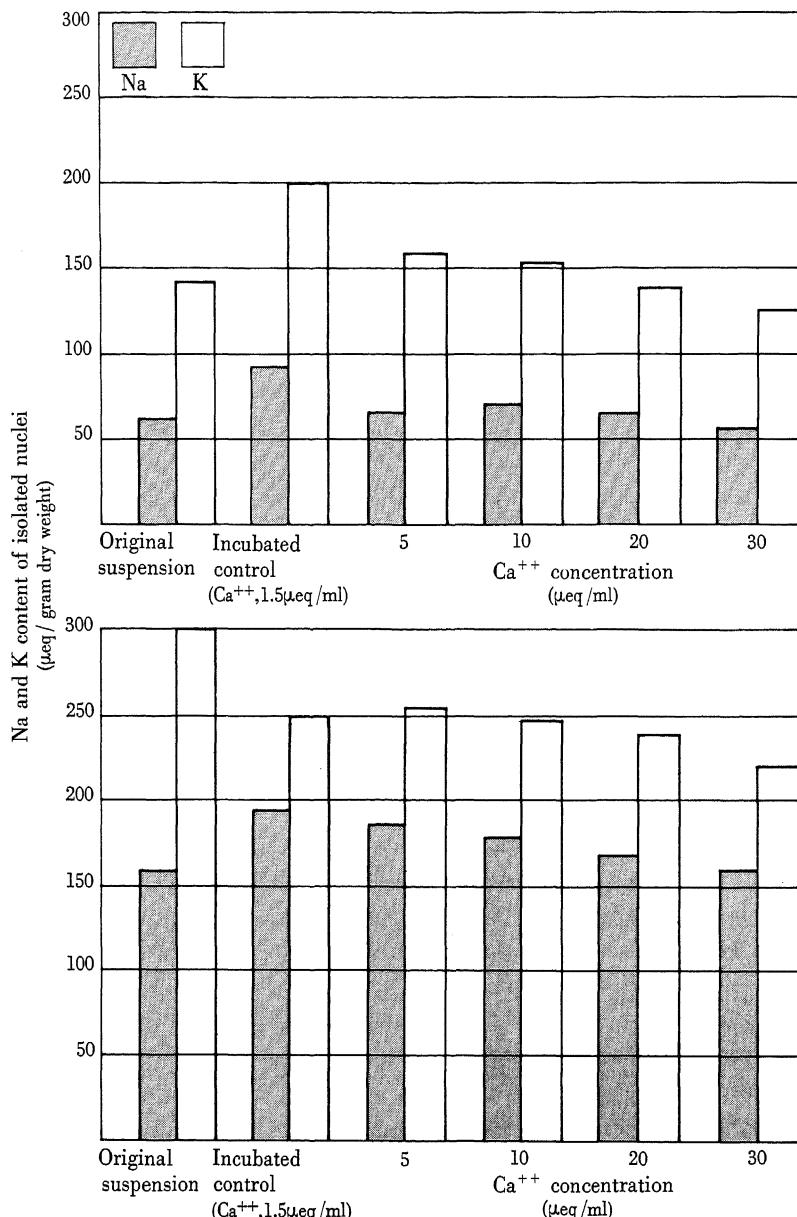
**Table 9.3.** K<sup>+</sup>- AND Na<sup>+</sup>-ION CONTENT OF WASHED MITOCHONDRIA, MITOCHONDRIAL FRAGMENTS, AND MICROSOMES. K:N or Na:N ratios refer to micromoles of K<sup>+</sup> or Na<sup>+</sup> ion per milligram of nitrogen. All particles were washed three times with electrolyte-free 0.25*M* sucrose before analysis. (Table from Gamble, 1957.)

removed or displaced by several washings with 0.25*M* sucrose or with 0.15*M* NaCl. Sodium, on the other hand, is almost completely removed by such washing procedures" (Gamble). Particularly important is the finding of Gamble that the mitochondrial structure (or membrane) need not be intact; fragments of mitochondria produced by treatment with digitonin retain the ability to bind the K<sup>+</sup> ion (Table 9.3). These authors point out that K<sup>+</sup>-ion retention seems to be related to the presence of ATP, suggesting a quantitative relation between ATP and K<sup>+</sup>-ion accumulation. This relation has been studied extensively by the author and will be discussed in detail in Section 9.5.

Thiers *et al.* (1960) studied the concentrations of various cations in rat liver mitochondria following poisoning with carbon tetrachloride. Their results, reproduced in Figure 9.6, show a fall of K<sup>+</sup>-ion concentration accompanied by a proportional rise of Ca<sup>++</sup>-ion concentration about 40 hours after the ingestion of carbon tetrachloride. Similar but smaller changes are also seen in the Mg<sup>++</sup>-ion and Na<sup>+</sup>-ion concentrations. All the changes are reversible, the ion levels returning to



**Figure 9.6. CATION CONCENTRATIONS IN RAT LIVER MITOCHONDRIA AFTER ADMINISTRATION OF CARBON TETRACHLORIDE TO THE ANIMAL.** The time course of the concentrations of various alkali-metal and alkaline-earth cations was followed after the administration of carbon tetrachloride. (Figure after Thiers *et al.*, 1960.)



**Figure 9.7. THE EFFECT OF THE Ca<sup>++</sup> ION ON THE Na<sup>+</sup>- AND K<sup>+</sup>-ION CONTENTS OF THYMUS NUCLEI.** Thymus nuclei were isolated and incubated for 30 minutes at 37°C in high (top) and low (bottom) K<sup>+</sup> media in the presence of different concentrations of Ca<sup>++</sup> ion. The high K<sup>+</sup> medium contained 64 microequivalents/ml of K<sup>+</sup> ion and 26 microequivalents/ml of Na<sup>+</sup> ion. The low K<sup>+</sup> medium contained 60 microequivalents/ml of Na<sup>+</sup> ion and only a trace of K<sup>+</sup> ion. (Figure after Itoh and Schwartz, 1957.)

normal after about 60 hours. In commenting on these results, the authors state, "Although the anionic sites which bind calcium in the mitochondria are unknown it seems clear that under normal circumstances many of them are occupied by other cations, among which is possibly potassium." These experiments strongly suggest that mitochondria behave as ion-accumulating fixed-charge systems, even in the isolated state.

### (3) Nuclei

Itoh and Schwartz (1957) showed that thymus nuclei, when isolated in sucrose, contain more  $K^+$  ion [ $244 \pm 12.2$  (S.E.) microequivalents/g dry weight] than  $Na^+$  ion ( $87 \pm 4.8$  microequivalents/g dry weight). This  $K^+$ -over- $Na^+$ -ion selectivity is retained even after the nuclei have been incubated for 30 minutes at  $37^\circ C$  in a medium containing 60 microequivalents/ml of  $Na^+$  ion and only a trace of  $K^+$  ion (Figure 9.7).

### (4) Erythrocyte ghosts

Human erythrocytes, when treated with distilled water, lose 90 per cent of their major protein, hemoglobin. Hoffman *et al.* (1960; see also Teorell, 1952) found that the remains of the cells, commonly known as "ghosts," retain the  $K^+$  ion at a concentration 12 times higher than that of the washing medium which contained  $170mM$  NaCl and  $5mM$  KCl. Washing was carried out at room temperature after a previous incubation of the ghosts at  $37^\circ C$  in another medium containing  $150mM$  KCl and  $30mM$  NaCl.

### (5) Broken cells

Cells broken under conditions in which the cytoplasm retains the characteristics of a true fixed-charge system, even though the specific ionic selectivity is altered,

Preparation	Approx. fiber length, mm	Intracellular Rb, $\mu M/ml$ cell water	$Rb_i/Rb_o$
"2 cut" (normal hemidiaphragm)	5-8	$47.0 \pm 1.71(16)^a$	9.2
"4 cut"	2.5-4	$37.8 \pm 2.77(8)$	7.4
"6 cut"	1.7-2.7	$30.4 \pm 1.97(8)$	6.0

**Table 9.4.**  $Rb^+$ -ION ACCUMULATION IN CUT MUSCLE FIBERS OF RAT DIAPHRAGM. Cut hemidiaphragms were incubated for one hour in a  $K^+$ -ion-free Ringer's solution containing  $5.1mM$   $Rb^+$  ion. (Table from Menozzi *et al.*, 1959.)

<sup>a</sup> Standard error of the mean; the figures in parentheses refer to the numbers of observations.

should show a high degree of ionic association. Such conditions apparently prevailed in a study made by Menozzi *et al.* (1959; also personal communication). Part of their data, reproduced in Table 9.4, shows that rat diaphragm muscles, cut six times to produce segments approximately 1.7 to 2.7 mm in length, when introduced into a Ringer's solution containing 5.1 mM Rb<sup>+</sup> ion and a normal concentration of Na<sup>+</sup> ion, can take up Rb<sup>+</sup> ion to an intracellular concentration six times that of the external Rb<sup>+</sup>-ion concentration after one hour of incubation.

### 9.3. Selective Ionic Accumulation in Living Cells

#### A. THE SELECTIVE ACCUMULATION OF ALKALI-METAL IONS

##### (1) The amphoteric nature of the living-cell fixed-charge system

When monomers are joined into a macroscopic three-dimensional network, the forces governing the dissociation of counterions are a function of the polarity of the fixed-charge system; if the fixed-charge system contains an equal number of fixed cations and fixed anions, under certain conditions a countercation and a counteranion can be removed simultaneously leaving no net charge. The free-energy change governing this complex dissociation is equal to the algebraic sum of three terms: the  $-\Delta F_1$  of the dissociation of the countercation, the  $-\Delta F_2$  of dissociation of the counteranion, and the  $+\Delta F_3$  of interaction between the fixed cation and the fixed anion.\* When the sum of these terms is sufficiently low, the amphoteric fixed-charge system may not accumulate free cations or anions at all, a condition especially favoring the formation of salt linkages between the fixed cations and the fixed anions (see Section 7.3A). However, if  $+\Delta F_3$  is approximately equal to  $-\Delta F_1$  and  $-\Delta F_2$  is large, the system behaves as a pure anionic fixed-charge system with respect to the selective adsorption of free countercations.† This appears to be the case for selective alkali-metal ion accumulation in living cells.

##### (2) The modal *c*-value of tissues and organs

The present model of the association-induction hypothesis makes it necessary to ask whether there are meaningful differences among the *c*-value spectra of various tissues. The experimental answer is affirmative; the data strongly suggest that

\* There are altogether six interaction energies between adjacent fixed ionic sites and their counterions; of these, all but  $-\Delta F_1$  and  $-\Delta F_2$  cancel out when the ions are associated and may be neglected. Of the neglected terms,  $+\Delta F_3$ , the interaction energy between the two fixed charges, becomes significant when both fixed charges lose their counterions.

† If, on the other hand,  $+\Delta F_3$  is approximately equal to  $-\Delta F_2$ , and  $-\Delta F_1$  is large, the system behaves as a pure cationic fixed-charge system with respect to selective accumulation of free counteranions.

different *c*-value ensembles are the foundation of the varying physiological functions of tissues (see Chapter 18 on growth and development and on carcinogenesis).

The most direct method of determining the modal *c*-value of a tissue is to determine the equilibrium distribution of the four cations, Cs<sup>+</sup>, Rb<sup>+</sup>, K<sup>+</sup>, and Na<sup>+</sup>, when these ions are fed to animals in fixed relative proportions at a constant rate. Differences among the equilibrium distributions of these ions in various organs of the same animal would indicate differences in the modal *c*-values (see Figure 4.9 for theoretical equilibrium distributions). Typical data from such experiments are presented in Tables 9.5 and 9.6\* and in Figure 9.8. According to the present theory, the fixed ionic groups on the cellular proteins are capable of a number of interactions and are, by nature, heterogeneous. Consequently, the *c*-value at any moment is a function of the ions adsorbed on the protein; variation in the ion content of the plasma will thus vary the modal *c*-value of the tissue. However, if we limit the ionic uptake to a relatively narrow range, we can still compare the *c*-value ensembles of different tissues. Most of our experiments were performed on rats and a few were done on rabbits. The results of the rabbit experiments are presented in Figure 9.9.† Although the details differ, the general pattern of the *c*-value ensemble of each rabbit organ appears similar to that for the corresponding rat organ. Notice that organs such as the lens and the brain, which are ectodermal in origin, appear to have uniformly high *c*-values. Tissues of endodermal origin, liver and parts of the gastrointestinal tract, have low modal *c*-values. Organs of mesodermal origin may have high modal *c*-values, as in spleen and blood, low *c*-values as in diaphragm muscle, or intermediate *c*-values as in voluntary muscle. The relation of the embryonic origin of these organs to the *c*-value will be discussed in Chapter 18.

## B. THE ACCUMULATION AND EXCHANGE OF H<sup>+</sup> IONS

The results of a series of experimental observations on the improvement of muscle function by external bicarbonate, the effect of pH in preventing potassium loss, and similar problems (Berg, 1911; Fenn and Cobb, 1934; Creese, 1949; Rona and Neukirch, 1912) have been explained as consequences of a proposed potassium-hydrogen ion exchange (Cooke *et al.*, 1952). However, the acceptance of the membrane theory engenders considerable difficulty in this explanation if one assumes, as in a Donnan system, that

$$\frac{f_{\text{H}}^{\text{in}}[\text{H}]_{\text{in}}}{f_{\text{H}}^{\text{ex}}[\text{H}]_{\text{ex}}} = \frac{f_{\text{K}}^{\text{in}}[\text{K}]_{\text{in}}}{f_{\text{K}}^{\text{ex}}[\text{K}]_{\text{ex}}} \gg 1$$

\* These experiments were performed by Kilpatrick *et al.* (1956) who studied the relative ratios of accumulated K<sup>42</sup> and Rb<sup>86</sup> ions in a variety of tissues.

† Equilibrium may not have been reached for all ions in these experiments. These 26-hour data should be compared with those for tissues from rats sacrificed after six days of isotope feeding (Table 9.5).

Animal No.		9070806		9070807		9071713		9071714	
Duration of feeding (days)		6		8		14		15	
Organ	Ion	Concentration, $\mu\text{M/g}$	[p] tissue	Concentration, $\mu\text{M/g}$	[p] plasma	Concentration, $\mu\text{M/g}$	[p] tissue	Concentration, $\mu\text{M/g}$	[p] tissue
Plasma	Na	142.84	—	128.89	—	150.98	—	151.00	—
	K	8.22	—	5.26	—	5.03	—	4.03	—
	Rb	0.22	—	0.24	—	0.12	—	0.31	—
	Cs	0.56	—	0.30	—	0.30	—	0.67	—
Brain	Na	59.84	0.42	44.84	0.35	38.53	0.26	53.72	0.36
	K	90.42	11.00	85.41	16.24	63.86	12.70	72.29	17.00
	Rb	0.64	1.14	2.56	8.53	2.49	8.30	—	—
	Cs	—	—	0.64	2.67	0.71	5.92	—	—
Lens	Na	—	—	—	—	28.06	0.19	36.41	0.24
	K	—	—	—	—	32.50	6.46	59.71	14.80
	Rb	—	—	—	—	2.83	9.43	6.64	21.40
	Cs	—	—	—	—	0.71	5.92	3.67	5.48
Gastrocnemius	Na	30.74	0.22	23.52	0.18	41.90	0.28	30.32	0.20
	K	88.15	10.72	85.04	16.17	83.80	16.66	71.54	17.80
	Rb	1.79	6.27	4.34	14.47	7.29	24.30	8.80	13.20
	Cs	1.38	3.20	2.39	9.96	4.86	40.50	7.64	24.60
Diaphragm	Na	41.42	0.30	29.71	0.23	31.50	0.21	46.30	0.33
	K	93.75	12.41	78.63	14.95	31.49	6.26	69.44	17.20
	Rb	2.16	3.86	4.10	13.67	4.81	28.75	8.47	18.20
	Cs	1.70	7.72	2.65	11.04	3.45	16.03	12.22	27.30
Heart	Na	59.07	0.41	62.05	0.48	50.53	0.34	49.18	0.33
	K	72.80	8.86	54.29	10.32	30.85	6.14	158.24	14.47
	Rb	1.15	2.05	3.70	10.53	3.12	17.50	8.15	12.16
	Cs	1.48	6.59	1.39	8.92	2.11	10.40	3.57	11.50
Spleen	Na	35.82	0.25	32.53	0.25	30.02	0.20	49.63	0.33
	K	95.56	11.63	84.86	16.13	62.99	12.52	70.59	17.50
	Rb	3.01	5.37	3.56	11.87	6.64	20.58	11.65	17.40
	Cs	1.12	5.09	2.04	8.50	2.47	22.13	3.97	12.80
Erythrocyte	Na	32.32	0.23	24.77	0.19	30.41	0.20	32.01	0.21
	K	70.41	8.57	68.96	13.11	67.70	13.45	61.96	15.40
	Rb	1.21	2.16	2.46	8.20	4.16	13.86	5.88	8.78
	Cs	0.42	1.91	0.48	2.00	0.59	4.92	1.25	4.03
Kidney	Na	80.00	0.56	60.89	0.47	98.56	0.65	68.31	0.45
	K	58.00	7.06	52.19	9.92	45.67	9.08	44.33	11.00
	Rb	1.22	2.19	3.36	11.20	8.46	28.20	8.98	13.40
	Cs	1.32	6.00	2.78	11.58	5.38	44.83	5.84	18.80
Liver	Na	38.25	0.27	30.63	0.24	62.22	0.41	43.99	0.29
	K	74.84	9.10	76.86	14.61	63.55	12.63	47.25	11.75
	Rb	3.14	5.61	5.62	18.73	8.90	29.83	12.32	18.40
	Cs	1.00	4.54	2.38	9.92	8.26	68.83	6.91	22.30

Table 9.5 (Continued on facing page)

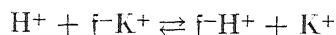
Animal No.		9070806		9070807		9071713		9071714	
Duration of feeding (days)		6		8		14		15	
Organ	Ion	Concen- tration, μM/g	[p] tissue [p] plasma						
Stomach	Na	63.40	0.44	45.31	0.35	67.54	0.45	—	—
	K	65.21	7.93	45.31	8.61	24.88	4.95	54.34	13.40
	Rb	2.28	4.07	3.36	11.20	6.74	22.47	16.17	24.10
	Cs	1.85	8.41	2.72	11.33	4.73	39.42	9.20	29.70
Duodenum	Na	66.66	0.67	53.19	0.41	86.79	0.57	67.26	0.45
	K	93.33	11.35	72.69	13.82	75.67	15.04	65.22	16.20
	Rb	2.60	4.64	4.89	16.30	10.42	34.74	11.86	28.40
	Cs	2.20	10.00	4.04	16.83	7.48	62.33	8.80	17.70
Large intestine	Na	54.32	0.38	45.63	0.35	44.94	0.30	58.14	0.39
	K	76.04	9.25	66.37	12.62	44.94	8.93	68.18	16.90
	Rb	3.30	5.89	3.48	11.60	5.39	17.97	10.29	15.40
	Cs	2.51	11.41	3.29	13.71	4.36	36.33	6.91	22.30
Adrenal gland	Na	66.16	0.46	—	—	78.09	0.52	63.83	0.42
	K	73.79	8.98	—	—	78.09	15.52	56.23	14.00
	Rb	—	—	—	—	9.37	31.23	6.66	9.90
	Cs	—	—	—	—	6.65	55.42	5.65	18.20
Testis	Na	—	—	—	—	—	—	51.41	0.34
	K	—	—	—	—	—	—	58.97	14.60
	Rb	—	—	—	—	—	—	6.77	10.10
	Cs	—	—	—	—	—	—	3.30	10.70

**Table 9.5. IONIC SELECTIVITY OF RAT ORGANS.** Rats were fed a low K<sup>+</sup>-ion diet to which Cs<sup>+</sup> and Rb<sup>+</sup> ions were added. After varying intervals of time the animals were sacrificed; the tissues were isolated and analyzed for Na<sup>+</sup>-, K<sup>+</sup>-, Rb<sup>+</sup>-, and Cs<sup>+</sup>-ion concentration by flame photometry (see Table 8.4 for details of procedure). The absolute concentrations are given in μM/g of wet tissue as are the ratios (concentration in tissue)/(concentration in plasma). These data must be regarded as exploratory because the toxicity of the Cs<sup>+</sup> and Rb<sup>+</sup> ions in the dosages used precluded maintenance of the animals for longer than two weeks. Thus, in many cases, and especially for the Cs<sup>+</sup> ion, equilibrium was not reached. The extremely low plasma Cs<sup>+</sup>-ion concentration maintained and its low rate of entry into tissue cells tend also to increase spuriously the over-all Cs<sup>+</sup>-ion selectivity ratio.

and that the activity coefficient of the intracellular H<sup>+</sup> ion  $f_H^{in}$  at concentration [H]<sub>in</sub> is equal to  $f_K^{in}$  of the intracellular K<sup>+</sup> ion at concentration [K]<sub>in</sub>. The difficulty is greatest in gastric mucosa cells and in yeast cells which thrive in a medium of very high acidity (*pH* 1 to 2). Maintenance of the Donnan ratio under these conditions would require an intracellular H<sup>+</sup>-ion concentration between 1 and 10*N*. At

such an H<sup>+</sup>-ion concentration, almost all metabolic enzymes cease to function and few proteins can resist denaturation.

According to the present hypothesis, intracellular hydrogen and potassium ions exist in the adsorbed form; the exchange reaction is of the form,

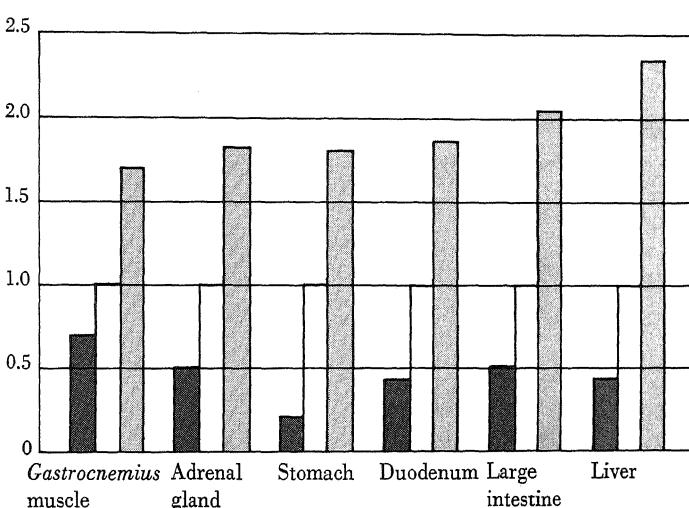
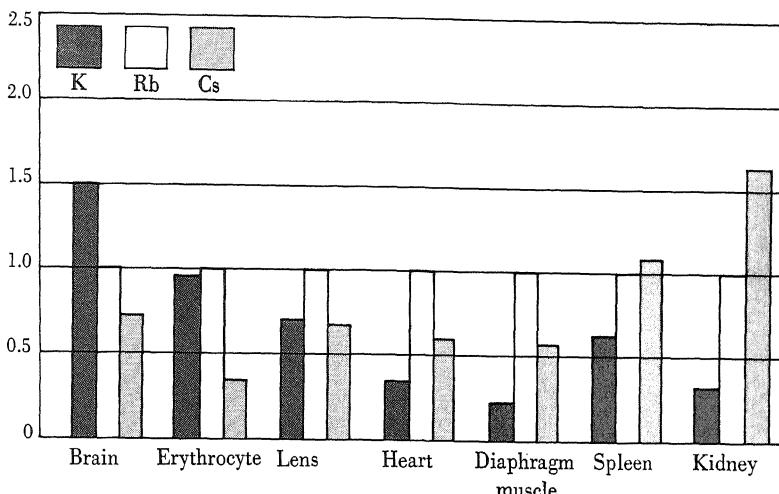


The H<sup>+</sup>-ion activity can be low when the H<sup>+</sup>-ion concentration is high because

Time after administration	8 hr	20 hr	39 hr	62 hr
No. of rabbits	5	3	3	5
Brain	0.67	0.60	0.62	0.63
Urine from bladder	0.77	—	—	0.65
Bone (femur)	0.82	0.58	0.59	0.81
Plasma	0.87	—	—	0.90
Muscle	0.79	1.09	1.04	0.99
Red blood cells	0.91	0.94	1.03	1.02
Heart	1.30	1.26	1.37	1.20
Spleen	1.16	1.34	1.30	1.23
Lung	1.30	—	—	1.26
Intestine	1.15	1.42	1.52	1.41
Kidney	1.79	1.49	2.00	1.88
Liver	2.46	2.55	2.70	2.43

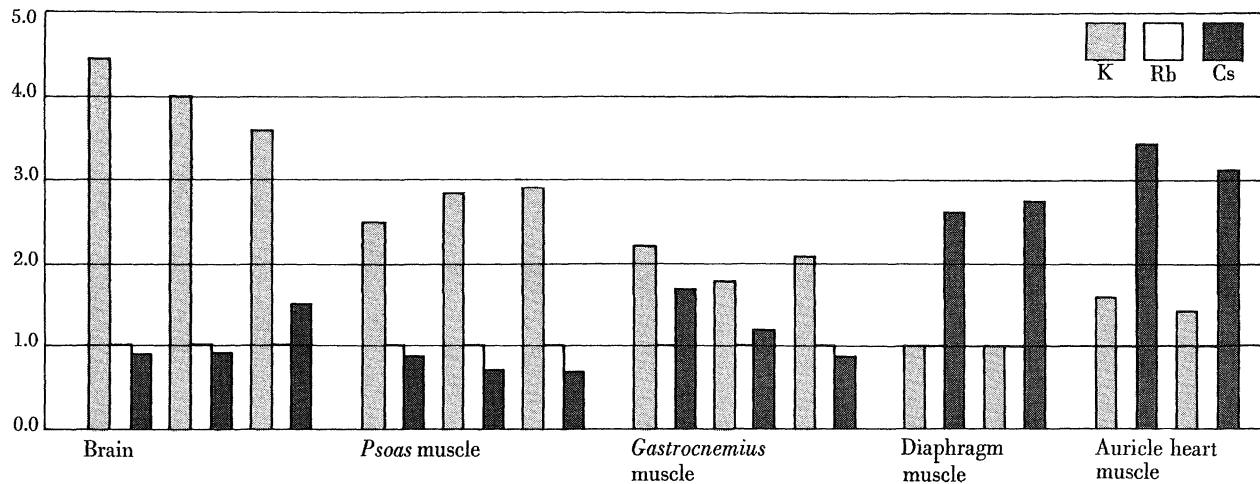
**Table 9.6. RATIO, ([Rb<sup>86</sup>]/[K<sup>42</sup>] IN TISSUE OR FLUIDS)/([Rb<sup>86</sup>]/[K<sup>42</sup>] IN INJECTION MIXTURE).** A value greater than unity indicates that Rb<sup>86</sup> is concentrated relative to K<sup>42</sup>; the reverse is true for values less than one. Analysis of variance shows that the differences among tissues are significant throughout as are the differences among groups sacrificed at various times, except between 39 and 62 hr. Isotopes were injected intravenously. (From Kilpatrick *et al.*, 1956.)

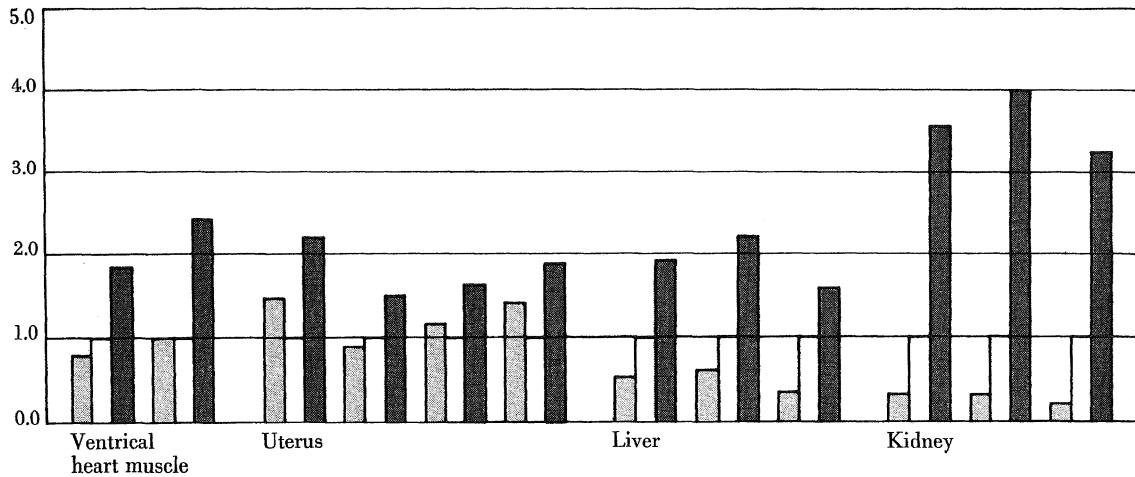
most of the H<sup>+</sup> ions may be adsorbed. Further, since the distribution ratio of a particular ion depends on its free energy of adsorption, the intracellular-to-extracellular ratios of the K<sup>+</sup> and H<sup>+</sup> ions need not be equal; equality of these ratios is a necessary consequence of other theories (Boyle and Conway, 1941) which is inconsistent with observation (see Section 11.1). We emphasize that a multiplicity of choices of counterions for fixed anions and cations exists. Although exchange might take place almost quantitatively between the H<sup>+</sup> ion and the K<sup>+</sup> ion under given conditions, the possibility of partial exchanges with other ions such as the Na<sup>+</sup> ion, fixed cationic groups, and alkaline-earth ions does exist. Thus, in yeast, the amount of H<sup>+</sup> ion secreted depends on the nature of alkali-metal ions present as shown by Rothstein and Demis (1953; see Figure 9.10). The relative



**Figure 9.8. IONIC SELECTIVITY OF RAT ORGANS.** Data from the 14-day rats used to collect the data presented in Table 9.5, plotted as  $([p^+]\text{tissue}/[p^+]\text{plasma})/([Rb^+]\text{tissue}/[Rb^+]\text{plasma})$ , where  $p^+$  represents the  $K^+$ ,  $Rb^+$ , or  $Cs^+$  ion.

effectiveness of these ions in causing  $H^+$ -ion release from yeast cells follows the order  $K^+ > Rb^+ > Na^+ > Li^+$ , an order roughly the same as that found for cation accumulation in many cells (see Table 9.5). These facts show that the  $H^+$  ion, before its emergence into the external solution, exists as the counterion on fixed anionic sites which are potentially able to adsorb  $K^+$ ,  $Rb^+$ , and other ions.



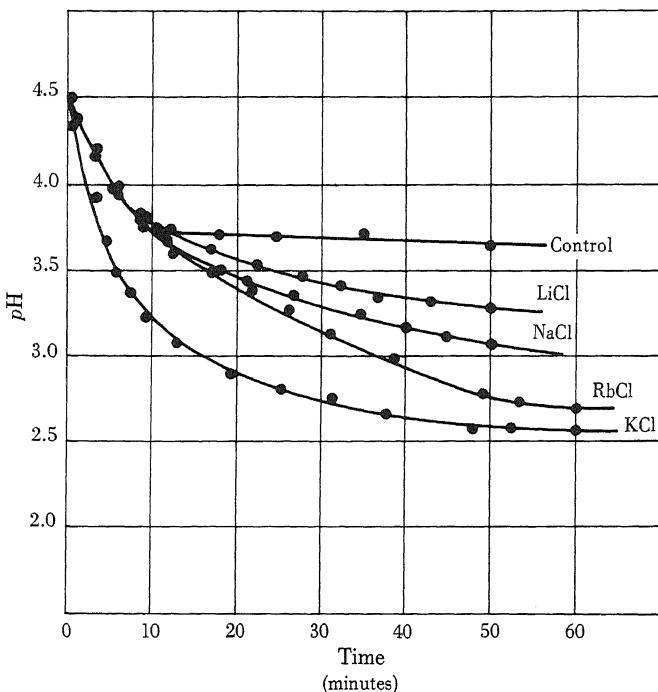


**Figure 9.9.** ACCUMULATION OF  $K^{42}$ ,  $Rb^{86}$ , AND  $Cs^{134}$  BY RABBIT TISSUES. Rabbits were sacrificed 26 hours after intravenous injection of a solution containing the isotopes, and the tissues were analyzed for their content of the three radioactive isotopes. The

ordinate gives the value of the ratio (distribution of  $p^+$ )/(distribution of  $Rb^+$ ), where the distribution ratio of ion  $p^+$  is equal to  $[p^+]_{\text{tissue}}/[p^+]_{\text{plasma}}$ . Compare these data with those presented in Table 9.5.

### C. THE SELECTIVE ACCUMULATION OF AMINO ACIDS IN CELLS

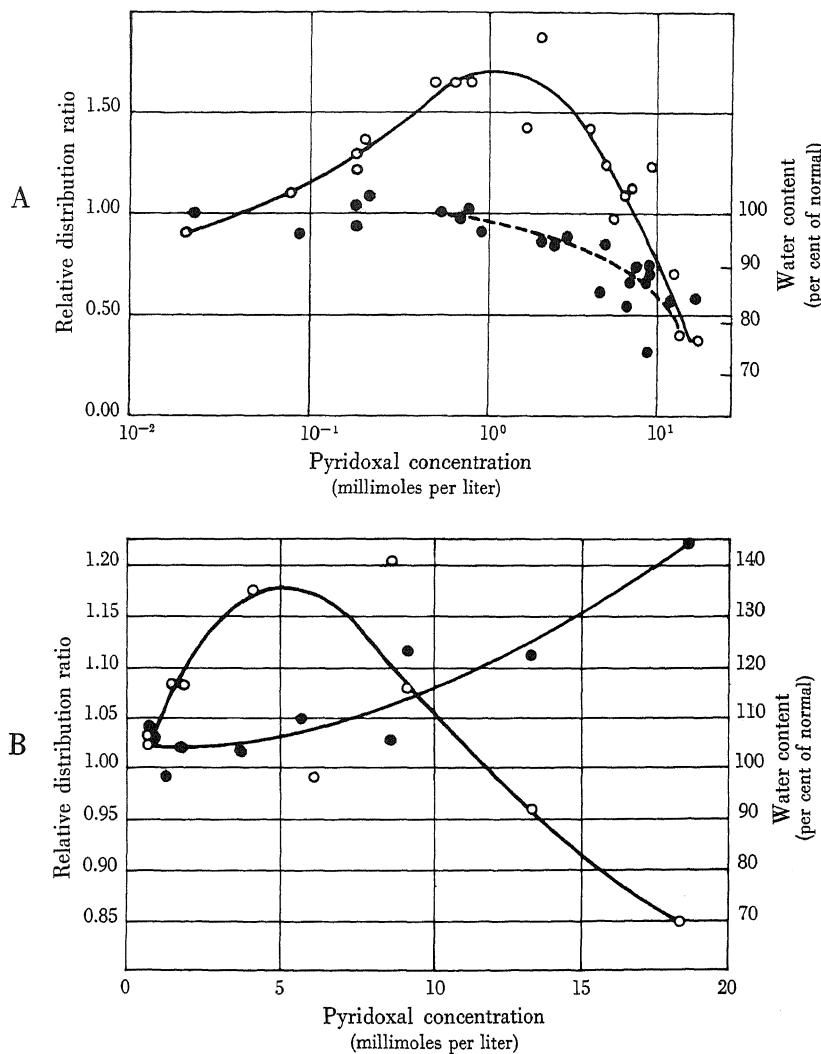
Amino acids form dipolar and multipolar ions. As ions, they tend to adsorb strongly onto suitable fixed ionic sites. For this reason, the man-made fixed-charge systems, synthetic ion exchange resins bearing sulfonate, carboxylate, and other



**Figure 9.10. EFFECTS OF ALKALI-METAL IONS ON HYDROGEN-ION SECRETION BY YEAST CELLS.**  $\text{H}^+$ -ion secretion determined by the change in  $\text{pH}$  of the medium during the fermentation of glucose. Yeast concentration, 100mg/ml; glucose, 0.1M; salts, 0.02M. (Figure after Rothstein and Demis, 1953.)

ionic sites, can be used in the separation and determination of various amino acids. The first step of this procedure involves the adsorption of the amino acids on the resin column; utilizing the differences among their free energies of adsorption, separation follows as the second step (W. E. Cohn, 1953). This and similar techniques have made possible the recent spectacular progress in the elucidation of exact studies of proteins (see Introduction).

These considerations lead one to anticipate that selective amino-acid accumulation in cells follows a pattern similar to that proposed for alkali-metal ion accumulation in the protoplasmic fixed-charge system—a pattern that depends on differences among free energies of adsorption on fixed sites.



**Figure 9.11. THE EFFECT OF PYRIDOXAL ON THE CONCENTRATION OF GLYCINE IN EHRLICH ASCITES CELLS (37°C).** Cells were soaked in Krebs-Ringer solution containing A, 2 mM glycine; B, 25 mM glycine. The curve joining the open circles represents the distribution ratios relative to those reached in the absence of pyridoxal (scale at left). Dots refer to the water content of the cells (scale at right). Note that in B the decrease in the distribution ratio of glycine in the right-hand part of the curve is due to the increased volume of the swollen cell and indicates a decrease of glycine concentration in the cell, not a decrease of glycine content. (Figure after Christensen *et al.*, 1954.)

(1) The selective accumulation of amino acids in higher organisms

Christensen and co-workers have extensively studied the accumulation of amino acids and alkali metal ions in a variety of cells (Christensen *et al.*, 1954; Christensen, 1955). These authors discovered that the addition of pyridoxal to the incubating medium increases glycine accumulation in Ehrlich ascites cells at the

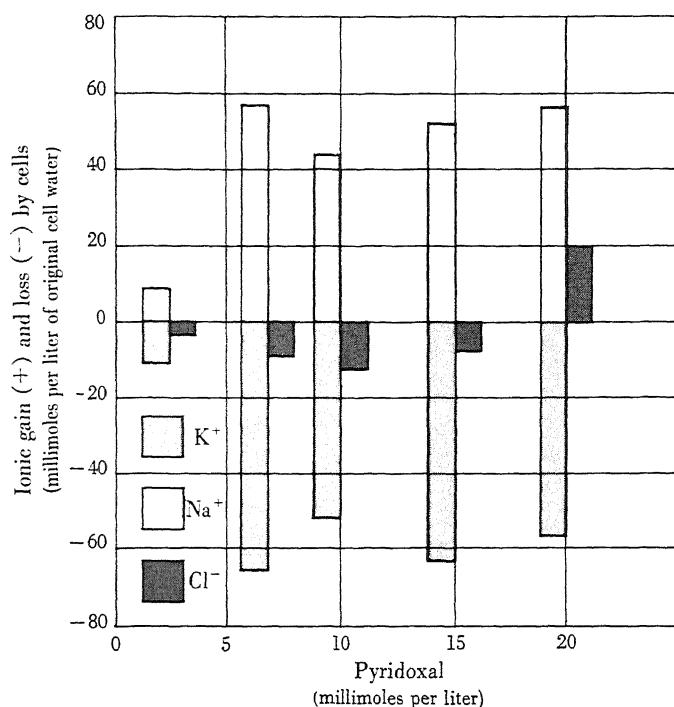
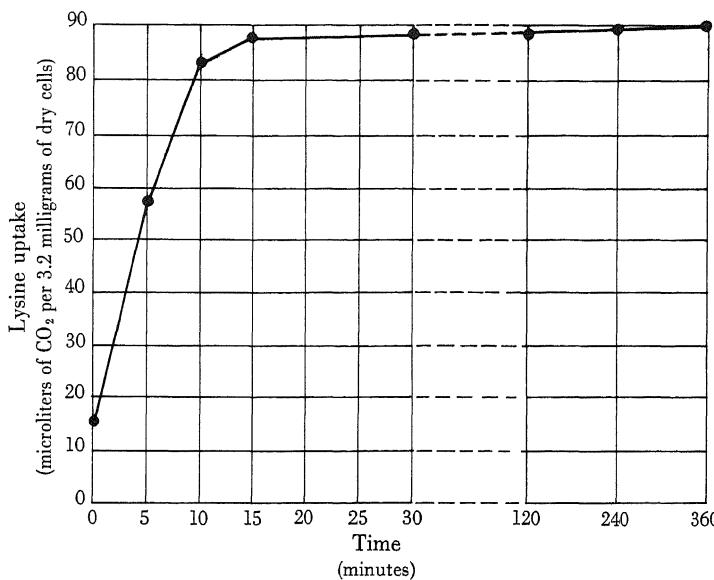


Figure 9.12. ION SHIFTS PRODUCED BY PYRIDOXAL. (Figure after Christensen *et al.*, 1954.)

expense of the  $K^+$  ion. They postulated that pyridoxal is an amino-acid "carrier" which transfers the amino acid inward from the extracellular phase and derives the necessary energy from metabolism. The association-induction hypothesis suggests the alternative explanation that pyridoxal, like other bioregulants, varies the  $c$ - and  $c'$ -values of polar groups on cell proteins. This enhances the ascites cells' preference for amino acids.

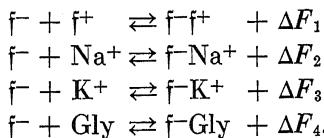
Christensen *et al.* (1952) further concluded that  $\alpha$ -amino acids enter the cells and accumulate there as free ionic molecules and that there is a common step in the accumulation process of  $K^+$  ion and amino acids. According to the present hypothesis, amino acids associate with anionic sites through their cationic  $\alpha$ -amino groups. At any given  $c$ -value (or pyridoxal concentration), in a milieu of

$\text{Na}^+$  ions,  $\text{K}^+$  ions, and  $\alpha$ -amino acids (such as glycine), an adsorption equilibrium exists between these free ions and salt linkages. The relative proportions of salt



**Figure 9.13. THE RATE OF ACCUMULATION OF LYSINE BY LYSINE-DEFICIENT *STREPTOCOCCUS FECALIS* CELLS (10°C).** The amino-acid concentrations in this graph as well as in Figure 9.14 and Table 9.7 are expressed in terms of the number of microliters of  $\text{CO}_2$  liberated in response to the enzymatic action of a specific decarboxylase. The external solution (pH 7.2) contained 300  $\mu\text{l}$  ( $\text{CO}_2$ ) of lysine/ml and no glucose. (Figure after Gale, 1947.)

linkages, of alkali-metal ions adsorbed, and of free glycine molecules adsorbed will be determined by the following relationships:



Here each  $\Delta F$  is a function of  $\Delta E$  as determined by the  $c$ -value (Figure 4.9), if the  $\alpha$ -amino group of glycine, similar to the fixed  $\epsilon$ -amino and guanidyl groups, behaves much like an  $\text{NH}_4^+$  ion.

When the concentrations of  $\text{f}^+$ ,  $\text{K}^+$ , and  $\text{Na}^+$  ions, and of glycine are constant and pyridoxal is added, a change in  $c$ -value occurs and a new equilibrium distribution of counterions results. If this change causes a specific increase in the preference for the fixed cationic groups there will follow: a decrease of glycine uptake,

a decrease in cellular K<sup>+</sup>-ion concentration, and a shrinkage of the cells with a loss of cell water through the formation of more intramolecular and intermolecular salt linkages  $\text{f}^-\text{f}^+$ . If, however, the concentration of glycine is increased, the glycine will compete with and decrease salt-linkage formation with concomitant swelling and increase of water content (see Section 9.4), and decrease the cellular K<sup>+</sup>-ion concentration. These events are those observed by Christensen and co-workers (Figures 9.11 and 9.12).

(2) The selective accumulation of amino acids in bacteria

Gale (1947, 1948) and his associates and others, in their studies of amino-acid accumulation in bacteria, found that lysine enters cells and is selectively accumulated only in the absence of glucose (Figure 9.13 and Table 9.7) while glutamic

External lysine concentration, μl/ml	Lysine uptake, μl CO <sub>2</sub> per 32.5mg cells	
	Glucose absent	Glucose present, 0.5%
25	23	3
50	38	4
100	42	4
400	45	3

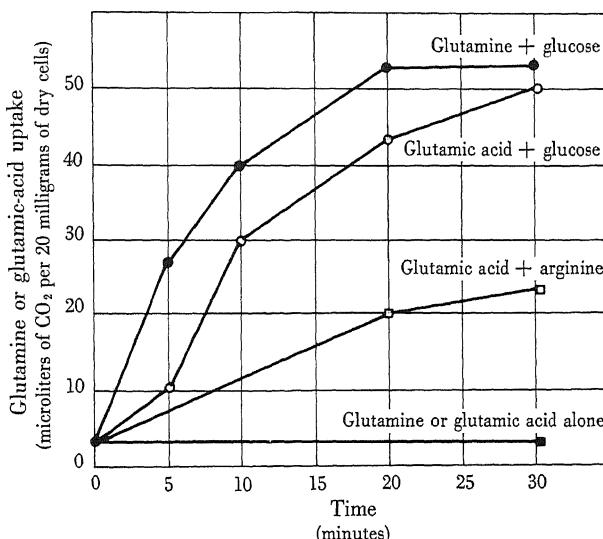
**Table 9.7. THE EFFECT OF GLUCOSE ON EQUILIBRIUM LYSINE CONCENTRATION IN LYSINE-DEFICIENT *STREPTOCOCCUS FECALIS* CELLS.** The cells were incubated for three hours at 25°C. Lysine-concentration units are described under Figure 9.13. (From Gale, 1947.)

acid is selectively accumulated only when glucose, the main metabolic substrate, is present (Figure 9.14). We interpret these phenomena in terms of the present hypothesis as follows: During metabolically quiescent periods there is a large number of anionic sites at *c*-values which favor lysine adsorption over, say, K<sup>+</sup>-ion adsorption; for glutamic-acid adsorption, the *c'*-value of the fixed NH<sub>3</sub><sup>+</sup> group must be altered by the cardinal adsorbent ATP, a product of glucose metabolism. The function of ATP in maintaining a proper *c*-value is discussed in Section 9.5.

Through a similar line of reasoning, we conclude that a chief difference between gram-positive and gram-negative microorganisms is a difference in the modal *c*-values of their cellular proteins; the gram-positive organisms accumulate both free lysine and free glutamic acid, while the gram-negative organisms do not (Table 9.8; Taylor, 1947).

#### D. THE SELECTIVE ACCUMULATION OF ANIONS

The thyroid gland selectively accumulates inorganic  $I^-$ ,  $Br^-$ , and  $Cl^-$  ions in the order  $I^- > Br^- > Cl^-$ .\* The less hydrated  $I^-$  ion is preferred over the more hydrated  $Br^-$  and  $Cl^-$  ions. The mechanism underlying specificity in the accumulation of monovalent cations should be applicable here and we can extend the association-induction hypothesis to anion accumulation.



**Figure 9.14. THE RATE OF ENTRY OF GLUTAMINE OR GLUTAMIC ACID INTO DEFICIENT CELLS IN THE PRESENCE AND IN THE ABSENCE OF GLUCOSE OR ARGININE (37°C).** The external concentration of glutamine and/or glutamic acid was 200 $\mu\text{l}$  ( $\text{CO}_2$ )/ml. The initial pH was 7.2 (see legend of Figure 9.13). (Figure after Gale, 1947.)

In contrast to the lability of  $K^+$ -ion preference (see Chapter 15), most isolated proteins show adsorption energies for halide ions which follow the order seen in living cells (see Table 7.5). The observation that strongly adsorbed anions inhibit iodide accumulation in thyroid in the order perchlorate > thiocyanate > iodide > nitrate (Wyngaarden *et al.*, 1952; 1953) is consistent with the parallelism between the energy of adsorption of anions on Dowex 1 (shown in Table 7.5) and the energy of adsorption on protein and cellular fixed-charge systems. In spite of this general parallelism, however, one must exercise caution in explaining selective halide accumulation in thyroid as equivalent to  $K^+$ -ion accumulation in muscle

\* Baumann and Metzger, 1949; Vanderlaan and Vanderlaan, 1947; Taurog *et al.*, 1947, 1951; Perlman *et al.*, 1942; Wyngaarden *et al.*, 1952.

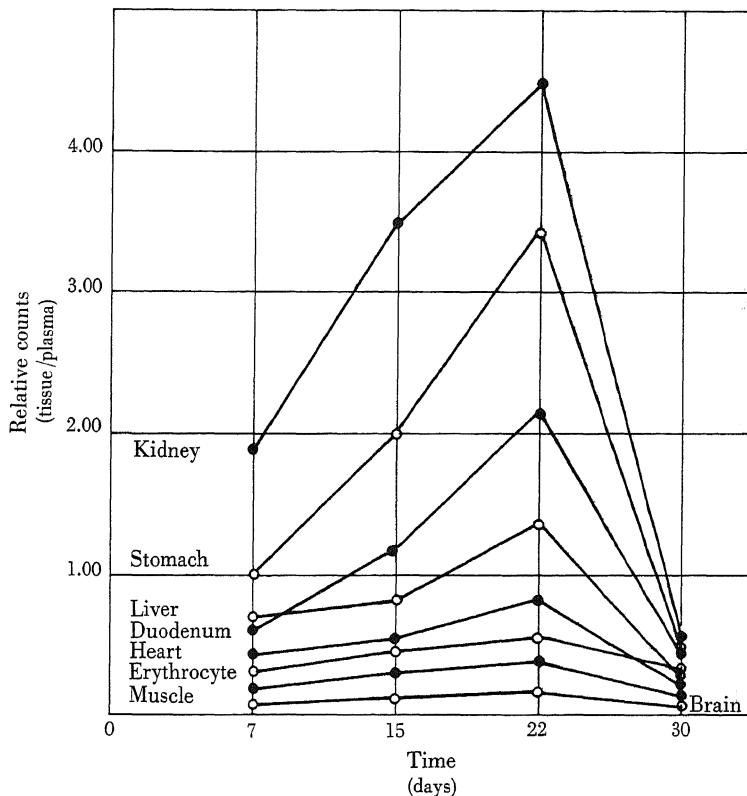
cells. If thyroid colloid behaves as a fixed-charge system, we can make this analogy. If it does not, the mechanism of accumulation would be similar to K<sup>+</sup>-ion accumulation in, say, plant vacuolar sap.

Organism	Time of growth, hr	Temperature of growth, °C	Glutamic acid, μl of CO <sub>2</sub> per 100mg	Lysine, μl of CO <sub>2</sub> per 100 mg
<b>Gram-positive organisms</b>				
<i>Yeast foam</i>	40	25	150	223
<i>Dutch top yeast</i>	40	25	378	543
<i>Saccharomyces carlsbergensis</i>	40	25	198	165
<i>Lactobacillus casei</i> , YCT 1	40	28	174	97
<i>L. delbrueckii</i> , B	40	37	80	103
<i>L. helveticus</i> , B	40	37	74	70
<i>Strep. fecalis</i> , ST	16	37	230	309
<i>Strep. fecalis</i> , SF	16	37	107	110
<i>Strep. haemolyticus</i> 'Richards'	16	37	99	83
<i>Staph. aureus</i> , A	16	37	293	59
<i>Staph. aureus</i> , D	16	37	456	99
<i>Sarcina lutea</i>	56	25	225	61
<i>Micrococcus lysodeikticus</i>	24	37	203	108
<i>Cl. sporogenes</i>	14	37	81	31
<i>Cl. septicum</i> , P3 (547)	14	37	7	36
<i>B. mesentericus</i>	14	37	14	70
<i>B. subtilis</i>	14	37	19	29
<b>Gram-negative organisms</b>				
<i>N. catarrhalis</i>	24	37	0	0
<i>B. brevis</i>	14	37	0	0
<i>E. coli</i> (36)	14	37	0	0
<i>E. coli</i> Taylor	14	37	0	0
<i>E. coli</i> (7020)	14	37	0	0
Organism N.C.T.C. No. 6578	14	37	0	0
<i>A. aerogenes</i> I	14	37	0	0
<i>A. aerogenes</i> II	14	37	0	0
<i>Proteus vulgaris</i>	14	37	0	0
<i>Pseudomonas pyocyannea</i> , G138	14	37	0	0

**Table 9.8. FREE AMINO-ACID CONTENTS OF VARIOUS GRAM-NEGATIVE AND GRAM-POSITIVE ORGANISMS.** The amino-acid concentrations are expressed as μl of CO<sub>2</sub>/100mg of dry cells evolved by specific enzymatic decarboxylations. (Data from Taylor, 1947.)

In Chapter 5 we discussed the fact that the *c*-, *c'*-value, or its analogue, of a functional group is determined by (1) the nature of the functional group, and the remainder of the side chain bearing the functional group, (2) the nature of the neighboring side chains and their counterions or adsorbents, (3) adsorbents on the polypeptide chain, (4) the length of the polypeptide chain separating the neighbor-

ing side chains and other adsorbing sites from the functional group under discussion, and other factors [see equation (5-6)]. We have also shown that while the polypeptide chain, because of its resonating structure, is highly polarizable and thus conducive to the inductive effect, elements of the saturated hydrocarbon



**Figure 9.15.**  $I^{131}$  INCORPORATION BY RAT TISSUE. Rats were injected with  $I^{131}$  at the beginning of the experiment. One rat was sacrificed approximately every seven days; the content of  $I^{131}$  in samples of several tissues was determined, and expressed as a ratio (tissue count per unit weight/plasma count per unit weight).

chain,  $-CH_2-$  often found on side chains are resistant to the transmission of inductive effects. Thus a functional group separated from the polypeptide chain by a long chain of  $CH_2$  groups will be effectively insulated from transmitted effects and will therefore be less variable in its  $c$ - or  $c'$ -values than functional groups separated from the peptide chain by fewer groups. Examination of the structural formulas of the amino acids (see Table 0.1) shows that fewer such  $CH_2$  groups intervene between the polypeptide chain and the sulfhydryl groups and between the poly-

peptide chain and the fixed anionic groups than intervene between the polypeptide chain and the fixed cationic groups. A high variability of the *c*- or *c'*-value connotes a high variability in the preferred counterions. We believe this to be the reason that anionic affinity is generally restricted to the order of preference shown in Table 7.5, while the affinity for cations varies widely. The stability of the *c'*-value relative to the *c*-value is shown by the invariant order of anion affinity found in adsorption on isolated proteins, in selective anionic accumulation by cells, and in the effect of anions on enzyme activity.

In discussing selective cationic accumulation, we have shown that various organs differ in their characteristic *c*-value ensembles. However, in the fixed-charge system of living cells, the bulk of the fixed cationic and anionic groups are associated with counterions; thus the whole protein molecule is essentially neutral. When a high proportion of the anionic sites on a protein possesses high *c*-values, on the average, the mobile electrons tend to congregate at the anionic residues. Consequently there is a paucity of electrons at the fixed cationic groups on the same protein.\* We, therefore, expect organs whose anionic sites have high *c*-values to have cationic sites of high *c'*-values. Thus, when I<sup>131</sup> (in competition with the Cl<sup>-</sup> ion of plasma) is accumulated by various organs of an animal injected with this isotope, the organ containing the largest fraction of highest *c*-value sites should accumulate the least I<sup>131</sup> and organs with lower modal *c*-value ensembles should accumulate proportionally more I<sup>131</sup>. By and large, this was found to be so (compare Figure 9.15 with Figure 9.8).

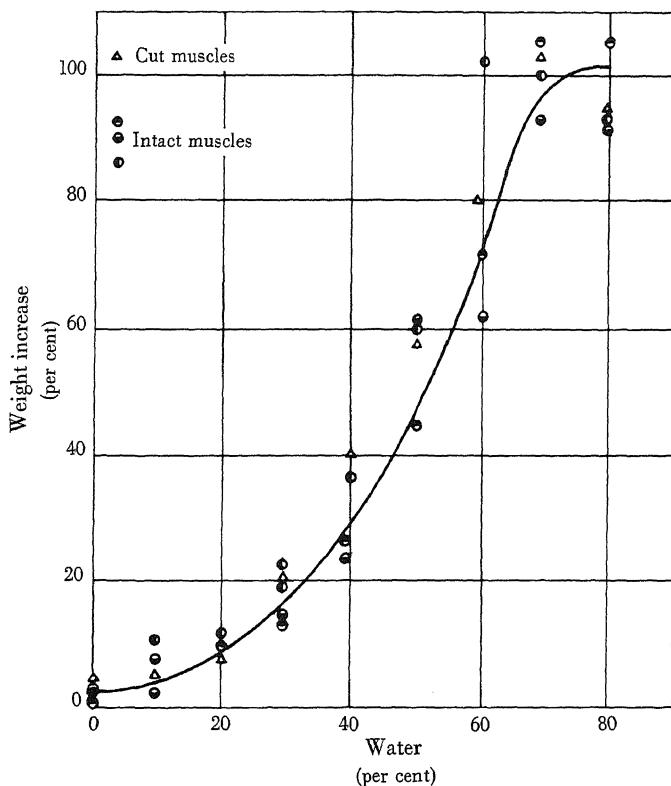
#### 9.4. The Osmotic Properties of Cells

According to the membrane concept, cells are essentially sacs of dilute aqueous solutions of salts and proteins. The cell membrane, according to this view, separates two aqueous phases of equal osmotic activity and maintains the structure of the cell (for an opposing view, see Steinbach, 1944). The association-induction hypothesis, in agreement with practically all careful observations that the typical cytoplasm is a gel (see Chambers and Kao, 1952; Hodgkin and Keynes, 1956), treats the cell content as a proteinaceous fixed-charge system; as such, the osmotic behavior of living cells should resemble that of a piece of gelatin (Ling, 1955a; Bozler and Lavine, 1958) more closely than that of a membrane-enclosed aqueous solution; the osmotic properties of a cell, therefore, must intimately reflect its

\* Another way of looking at this is to recall that for a group of, say, three cationic and three anionic sites the lowest over-all *c*-value ensemble is obtained by arranging them + - + - + -. The highest over-all *c*-value ensemble obtains when the three anionic charges, ---, are placed together as a subgroup and this subgroup is removed as far from the cationic sites as possible. In this case, the segregated cationic groups, whether isolated or also in groups, would have higher over-all *c'*-values than they would if they were in the sequence, + - + - + -.

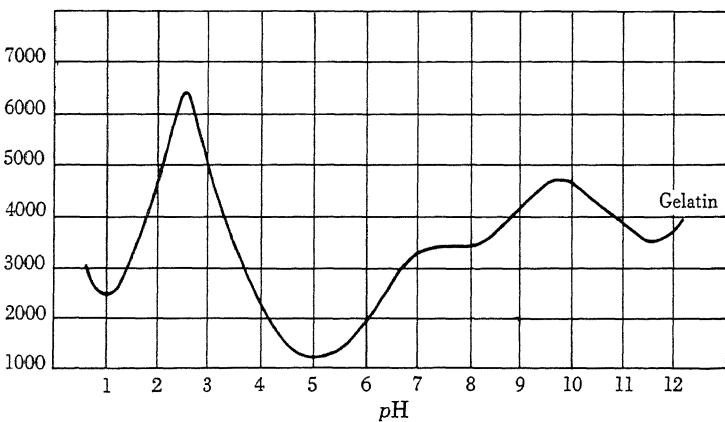
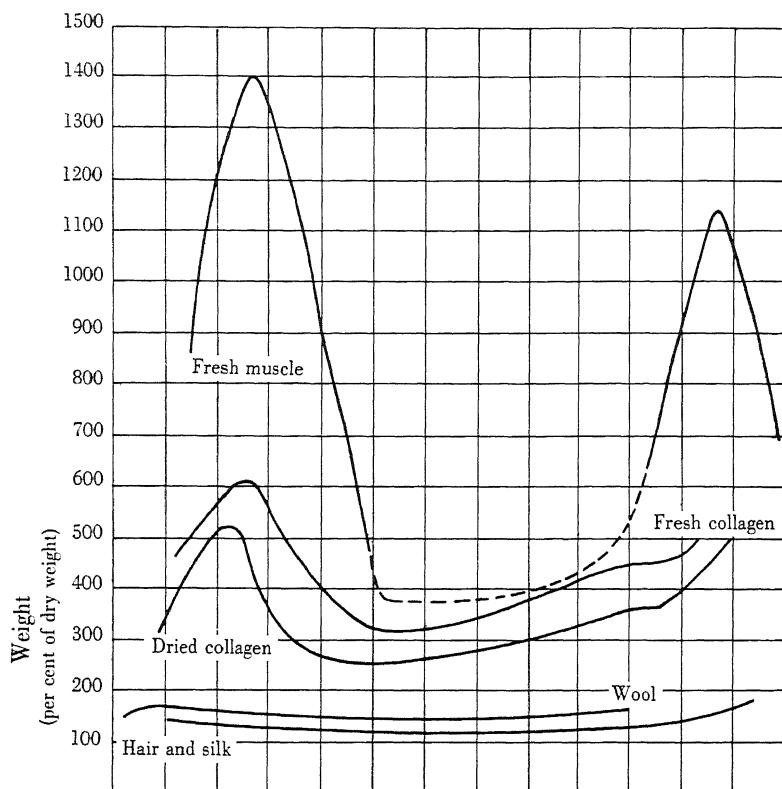
ion-adsorption properties. Some experiments are presented here as evidence in support of this point of view.

If the membrane theory is correct, rupture of the membrane should profoundly alter the osmotic behavior of frog muscle cells. That this is not the case is shown



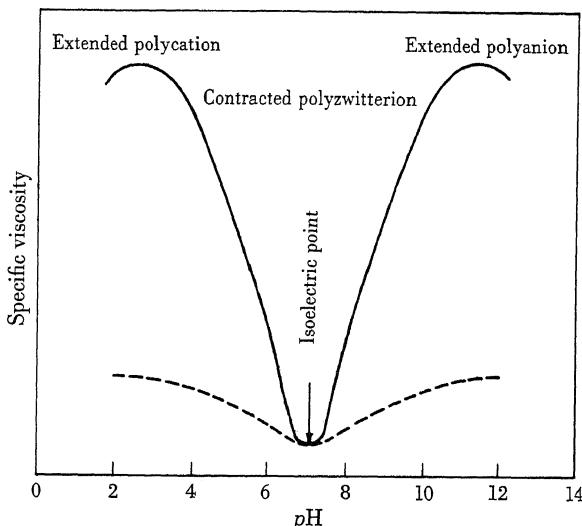
**Figure 9.16.** WEIGHT CHANGES OF INTACT AND CUT FROG SARTORIUS MUSCLES IN VARIOUS MIXTURES OF RINGER'S SOLUTION AND WATER ( $29.6^{\circ}\text{C}$ ). Intact sartorius muscle and muscles cut two millimeters from the pelvic end were allowed to equilibrate for three hours. The cut muscles and those indicated by circles with left halves shaded were bubbled with 5 per cent  $\text{CO}_2$  and 95 per cent  $\text{O}_2$  while equilibrating. Those indicated by circles with upper and lower halves shaded were not bubbled while equilibrating.

by Figure 9.16 from which it is apparent that the osmotic behavior of this tissue is not appreciably affected by one or two transections across it. Having thus demonstrated the secondary role of an intact cell surface (membrane) in the maintenance of the equilibrium shape and size of the living cell, we can consider the present theory. According to this theory, equilibrium shape and size depend pri-



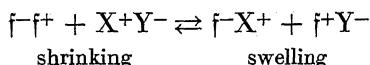
**Figure 9.17.** SWELLING OF VARIOUS STRUCTURED PROTEINS AS A FUNCTION OF pH. Note that the gelatin curve is plotted on a reduced ordinate. (Figure after Jordan-Lloyd, 1933.)

marily on a balance between cohesive forces in the S—S bonds, the H-bonds, and, particularly, the salt linkages, and on dispersive forces such as the electrostatic repulsion between similarly charged groups and the entropy gain associated with the dissolution of intramolecular and intermolecular bonds. Since salt linkages are



**Figure 9.18.** THE EFFECT OF pH ON THE SPECIFIC VISCOSITY OF AN AMPHOTERIC POLYELECTROLYTE. A copolymer of methacrylic acid and diethylaminoethylmethacrylate. Extension and contraction of the linear chains are reflected by large changes in the viscosity of the solution. The dotted curve represents the change produced by increasing the ionic strength through the addition of salt. When linear chains are cross linked into a gel, extension and contraction are seen as swelling and shrinking. (Figure after Alfrey and Gurnee, 1957.)

among the most important cohesive forces, the breaking and reforming of these should affect the osmotic behavior of cells, causing swelling and shrinking respectively.



where  $f^-$  and  $f^+$  are fixed anions and cations, and  $X^+$  and  $Y^-$  represent free counterions ( $K^+$ ,  $Cl^-$ , for example). This relation indicates that the greatest swelling should occur when  $f^-$  and  $f^+$  are "neutralized" by free counterions. Such a neutralization is achieved readily through a decrease or an increase of  $pH$ . Thus, if the salt linkages of the cellular proteins are important cohesive factors, a plot of the equilibrium weight of muscle tissue against  $pH$  should yield U-shaped curves; the midpoints of the limbs of each U should coincide roughly with the  $pK$  value of the  $f^-$

(carboxyl,  $pK = 4$ ) and of the  $\text{f}^+$  ( $\epsilon$ -amino,  $pK = 10$ ) groups. Jordan-Lloyd (1933) performed an experiment of this type with the results shown in Figure 9.17. Alfrey and Gurnee (1957) showed parallel behavior in the viscosity of a synthetic amphoteric electrolyte carrying both fixed anionic and fixed cationic groups (Figure 9.18).

## 9.5. The Role of Metabolism in the Maintenance of Selective Ionic Accumulation

According to the association-induction hypothesis, metabolism plays its role in the maintenance of selective ionic accumulation in muscle cells through the production of adenosinetriphosphate (ATP) and other metabolically derived organic anions such as creatine phosphate (CrP) and hexose phosphates (Ling, 1951, 1952). According to the 1952 version of the theory, the action of ATP was due to mutual electrostatic repulsion of the charged groups on ATP and on other groups which thus maintained an unfolded configuration.. This view was akin to those postulated by Meyer and Mark (1930), Riseman and Kirkwood (1948), and in particular to that of Morales and Botts (1953, 1956; see also Morales, 1956; Morales *et al.*, 1955; Botts and Morales, 1951). According to the present version of the theory, ATP has a twofold function. (1) In Section 7.3 we demonstrated that in an amphoteric fixed-charge system the fixed anions and cations tend to form salt linkages. When salt linkages are formed, the accumulation of free alkali-metal ions is decreased—often until virtually none are accumulated. We can transform such a system into one which effectively accumulates free cations by introducing into it anions of sufficiently great association energy. This function is fulfilled by ATP and a number of other metabolically derived products such as creatine phosphate in muscle (Ling, 1952), glutamate in retina and brain (Terner *et al.*, 1950) and in gram-positive bacteria (Gale, 1947), hexose phosphate in the gram-negative bacteria, *Escherichia coli* (R. B. Roberts *et al.*, 1949), and metaphosphate in yeast (G. Schmidt *et al.*, 1949).

Two groups of experiments deserve separate discussion. (a) The data of Terner, Eggleston, and Krebs (1950) are illustrated in Table 9.9. Brain slices and retina will resorb lost  $\text{K}^+$  ion if incubated in a medium containing glucose,  $\text{K}^+$ , and glutamate ion under aerobic conditions; they will not resorb  $\text{K}^+$  ion if the medium contains only  $\text{Cl}^-$  as the anion. This is a counterpart of the experiment of Steinhardt *et al.* (1942) on the adsorption of  $\text{H}^+$  ion by wool protein (Figure 7.5). Because wool is a macroscopic amphoteric fixed-charge system, the uptake of  $\text{H}^+$  ion is always accompanied by the uptake of an equivalent quantity of anion. At  $\text{H}^+$ -ion activity of  $10^{-4}$ , wool adsorbed little  $\text{H}^+$  ion (less than 50 millimoles per kilogram) when the anion present was  $\text{Cl}^-$ ; 350 millimoles per kilogram were taken up when the  $\text{H}^+$  ion was present at the same activity and its anion was the strongly

adsorbed picrate. Thus, the demonstration of a high degree of selectivity of free cations over fixed cations ( $H^+$  ion over fixed amino group in wool,  $K^+$  ion over fixed amino group in brain slice and in retina) depends upon the simultaneous accumulation in the fixed-charge system of an equimolar amount of strongly bound anion. In neither system was the  $K^+$  or the  $H^+$  ion taken up in substantial

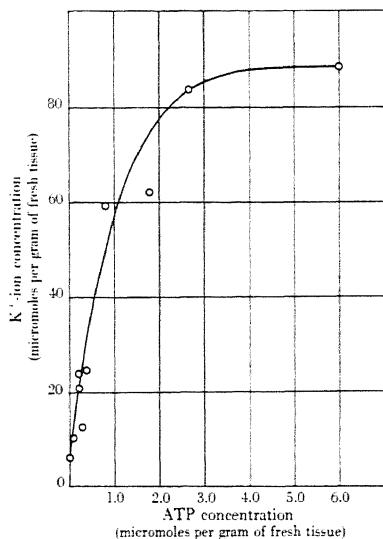
Experiment	Portion of tissue	Substrates added	Experimental condition	$K^+$ concentration in tissue, $\mu M/g$		
				Initial	Final	Change
A	1	—	—	90.04	—	—
	2	none	anaerobic	90.04	9.21	-80.83
	3	glucose	anaerobic	90.04	20.21	-69.83
	4	glucose; L-glutamate	anaerobic	90.04	12.28	-77.76
	5	glucose; L-glutamate; ATP	anaerobic	90.04	18.42	-71.62
	6	glucose; L-glutamate	aerobic	90.04	94.65	+4.60
B	1	—	—	31.21	—	—
	2	glucose	aerobic	31.21	35.81	+4.60
	3	glucose; L-lysine	aerobic	31.21	30.70	-0.51
	4	glucose; L-tyrosine	aerobic	31.21	27.63	-3.58
	5	glucose; L-leucine	aerobic	31.21	36.83	-5.63
	6	glucose; L-methionine	aerobic	31.21	40.42	-9.21
	7	glucose; L-glutamate	aerobic	31.21	60.62	-29.41

**Table 9.9. THE EFFECT OF GLUCOSE AND AMINO ACIDS ON  $K^+$ -ION LOSS OR GAIN BY RETINA OR BRAIN SLICES.** A, guinea pig brain cortex slices or B, ox retina, were incubated in bicarbonate saline at 40°C for 60 minutes with various substances: glucose, 0.02M; ATP, 0.001M; and amino acids, 0.01M. Initial and final  $K^+$ -ion contents were determined on portions of the same tissue and expressed as  $\mu M$  of  $K^+$  ion/g of fresh tissue. Tabulated values were corrected for weight changes and for the  $K^+$ -ion content of the adhering medium. (Data from Terner *et al.*, 1950.)

quantity when the only anion available was the weakly preferred\*  $Cl^-$  ion (see Table 7.5). (b) On the basis of equivalents, CrP and ATP constitute 60 per cent of the free anionic component of living muscle (see Table 9.2). Both of these constituents depend on the intact metabolic machine for their continued resynthesis. This accounts for the widely observed loss of ion-accumulating ability under conditions that hinder cellular metabolism while permitting continued degradation of these compounds (by the combined action of cyanide and iodoacetate).

\* Due to the relatively small variability of the  $c'$ -value of fixed cationic groups (discussed in Section 7.4D and Section 9.3D), the  $Cl^-$  ion is weakly adsorbed in most living systems (see Table 7.5). This does not preclude the possibility of some high  $c'$ -value sites that strongly adsorb the  $Cl^-$  ion (see Figure 14.8).

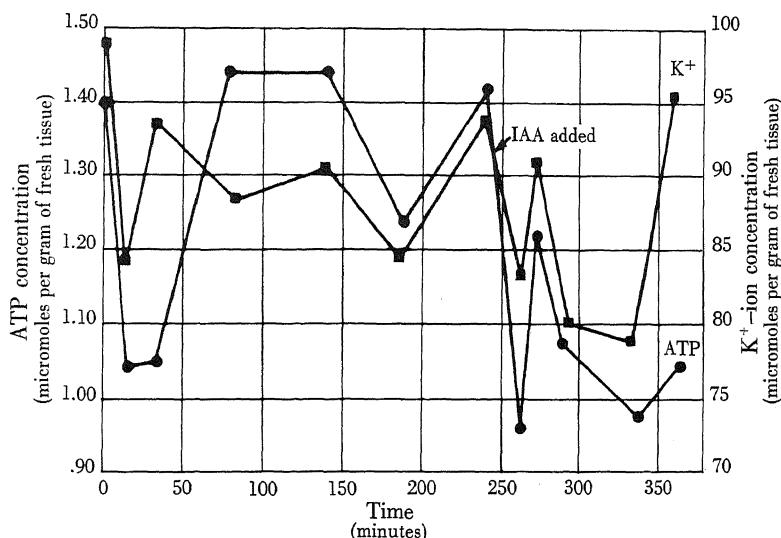
tate at room temperature, for example). Muscle cells so treated go into rigor and a large proportion of their fixed cationic and anionic sites become locked in salt linkages. In this condition, they closely resemble a piece of wool, which, despite its 30 per cent water content, takes up exceedingly little  $K^+$  or  $Na^+$  ion from Ringer's solution (unpublished data).



**Figure 9.19. THE CORRELATION OF INTRACELLULAR  $K^+$ -ION CONCENTRATION AND ATP CONCENTRATION IN FROG VOLUNTARY MUSCLES.** Muscles were treated with 5mM IAA for varying lengths of time at room temperature, then chilled in the same bathing solution to 0°C and allowed to equilibrate at this lower temperature for one hour, after which they were analyzed for both their  $K^+$ -ion and ATP contents. ATP analyses were made according to a method modified from Kalckar (1947). The details of the modified procedure are given in Appendix D. The  $K^+$ -ion concentration was determined by flame photometry.

(2) Second, ATP, like other bioregulants, regulates the  $c$ - and  $c'$ -values of the polar groups of the proteins through an inductive effect. It thus influences ionic preference. That ATP is strongly adsorbed onto muscle proteins is well established (Szent-Györgyi, 1947; Nanninga *et al.*, 1957; Asakura, 1961). Through the direct or indirect  $F$ -effect, its adsorption leads to changes of the  $c$ -value of neighboring anionic sites and increased  $K^+$ -ion accumulation as predicted on the basis of the theoretical curves shown in Figures 4.9 to 4.11. This hypothesis requires the existence of a quantitative relationship between the concentration of ATP present in the living cell and the concentration of selectively accumulated ions in the system. To study this problem, frog muscles were poisoned with iodoacetate at room temperature over various periods of time. These muscles and their bathing

solutions were then chilled and kept for several hours at 0°C to equilibrate; after this, the ATP and K<sup>+</sup>-ion concentrations were determined.\* In Figure 9.19, the ATP concentrations found are plotted against the K<sup>+</sup>-ion concentrations in the same muscles (for abstract, see Ling, 1951). The result indicates that, within the range, 0 to 2.5 millimoles of ATP per kilogram of fresh muscle, each



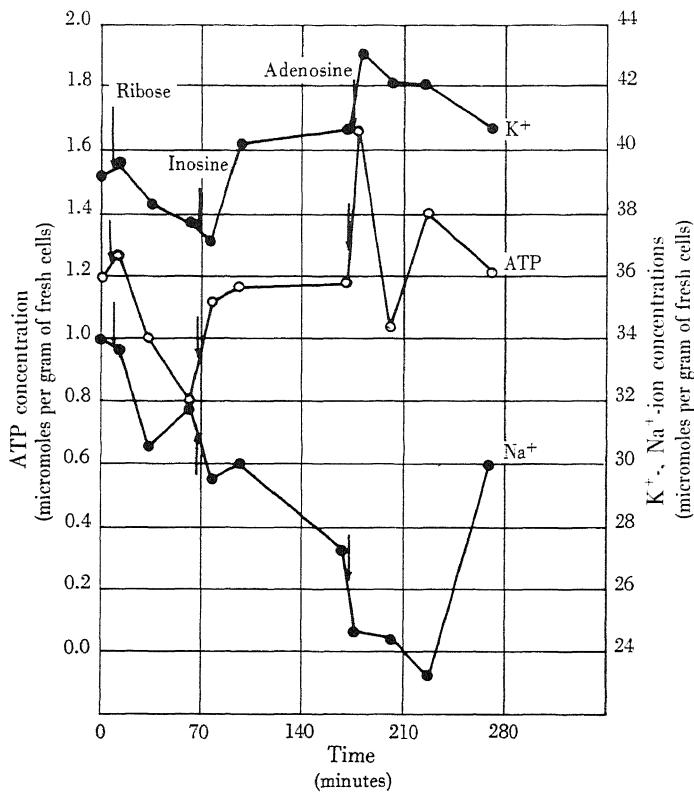
**Figure 9.20. THE COVARIATION OF THE K<sup>+</sup>-ION AND ATP CONTENTS IN HUMAN ERYTHROCYTES (25°C).** The cells were stored at 4°C and washed in Krebs-Ringer solution before use. The experiment was carried out in Ringer's solution to which glucose was added to a final concentration of 5mM. Isotonic IAA was added to a final concentration of 1mM. To effect complete separation of erythrocytes, a bomb-shaped apparatus was devised (see Dowben and Holley, 1959). For the method of ATP analysis, see Appendix D.

mole of ATP critically determines the selective accumulation of about 34 moles of K<sup>+</sup> ion. This is analogous to the experimental results of Steinhardt and Zaisser (1951, 1955), who found that the addition of 4 moles of H<sup>+</sup> ion to hemoglobin, changed the preference of 36 carboxyl groups from fixed amino groups to H<sup>+</sup> ions.

In red blood cells, an even more striking quantitative correlation exists between the ATP and K<sup>+</sup>-ion contents. This close parallel is preserved whether the ATP content is made to vary in response to metabolites such as ribose, inosine, and adenosine or in response to poisons such as iodoacetate (Figures 9.20 and 9.21). The Na<sup>+</sup>-ion content in red blood cells was found to vary directly or inversely with the K<sup>+</sup>-ion concentration change. This phenomenon may be

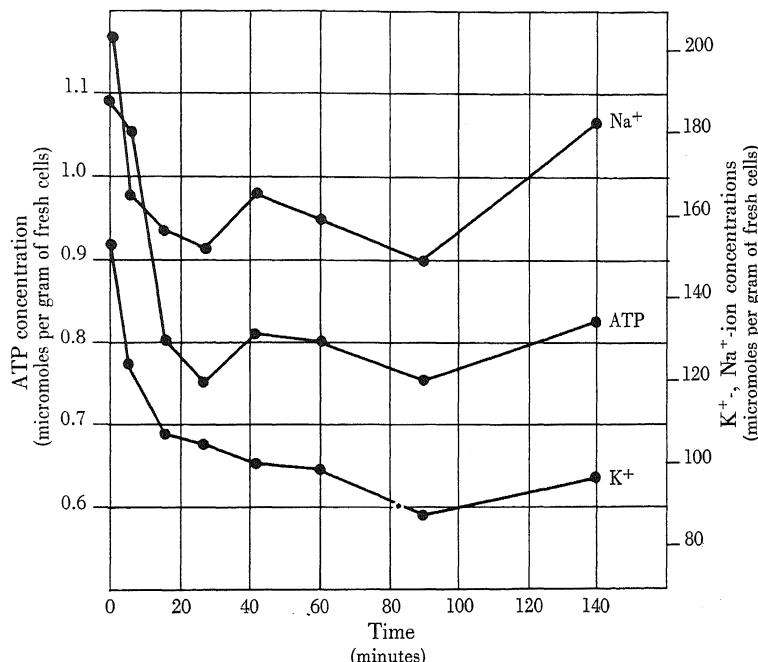
\* For the improved method of ATP assay, see Appendix D.

interpreted as dependent on whether the initial *c*-value of the cell is such that the K<sup>+</sup>-ion content varies at the expense of salt linkages, or at the expense of adsorbed Na<sup>+</sup> ions (see Section 16.2A for the similar action of digitalis on the K<sup>+</sup>-ion content of heart muscle). In the bacteria, *Escherichia coli*, which show



**Figure 9.21. THE COVARIATION OF ATP, K<sup>+</sup>-, AND Na<sup>+</sup>-ION CONTENTS IN HUMAN ERYTHROCYTES (37°C).** Isotonic ribose, inosine, and adenosine were added to the incubation solutions to reach the following final concentrations: ribose, 10mM; inosine, 2mM; adenosine, 2mM (experimental procedures were similar to those given for Figure 9.20).

a somewhat lower selectivity for the K<sup>+</sup> ion, there is a more pronounced correlation between ATP content and the Na<sup>+</sup>-ion concentration of the cell than between ATP and the K<sup>+</sup> ion (Figure 9.22). Although ATP plays a critical role in determining cationic selectivity in many cells, in other tissues and cells, different metabolic products can be equally important or more important than ATP in determining selective ionic accumulation. In these cases, the controlling role of ATP in ionic accumulation may be less prominent.



**Figure 9.22. THE COVARIATION OF ATP, K<sup>+</sup>-, AND Na<sup>+</sup>-ION CONTENTS IN *ESCHERICHIA COLI* DURING FERMENTATION.** Glucose was added at zero time to give a concentration of 6mM in the phosphate-buffer medium. Experimental procedures were similar to those given for Figure 9.20.

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#### REFERENCES TO THE MEMBRANE THEORY OF CELLULAR PERMEABILITY AND ACCUMULATION OF IONS AND NONELECTROLYTES

According to the classical membrane theory, the major physiological properties of cells reside in a microscopically thin "plasma membrane"; this membrane encloses the cellular cytoplasm which is regarded as having the properties of a dilute aqueous solution of protein and salt. As the membrane theory evolved, the idea took root that this hypothetical plasma membrane was similar in its physiological function and mechanism to frog skins, kidney tubule cells, and the cytoplasmic layer of *Valonia* cells where there is little doubt that an asymmetrical ionic distribution is maintained in two essentially similar aqueous phases. The mechanism (often referred to as "active transport") for the creation of such asymmetrical ionic distributions is supposedly an as yet unidentified "pump" energized by metabolic reactions. Classically, active transport includes the maintenance of asymmetrical distribution patterns; and also several phenomena which, according to the association-induction hypothesis, are essentially distinct; these are, selective accumulation of ions and nonelectrolytes, selective permeability, and diffusion.

Each of the references which follow presents an aspect of the classical membrane theory. Although this concept is no longer considered valid by the present author, as a former adherent and student of the membrane theory, he would like to point out that much of our present knowledge of cellular physiology has been obtained by workers following the main outlines of this view. The following list of references is given for the reader who desires to acquaint himself with the broader aspects of cellular physiology from more than one point of view.

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# 10

## THE MECHANISM OF CELLULAR POTENTIALS

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### 10.1. The Classical Membrane Theories

More than a century after Galvani's discovery of bioelectricity, Ostwald (1890) suggested a mechanism to explain cellular electrical potentials.\* McDonald (1900, 1902) and Bernstein (1902) independently developed this idea into a theory of membrane potentials. They postulated that the cellular electrical potential arises from the semipermeability of the cell membrane which was thought to bar the passage of negatively charged anions, while permitting some

\* Studying electrical potentials generated across semipermeable precipitation membranes, Ostwald wrote, "At this time it is perhaps not too presumptuous to predict that not only the current in muscle and nerve, but also the mysterious action of the electric fish, can be interpreted in terms of the characteristics of semipermeable membrane which have been dealt with here." (Free translation by the present author.)

cations to pass through. If this mechanism were operative, the potential  $\psi$  should obey the Nernst equation,

$$\psi = \frac{RT}{\mathfrak{F}} \ln \frac{[p^+]_{in}}{[p^+]_{ex}} \quad (10-1)$$

where  $[p^+]_{in}$  and  $[p^+]_{ex}$  represent the intracellular and extracellular concentrations of the permeant cation;  $R$  and  $\mathfrak{F}$ , the gas constant and Faraday constant, respectively; and  $T$ , the absolute temperature. Since the  $K^+$  ion is the major cation found inside muscle and nerve cells, the cell membrane must be permeable to this ion. The equation can then be written

$$\psi = \frac{RT}{\mathfrak{F}} \ln \frac{[K]_{in}}{[K]_{ex}} \quad (10-2)$$

where  $[K]_{in}$  and  $[K]_{ex}$  are the intracellular and extracellular  $K^+$ -ion concentrations.

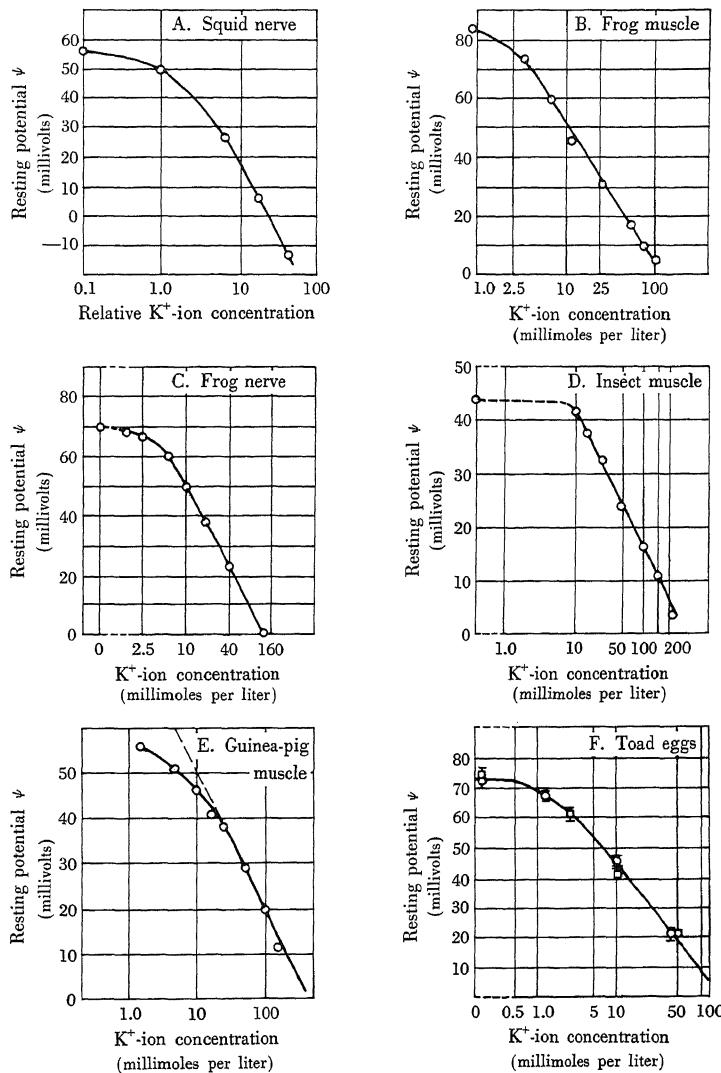
The theory which Ostwald, McDonald, and Bernstein based on the concept of the cell membrane's selective ionic permeability is quite commonly called the membrane theory.\* This theory and its variations must be distinguished from other theories bearing the same name.† Thus, Loeb and Beutner, 1911 and 1912, (see also Beutner, 1920, 1944) in their phase-boundary potential theory, postulated that the cell membrane represents a nonaqueous homogeneous phase and ascribed the origin of the potential to the two-phase boundaries separating the membrane from the extracellular and intracellular aqueous phases. The equation they deduced for the cellular potential is identical with equation (10-2).

In following years, the logarithmic relation between the resting potential and the external  $K^+$ -ion concentration, originally observed by McDonald, was repeatedly confirmed for the cells of a number of tissues; Figure 10.1 shows this relation for several tissues (nerve: Curtis and Cole, 1942; A. F. Huxley and Stämpfli, 1951; see also Cowan, 1934; muscle: Ling and Gerard, 1950; D. W. Wood, 1957; Holman, 1958; egg cells: Maéno, 1959). The relation between  $\psi$  and  $T$  predicted by equation (10-2) was also confirmed; two examples of this relation are shown in Figure 10.2 (Ling and Woodbury, 1949; Hodgkin and Katz, 1949a). The validity of the equation, however, was by no means proven. In fact, two important difficulties indicated the need for major revisions.

The first of these difficulties arose when it became known that, contrary to earlier conclusions (for example, Boyle and Conway, 1941), the membrane is permeable to the  $Na^+$  ion as shown by Table 9.1 (see Heppel and Schmidt, 1938;

\* Later, Donnan (1911) provided a thermodynamic treatment of the equilibrium state in a two-phase system separated by a semipermeable membrane. The membrane equilibrium has since been called the Donnan equilibrium and the potential, a membrane or Donnan potential.

† Osterhout (1933) postulated a nonaqueous homogeneous cell membrane similar to that of Loeb and Beutner but regarded the potential as a diffusion potential; in this aspect, his view resembles that of Ostwald, McDonald, and Bernstein.

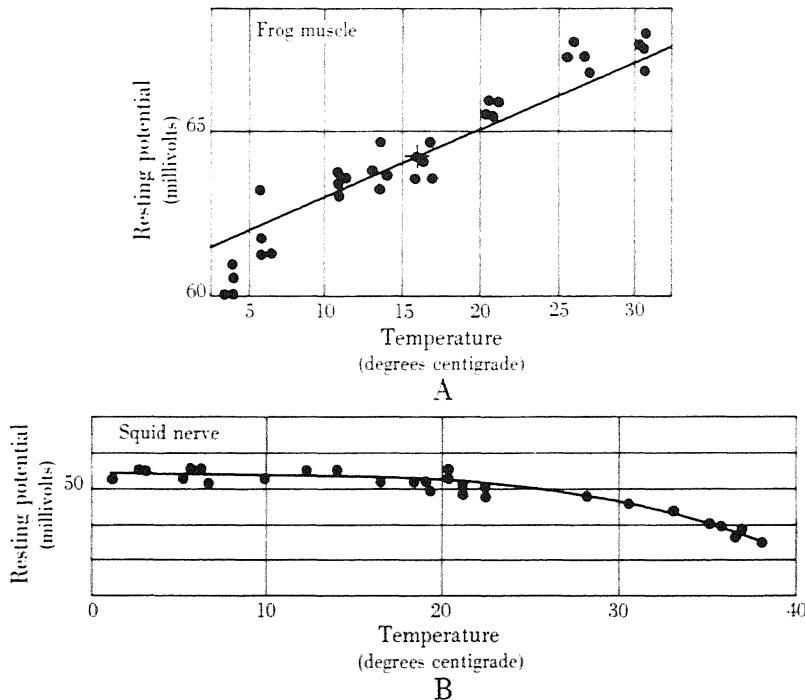


**Figure 10.1. THE RELATION BETWEEN THE EXTERNAL K<sup>+</sup>-ION CONCENTRATION AND THE RESTING POTENTIAL IN A VARIETY OF CELLS.** In each case the potential in millivolts is plotted on the ordinate and the concentration of external K<sup>+</sup> ion is plotted on the abscissa. All external K<sup>+</sup>-ion concentrations are represented as millimoles per liter of solution except that in part A, the concentration is given as a multiple of the concentration of a standard solution (13mM). A, squid nerve axon (Curtis and Cole, 1942); B, frog *sartorius* muscle (Ling and Gerard, 1950); C, frog myelinated nerve (A. F. Huxley and Stämpfli, 1951); D, insect muscle (D. W. Wood, 1957); E, guinea-pig smooth muscle (Holman, 1958); F, toad eggs (Maéno, 1959).

Heppel, 1940; Steinbach, 1951). Consequently, equation (10-1) had to take the form

$$\psi = \frac{RT}{\bar{f}} \ln \frac{[K]_{in} + [Na]_{in}}{[K]_{ex} + [Na]_{ex}}. \quad (10-3)$$

However, since the sum,  $[K]_{in} + [Na]_{in}$ , is approximately equal to the sum,

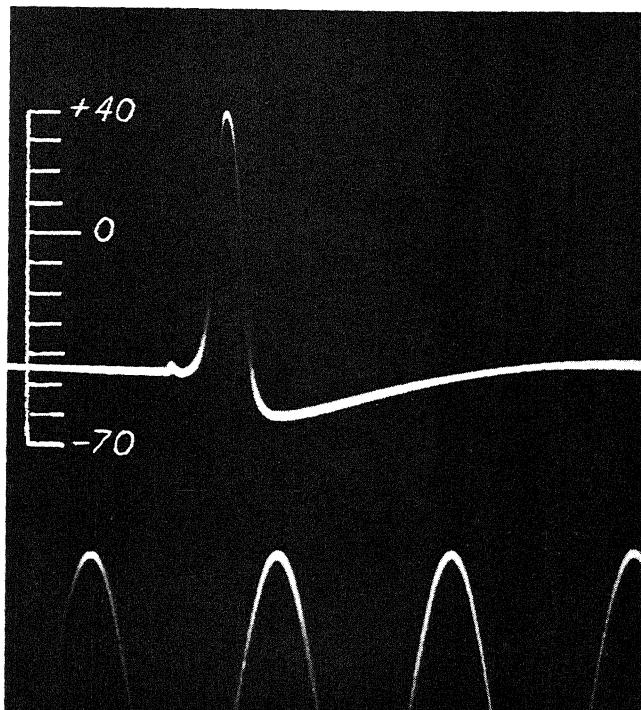


**Figure 10.2. THE EFFECT OF TEMPERATURE ON THE MAGNITUDE OF THE RESTING POTENTIAL.** Data from A, frog voluntary muscle fibers (Ling and Woodbury, 1949), and B, squid axon (redrawn from Hodgkin and Katz, 1949a) are presented. At temperatures above 35°C, the resting potential of frog muscles declines irreversibly (see Ling and Woodbury, 1949). In A, the point through which a cross is drawn indicates the mean value of all points. The line is theoretical for the potential, proportional to absolute temperature.

$[K]_{ex} + [Na]_{ex}$  (Table 9.2), equation (10-3) predicts no potential whatever. But sizable potential differences do exist. The problem was approached by Hodgkin and Katz (1949b) who adopted the treatment of Goldman (1944) and wrote the equation

$$\psi = \frac{RT}{\bar{f}} \ln \frac{P_K[K]_{in} + P_{Na}[Na]_{in} + P_{Cl}[Cl]_{in}}{P_K[K]_{ex} + P_{Na}[Na]_{ex} + P_{Cl}[Cl]_{in}} \quad (10-4)$$

where  $P_K$ ,  $P_{Na}$ , and  $P_{Cl}$  represent permeability constants. If, in the resting state  $P_{Na} \ll P_K$ , then the problem of the effect of  $Na^+$ -ion permeability on the magnitude of the resting potential is resolved. Even more important, this equation offers an explanation for the important discovery of Hodgkin and Katz (1949b;



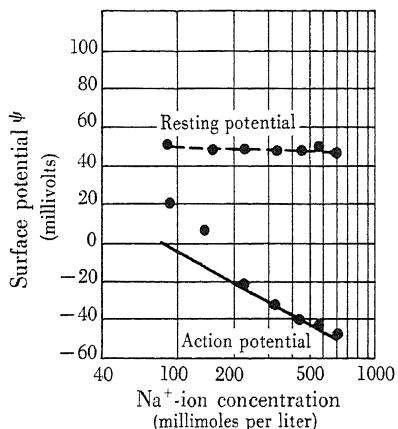
**Figure 10.3.** ACTION POTENTIAL RECORDED BETWEEN THE INSIDE AND OUTSIDE OF A SQUID NERVE AXON. The scale indicates the electrostatic potential difference between the inside of the axon and the external sea water which is taken as zero potential. The oscillographic recording shows an initial resting potential of -44 millivolts and an action spike 84 millivolts in height, at which peak the internal microelectrode becomes positive with respect to the sea water. (Figure from Hodgkin and Huxley, 1945.)

see also Hodgkin, 1951) that the external  $Na^+$ -ion concentration  $[Na]_{ex}$  is logarithmically related to the magnitude of the overshoot\* of the action potential as shown in Figure 10.3 (see also Overton, 1904). This type of relation has since been observed in many living systems (see Figure 10.4). However, the concept on which equation (10-4) is based does not directly suggest a mechanism for maintenance of the low intracellular  $Na^+$ -ion concentration found in resting

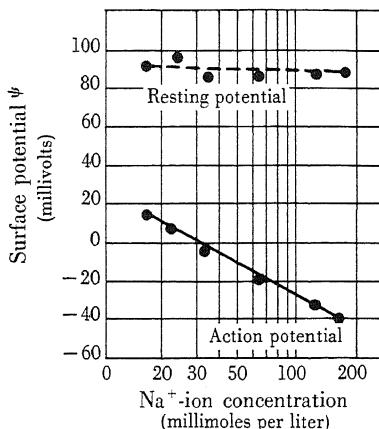
\* Curtis and Cole (1942) first discovered that, at the height of the action potential, the potential reverses its polarity.

cells. This function has thus been delegated to a hypothetical sodium "pump" energized by metabolic reactions (see Hodgkin, 1951). Experimental evidence incompatible with the pump concept, some of which was presented in Chapter 8, led the present author to question the validity of this concept and eventually to reject the sodium pump and develop the earlier version of the association-induction hypothesis (Ling, 1951, 1952, 1955a).

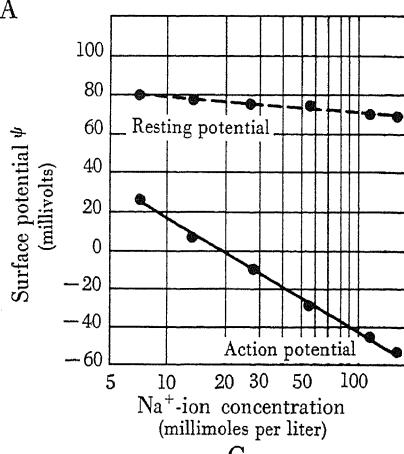
The second major difficulty arose from discrepancies between the actual and predicted relationships of the potential to the internal  $K^+$ -ion concentration  $[K]_{in}$  in equation (10-2). As the examples quoted below illustrate, recent experimental evidence showing a lack of correlation between  $\psi$  and  $[K]_{in}$  has been collecting as rapidly and consistently as evidence proving the existence of an inverse correlation between  $\psi$  and  $[K]_{ex}$ .



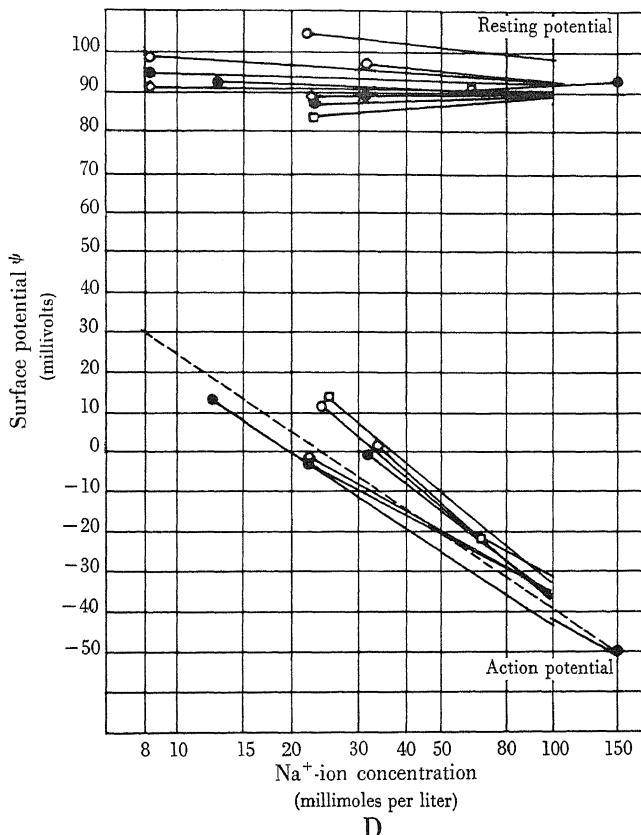
A



B



C

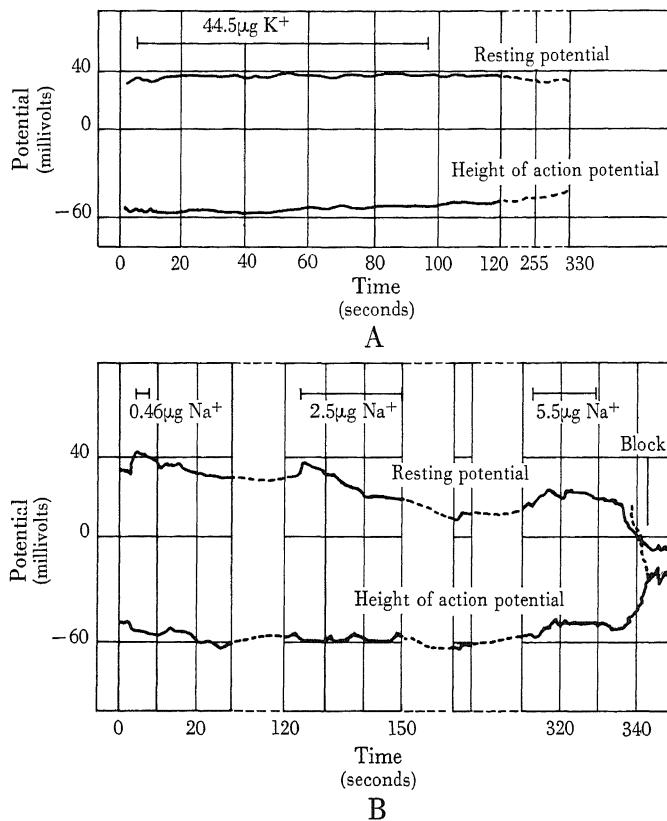


**Figure 10.4. RELATION BETWEEN SODIUM CONCENTRATION IN EXTERNAL SOLUTION AND POTENTIAL OF RESTING AND ACTIVE CELLS.** A, giant squid axon (Hodgkin and Katz, 1949b); B, frog *sartorius* muscle (Nastuk and Hodgkin, 1950); C, frog myelinated nerve (A. F. Huxley and Stämpfli, 1951); D, Purkinje fibers of the kid heart (circles) and false tendon of dog heart (squares) (Draper and Weidmann, 1951). The abscissa shows the  $\text{Na}^+$ -ion concentration on a logarithmic scale. The ordinate gives the magnitude of the potentials which are regarded as positive if the extracellular phase is positive with respect to the intracellular phase. Values of action potentials refer to their peak values. Parts A, B, and C were redrawn from figures reproduced by Hodgkin (1951); Part D was redrawn from Draper and Weidmann (1951).

(1) According to the membrane-pump concept, injection of sufficient 2*M* potassium aspartate or glutamate\* to cause a fivefold increase of intracellular  $\text{K}^+$ -ion concentration should increase the resting potential by about 53mv [equation (10-4)]. Using the values of  $P_{\text{K}}$ ,  $P_{\text{Na}}$ , and  $P_{\text{Cl}}$  prescribed by the membrane-pump hypothesis, a massive injection of 2*M* KCl should lead to a decrease of  $\psi$

\* In neurons, free ions carrying net negative charges are often found in the form of dicarboxylic amino acids. Thus mammalian brain and retina accumulate glutamate ion (see Table 9.9) and squid axon accumulates both aspartate and glutamate ions (see Table 1 of Deffner, 1961). According to the membrane-pump concept, these must be considered the "impermeant" anions of the Donnan system.

by 38mv.\* Figure 10.5 shows that neither expectation is confirmed by the experimental evidence (Grundfest *et al.*, 1954). Similarly, there is no change of the resting potential following the microinjection of concentrated KCl, NaCl, or sodium glutamate into muscle fibers (Falk and Gerard, 1954).



**Figure 10.5. THE EFFECT OF POTASSIUM AND SODIUM SALTS INJECTED INTO GIANT SQUID AXONS.** A, 1.3M potassium aspartate in 0.2M KCl was injected over a period of 100 seconds. The total amount of K<sup>+</sup> ion injected, 44.5 $\mu\text{g}$  leads to negligible change in the resting potential (27.5°C). B, 2.0M NaCl was injected over periods of 2 to 20 seconds. The total amount of Na<sup>+</sup> ion injected each time is indicated. No significant change in the height of action potential was observed (25.5°C). (Figure after Grundfest *et al.*, 1954.)

(2) Koketsu and Kimura (1960) found that soaking frog *sartorius* muscles in 0.112M sucrose solutions for various lengths of time produced a large drop

\* Because of the rapidity of ionic diffusion over short distances in free solution, according to the membrane concept, injected salt solutions should equilibrate rapidly and the predicted changes of  $\psi$  should be completed in a few seconds.

(up to 80 per cent) in the total intracellular K<sup>+</sup>-ion content with very little change of the resting potential of the surface muscle fibers when measured after three minutes of soaking in a normal Ringer's solution that contained the usual K<sup>+</sup>-ion concentration. Mrs. Margaret Neville, in this laboratory, has confirmed the results quoted in Table 10.1. A similar lack of correlation between [K]<sub>in</sub> and

Experiment No.	Soaking solution	Soaking		Wet weight, mg		Intracellular K content, mM/l	Resting potential, mv
		hr	temp	initial	final		
36	Sucrose	16	22	96	98	11.3	84.1 ± 1.4
36	Sucrose	16	22	79	87	15.9	87.0 ± 1.1
36	Sucrose	16	22	70	77	15.2	87.9 ± 0.8
37	Sucrose	16	22	73	81	27.4	88.6 ± 1.3
37	Sucrose	16	22	93	100	23.4	84.6 ± 1.5
37	Sucrose	16	22	92	101	17.6	91.0 ± 1.3
51	Sucrose	16	10	218	306	45.7	87.0 ± 1.5
51	Sucrose	16	10	102	152	47.8	85.0 ± 1.9
51	Sucrose	16	10	166	245	60.7	87.0 ± 1.6
52	Sucrose	24	10	144	190	23.9	85.6 ± 1.2
52	Sucrose	24	10	113	149	36.2	98.6 ± 1.8
52	Sucrose	24	10	104	120	52.6	95.5 ± 1.0
54	Sucrose	40	10	112	137	21.3	85.0 ± 1.9
54	Sucrose	40	10	83	96	15.5	85.9 ± 0.6
54	Sucrose	40	10	113	141	25.3	88.7 ± 1.6
36	Ringer	16	22	73	73	117.0	92.5 ± 0.8
37	Ringer	16	22	78	75	113.2	96.5 ± 0.5
51	Ringer	16	10	107	110	123.3	96.0 ± 0.7
52	Ringer	24	10	155	149	122.0	95.7 ± 0.6
54	Ringer	40	10	92	86	122.6	92.5 ± 0.7

**Table 10.1. THE RESTING POTENTIAL AND THE INTRACELLULAR K<sup>+</sup>-ION CONCENTRATION IN FROG MUSCLE CELLS TREATED WITH 0.112M SUCROSE SOLUTIONS.** After soaking for various lengths of time, frog *sartorius* muscles were immersed in a normal Ringer's solution and equilibrated for three minutes before the resting potential was measured on 10 single fibers chosen at random. The muscles were then analyzed for their gross intracellular K<sup>+</sup>-ion content. The results are expressed as millimoles of K<sup>+</sup> ion per liter of intracellular water; the quantity of intracellular water was calculated as the difference between the final wet weight and 21 per cent of the initial wet weight. (Koketsu and Kimura, 1960.)

$\psi$  in frog muscle was earlier observed by Tobias (1950), and by Shaw and co-workers (1955, 1956). The latter authors, in addition, found no correlation between [Na]<sub>in</sub> and the magnitude of the overshoot.

There is a third difficulty with the membrane theory of cellular potentials. Conceptually, the sodium theory marks an important advance in this field for it deals with the role of the Na<sup>+</sup> ion which, until the work of Hodgkin and Katz,

usually had been considered unimportant or, at best, elusive. Quantitatively, however, equation (10.4) does not predict the correct relation between the theoretically predicted and the experimentally measured resting potentials. Thus, the data of Cole and Curtis on the variation of the squid axon potential with external cations (Figure 10.1A) can be described by equation (10.4) if  $P_K:P_{Na}:P_{Cl} = 1:0.04:0.45$  (Hodgkin, 1951). However, the lack of a lasting change in the measured potential when the  $Cl^-$  ion is substituted by various other ions indicates the minor role of this ion in determining the potential (see Section 10.4). Eliminating the chloride term, for frog muscle at  $0^\circ C$  and an external  $K^+$ -ion concentration of  $0.5mM$ , the ratio  $P_K/P_{Na}$  has to be at least as high as 40 in order to give a resting potential of  $60mv$  (see Figure 10.2 and Ling and Woodbury, 1949); results of actual measurements of the permeabilities shows that this ratio is lower than 10 (see Table 11.2).

A further difficulty is that  $P_K$ ,  $P_{Na}$ , and  $P_{Cl}$ , referred to as permeability constants, are not constants, but are essentially diffusion coefficients. Thus, each value must contain, as in all rate processes (see Glasstone *et al.*, 1941), the term,  $\exp(-\epsilon_i/RT)$ , where  $\epsilon_i$  is the activation energy of the  $i$ th ion involved. When  $P_K/P_{Na}$  is relatively low, at constant ionic concentration, the term

$$\varphi = \frac{P_K[K]_{in} + P_{Na}[Na]_{in}}{P_K[K]_{ex} + P_{Na}[Na]_{ex}}$$

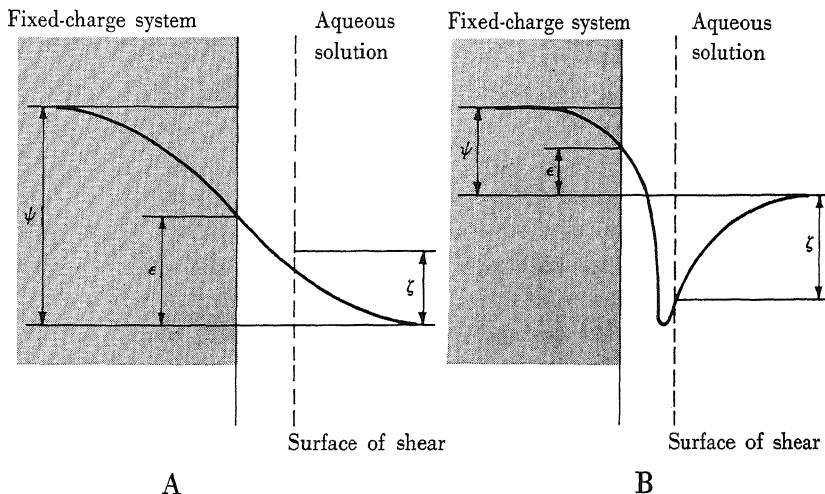
will be temperature dependent unless  $\epsilon_K = \epsilon_{Na}$ . The quantity  $\epsilon_K$  as well as the quantity  $\epsilon_{Na}$  may be derived from data given in Table 11.2 which show that  $\epsilon_{Na} \neq \epsilon_K$ . Now if  $\varphi$  is temperature dependent, the over-all potential  $\psi$  will not be proportional to the absolute temperature. Figure 10.2 clearly shows, however, that, in muscle as in nerve, the resting potential is directly proportional to the absolute temperature.

*Collectively, this evidence shows that the measured cellular potential does not directly depend on the gross internal concentration of the  $K^+$  or  $Na^+$  ion, nor on the surface permeabilities of these ions.* These results have been confirmed in other laboratories; they can be accepted as established. With the disproof of these fundamental assumptions of the membrane potential theory, a major revision of the current concept of the mechanism of cellular potentials becomes a necessity.

## 10.2. The Association-Induction Hypothesis of Cellular Potentials

Although the concept of membrane potentials has long been taught as a topic of physical chemistry, the membrane potential is not the only form of electrical potential that can arise at an interface. Its existence has, in fact, been demonstrated in only a few physical systems. The potential observed even in some of these systems is not necessarily a true membrane potential (see Section 10.4). A

far more universal type of potential, sometimes called a surface potential, usually exists at the junction of two dissimilar phases. The contact potential across an interface between two different metals is one example of such a potential (see Slater, 1939, p. 467). The potentials between a gas phase, air, and a liquid phase, an aqueous solution (see Butler, 1951, p. 68), and those measured across many



**Figure 10.6. THE POTENTIALS AT A LIQUID-SOLID INTERFACE.** We suggest that the cellular resting and action potentials correspond to the potential difference between a point far in the interior of the fixed-charge system and an infinitely distant point within the liquid phase. This potential difference is represented by  $\psi$  in the figure. The  $\epsilon$ -potential is the total potential drop within the liquid phase. The  $\zeta$ -potential, which determines the electrophoretic mobility of a solid particle, is the potential drop from the surface of shear to an infinitely distant point within the solution. The term  $\epsilon$ -potential is of historical interest only; its physical significance can, in many instances, probably be represented as  $\psi$ . A and B represent two possible potential profiles.

types of liquid-solid interfaces are examples of surface potentials. Von Helmholtz, in 1879, suggested that an electrical double layer is generally formed at the surface of separation between two different phases. Gouy (1910), Chapman (1913), and Otto Stern (1924) developed and extended this concept. At a solid-liquid interface, the double layer consists of one sharply defined layer that remains fixed to the solid phase and a second diffuse layer that extends into the solution. The total surface potential  $\psi$  between a fixed-charge system and a free solution is the potential difference between the interior of the fixed-charge system and an infinitely distant point in the solution (Figure 10.6); the fraction of this potential that represents the total potential drop within the liquid phase has generally been called the

$\epsilon$ -potential.\* The potential drop from the surface of shear  $F$  to an infinitely distant point in the solution, has been called the  $\zeta$ -potential (Freundlich, 1926) and is intimately related to electrokinetic phenomena. The  $\zeta$ -potentials of various kinds of inanimate solid particles (glass particles, for instance) and of living cells (bacteria and red blood cells suspended in water or salt solutions) have been studied extensively (Abramson, 1934). However, the first suggestion that the resting potential of living cells corresponds to the  $\psi$ -potential at the cell surface† (referred to as the thermodynamic adsorption potential) was not made until 1952 (Ling, 1952, p. 788). This hypothesis was stated more clearly in 1955 and 1959 (Ling, 1955b, 1959), and presented in detail in 1960 (Ling, 1960). It was based on the concept that the entire cell is a fixed-charge system and, as such, constitutes a phase separate from the plasma or Ringer's solution.

A macroscopic potential difference between the two phases arises whenever a fixed-charge system bounds a free solution. To understand this statement we must recall that the dissociation constants of the two chemically indistinguishable carboxyl groups of a compound like oxalic acid differ. The electrostatic field produced by the dissociation of the first proton contributes greatly to this difference; the field acts on the second proton to increase its effective dissociation energy. Such charged groups, fixed into an aggregate of macroscopic dimensions, form a fixed-charge system. The dissociation of a counterion from a fixed-charge system produces a net electrostatic field which increases the dissociation energy of a large number of counterions in its vicinity. The presence of a relatively small number of vacant fixed sites on and near the surface of the fixed-charge system (sites whose counterions are in free solution) both increases the dissociation energy of the remaining counterions and engenders a macroscopic potential difference between the fixed-charge phase and the external free solution. We have designated this potential difference by  $\psi$ . In the case of muscle and nerve cells, this potential difference is called the resting potential.

\* Nernst (1892), Haber (1908), and Michaelis (1909) worked out the relation between the  $\epsilon$ -potential and what they called the solution tensions of the anion and of the cation,  $\Pi_A$  and  $\Pi_K$ , respectively, and the osmotic pressures of the anion and of the cation,  $P_A$  and  $P_K$ :

$$\epsilon = \frac{RT}{\mathfrak{f}} \ln \frac{\Pi_K}{P_K} = - \frac{RT}{\mathfrak{f}} \ln \frac{\Pi_A}{P_A}.$$

It is interesting to note that by taking into account the gradual drop of potential in the liquid phase, the original Helmholtz double layer was transformed into the improved model of Stern. The total potential drop in Stern's model clearly corresponds to the  $\epsilon$ -potential. The  $\psi$ -potential, which we have proposed for the surface of a fixed-charge system, also takes into account the gradual drop of potential in the fixed-charge phase. However, in spite of this interesting aspect of the evolution of details, it is clear that all refer to the same surface potential.

† Interpreted in the light of what has been presented in this chapter, Loeb and Beutner, in their phase-boundary potential theory (see Section 10.1) proposed that there are two  $\psi$ -potentials, one at the phase boundary between the cell membrane and the external solution, and another one at the phase boundary between the cell membrane and the intracellular water phase. As another variation of the basic membrane concept, this theory is disproved by the facts presented in Section 10.1. In its details relating to the origins of the potentials, it does not conflict with the present thesis.

An ion within a fixed-charge system may exist as an adsorbed ion occupying a site with partition function,  $(\text{p.f.})^{\text{ads}}$ . At equilibrium, the adsorbed  $\text{K}^+$ -ion concentration  $[\text{K}]^{\text{ads}}$  is related to the external  $\text{K}^+$ -ion concentration  $[\text{K}]_{\text{ex}}$  by the equation,

$$[\text{K}]^{\text{ads}} = [\text{K}]_{\text{ex}} \frac{(\text{p.f.})_K^{\text{ads}}}{(\text{p.f.})_K^{\text{fr}}} \exp \frac{(-\Delta E_K + \mathfrak{F}\psi)}{RT}. \quad (10-5)$$

Similarly,

$$[\text{Na}]^{\text{ads}} = [\text{Na}]_{\text{ex}} \frac{(\text{p.f.})_{\text{Na}}^{\text{ads}}}{(\text{p.f.})_{\text{Na}}^{\text{fr}}} \exp \frac{(-\Delta E_{\text{Na}} + \mathfrak{F}\psi)}{RT}. \quad (10-6)$$

We have shown that, within a fixed-charge system at the appropriate  $c$ -value, most ions are adsorbed. Consequently, if we represent the concentration of fixed anionic sites on the fixed-charge surface by  $[\text{f}^-]_{\text{surf}}$  and neglect the few vacant sites mentioned above, we can make the approximation,

$$[\text{f}^-]_{\text{surf}} = [\text{K}]_{\text{surf}}^{\text{ads}} + [\text{Na}]_{\text{surf}}^{\text{ads}}. \quad (10-7)$$

By substituting equations (10-5) and (10-6) into equation (10-7), we derive the relation

$$\begin{aligned} \psi = \frac{RT}{\mathfrak{F}} \ln [\text{f}^-]_{\text{surf}} - \frac{RT}{\mathfrak{F}} \ln \left[ \frac{(\text{p.f.})_K^{\text{ads}}}{(\text{p.f.})_K^{\text{fr}}} [\text{K}]_{\text{ex}} \exp \left( \frac{-\Delta E_K}{RT} \right) \right. \\ \left. + \frac{(\text{p.f.})_{\text{Na}}^{\text{ads}}}{(\text{p.f.})_{\text{Na}}^{\text{fr}}} [\text{Na}]_{\text{ex}} \exp \left( \frac{-\Delta E_{\text{Na}}}{RT} \right) \right]. \end{aligned} \quad (10-8)$$

If we make the simplifying assumptions

$$(\text{p.f.})_K^{\text{ads}} = (\text{p.f.})_{\text{Na}}^{\text{ads}} = (\text{p.f.})^{\text{ads}} \text{ and } (\text{p.f.})_K^{\text{fr}} = (\text{p.f.})_{\text{Na}}^{\text{fr}} = (\text{p.f.})^{\text{fr}} \quad (10-9)$$

we can write equation (10-8) in the form

$$\begin{aligned} \psi = \frac{RT}{\mathfrak{F}} \ln [\text{f}^-]_{\text{surf}} - \frac{RT}{\mathfrak{F}} \ln \frac{(\text{p.f.})^{\text{ads}}}{(\text{p.f.})^{\text{fr}}} - \frac{RT}{\mathfrak{F}} \ln \left[ [\text{K}]_{\text{ex}} \exp \left( \frac{-\Delta E_K}{RT} \right) \right. \\ \left. + [\text{Na}]_{\text{ex}} \exp \left( \frac{-\Delta E_{\text{Na}}}{RT} \right) \right]. \end{aligned} \quad (10-10)$$

Recalling the linearity of the  $c$ -versus- $\Delta E$  plots shown in Figures 4.9 to 4.11, for a short range of  $c$ -value variation, the approximations

$$\Delta E_K = A_K c + B_K \quad \text{and} \quad \Delta E_{\text{Na}} = A_{\text{Na}} c + B_{\text{Na}} \quad (10-11)$$

can be made, where  $A_K$ ,  $B_K$ ,  $A_{\text{Na}}$ , and  $B_{\text{Na}}$  are constants. Substitution into equation (10-10) yields:

$$\begin{aligned} \psi = \frac{RT}{\mathfrak{F}} \ln [\text{f}^-]_{\text{surf}} - \frac{RT}{\mathfrak{F}} \ln \frac{(\text{p.f.})^{\text{ads}}}{(\text{p.f.})^{\text{fr}}} \\ - \frac{RT}{\mathfrak{F}} \ln \left\{ [\text{K}]_{\text{ex}} \exp \left[ \frac{-(A_K c + B_K)}{RT} \right] + [\text{Na}]_{\text{ex}} \exp \left[ \frac{-(A_{\text{Na}} c + B_{\text{Na}})}{RT} \right] \right\}. \end{aligned} \quad (10-12)$$

The new equation, (10-10) or (10-12), is a counterpart of the original Nernst equation. The Nernst equation describes the potential difference between two similar ionic phases (both dilute solutions). Equation (10-12) deals with the potential difference at an interface separating any two phases. Several interesting features of the present theory as expressed in equation (10-12) should be mentioned.

(1) Since ionic selectivity is due to differential adsorption of various cations onto fixed anionic charges and since it arises under equilibrium conditions, a metabolic pump is not necessary.\*

(2) The form of our equation is identical with that of the Hodgkin-Katz-Goldman equation if the chloride term is excluded. (The justification for this exclusion will be discussed in Section 10.5.) In equations (10-10) and (10-12),  $P_K$  and  $P_{Na}$  are replaced by the equilibrium constants of adsorption of the respective ions onto the surface ionic sites of the fixed-charge system.

(3) The magnitude of the potential is related to the concentration of the surface fixed anionic charges,  $[f^-]_{surf}$  and to the nature of these charges. It is not a function of the macroscopic bulk-phase  $K^+$ -ion concentration, although, under special conditions, the surface concentration and the bulk-phase concentration can be proportional or even equal. In fact, the concept of a single, homogeneous, intracellular ion concentration is a corollary of the membrane theory. The necessity of this concept is eliminated by the association-induction hypothesis. According to the association-induction hypothesis, the density, the sign of the charge, and the  $c$ - and  $c'$ -values of fixed ionic sites can vary from one microscopic area to another, corresponding to various cytological structures within the cell. Since, however, it is only surface dissociations which give rise to resting potentials, an inhomogeneity within the cell is, in this regard, of no consequence. For this reason, the observed lack of correlation between  $\psi$  and  $[K]_{in}$ , which directly contradicts the membrane-potential theory, can be used to support the association-induction hypothesis. The differences between the surface sites and, say, the average bulk-phase fixed site will be presented in Section 10.5. Equation (10-12) can be written in the form,

$$\psi = \frac{RT}{\mathfrak{F}} \ln [f^-]_{surf} + \frac{\Delta E_K}{\mathfrak{F}} - \frac{RT}{\mathfrak{F}} \ln \frac{(p.f.)^{ads}}{(p.f.)^{fr}} - \frac{RT}{\mathfrak{F}} \ln \left\{ [K]_{ex} + [Na]_{ex} \exp \frac{(A_K - A_{Na})c + (B_K - B_{Na})}{RT} \right\}. \quad (10-13)$$

At the resting  $c$ -value of  $-2.7\text{\AA}$ , from Figure 4.9, we find,

$$[Na]_{ex} \exp \left[ \frac{(A_K - A_{Na})c + (B_K - B_{Na})}{RT} \right] \ll [K]_{ex}. \quad (10-14)$$

\* The question has frequently been raised that if the cellular ionic distribution and electrical potential represent equilibrium conditions, how is it possible to reconcile this with the repeated demonstration that a current, the demarcation current, can be shown to flow from intercellular phase to extracellular phase for long periods of time. This question is discussed at length in Appendix F. It has been shown that the current arises as an artifact of experimental design and is not due to metabolic work on the part of the cell.

Equation (10-13) is thus reduced to

$$\psi = -\frac{RT}{F} \ln \frac{(\text{p.f.})^{\text{ads}}}{(\text{p.f.})^{\text{fr}}} + \frac{\Delta E_K}{F} + \frac{RT}{F} \ln \frac{[f^-]_{\text{surf}}}{[K]_{\text{ex}}}. \quad (10-15)$$

Since almost all the surface anionic sites are now occupied by  $K^+$  ions, the equation simplifies to

$$\psi = -\frac{RT}{F} \ln \frac{(\text{p.f.})^{\text{ads}}}{(\text{p.f.})^{\text{fr}}} + \frac{\Delta E_K}{F} + \frac{RT}{F} \ln \frac{[K]_{\text{surf}}}{[K]_{\text{ex}}}. \quad (10-16)$$

In some cases, the surface fixed-charge density,  $c$ -value, and other characteristics interact so that  $[K]_{\text{surf}}$  approaches the value of  $[K]_{\text{in}}$ . Equation (10-16) then reduces to

$$\psi = \text{const} + \frac{RT}{F} \ln \frac{[K]_{\text{in}}}{[K]_{\text{ex}}} \quad (10-17)$$

which is the original Nernst equation. This accounts for the gross correlation sometimes observed between  $\psi$  and the intracellular  $K^+$ -ion concentration in cells (for instance, high potential and high  $[K]_{\text{in}}$  in living cells, low potential and low  $[K]_{\text{in}}$  in dead cells; see also Desmedt, 1953).

Let us approximate the value of  $\psi$ . We assume that the fixed anionic sites at the surface of a muscle cell have the same average density as those in the bulk phase; then  $[f^-]_{\text{surf}}$  must be about 150 mM/l (Chapter 3). At  $[K]_{\text{ex}} = 2.5$  mM/l,  $(RT/F) \ln ([f^-]_{\text{surf}}/[K]_{\text{ex}})$  must be approximately 100mv, or 2.5 kcal/mole. From Figure 4.9 and the considerations in Section 4.4A,  $\Delta E_K$  at a  $c$ -value of  $-2.7\text{\AA}$  should be approximately  $-6$  kcal/mole. Since  $\psi$  as actually measured is some 100mv or 2.5 kcal/mole,  $RT \ln [(\text{p.f.})^{\text{ads}}/(\text{p.f.})^{\text{fr}}]$  approximates  $-6.0$  kcal/mole, which gives  $(\text{p.f.})^{\text{fr}}/(\text{p.f.})^{\text{ads}} = 2.2 \times 10^4$ , a reasonable figure.

Since the potential is determined only by microscopic events at a surface, we may expect any new equilibrium potentials to be reached instantaneously (see below). Thus, if we bring about an abrupt  $c$ -value shift of the surface sites by an indirect  $F$ -process, the new potential may again be represented by equation (10-12). Let us say that a  $c$ -value shift leads to a new average value of  $-2.1\text{\AA}$ . We write equation (10-12) in the form,

$$\begin{aligned} \psi &= \frac{RT}{F} \ln [f^-]_{\text{surf}} + \frac{\Delta E_{Na}}{F} - \frac{RT}{F} \ln \frac{(\text{p.f.})^{\text{ads}}}{(\text{p.f.})^{\text{fr}}} \\ &\quad - \frac{RT}{F} \ln \left\{ [Na]_{\text{ex}} + [K]_{\text{ex}} \exp \frac{(A_{Na} - A_K)c + (B_{Na} - B_K)}{RT} \right\}. \end{aligned} \quad (10-18)$$

At this new  $c$ -value,

$$[Na]_{\text{ex}} \gg [K]_{\text{ex}} \exp \frac{(A_{Na} - A_K)c + (B_{Na} - B_K)}{RT}. \quad (10-19)$$

Thus, we arrive at the equation,

$$\psi = -\frac{RT}{F} \ln \frac{(\text{p.f.})^{\text{ads}}}{(\text{p.f.})^{\text{fr}}} + \frac{\Delta E_{Na}}{F} + \frac{RT}{F} \ln \frac{[f^-]_{\text{surf}}}{[Na]_{\text{ex}}}. \quad (10-20)$$

In frog Ringer's solution,  $[Na]_{surf}$  is about 100mM/l;  $[f^-]_{surf}$  is 150 mM/l;  $(RT/3) \ln ([f^-]_{surf}/[Na]_{ex})$  is then 10mv (0.2 kcal/mole). We have already calculated  $RT \ln [(p.f.)^{ads}/(p.f.)^{fr}]$  to be -6.0 kcal/mole. At  $c = -2.1\text{\AA}$ ,  $\Delta E_{Na}$  should be about -8 kcal/mole. Substituting into equation (10-20) one obtains  $\psi = -90\text{mv}$ . A small shift of  $c$ -value will thus create a reversed potential of nearly 100mv with the intracellular phase positive. This is known as the "overshoot."

In the above model, the alternative metastable states are such that in one, the resting state, the fixed anions have a  $c$ -value such that they prefer the  $K^+$  ion exclusively and in the other, the active state, the fixed anions have a  $c$ -value such that they prefer the  $Na^+$  ion exclusively. While this model is adequate to describe the electrical behavior of certain tissue cells (frog *sartorius* muscle fibers and squid axons, for example) the present theory in no way prescribes that the various metastable states available to other types of cells must be identical with those above. Indeed, flexibility of the  $c$ -value in both the resting and the active state in various types of cells must be anticipated. Thus, among the ions considered above, the preference for the  $Na^+$  ion by the surface of the activated cell is overwhelmingly large when compared with the preference for the  $K^+$  ion. However, we expect ions with attributes similar to those of the  $Na^+$  ion to be capable of producing similar action potentials when substituted for the  $Na^+$  ion in the external solution. For this reason, it is entirely reasonable that frog nerve remains excitable and capable of producing an action potential when all the external  $Na^+$  ions have been replaced by quaternary ammonium ions and onium ions (Lorente de Nó, 1949, 1957; for similar observations on other tissues, see Koketsu and Nishi, 1959). Other observations explicable in terms of the present theory are the lack of a large  $Na^+$ -ion concentration gradient across the surfaces of the muscles of herbivorous insects and the apparent dependence of the action potentials of these muscles, not so much on the concentration of  $Na^+$  ion in the hemolymph as on a high magnesium-ion concentration:  $[K]_{ex} = 18\text{mM}$ ;  $[Na]_{ex} = 15\text{mM}$ ;  $[Ca]_{ex} = 7.5\text{mM}$ ;  $[Mg]_{ex} = 53\text{mM}$ , in the stick insect (D. W. Wood, 1957).

### 10.3. Experimental Evidence in Support of the Present Model of Cellular Potentials

We have shown how the fixed-charge system can possess a surface potential and yet retain most of its accumulated countercations within the bulk phase as adsorbed ions. Now we discuss two physical systems that have long been used to demonstrate membrane potentials as given by the Nernst equation (for earlier abstracts, see Ling, 1955b, 1959).

The glass membrane of the glass pH electrode has been considered selectively permeable to the  $H^+$  ion and impermeable to other ions such as  $K^+$  in the solu-

tion. As apparent confirmation, the potential follows the equation,

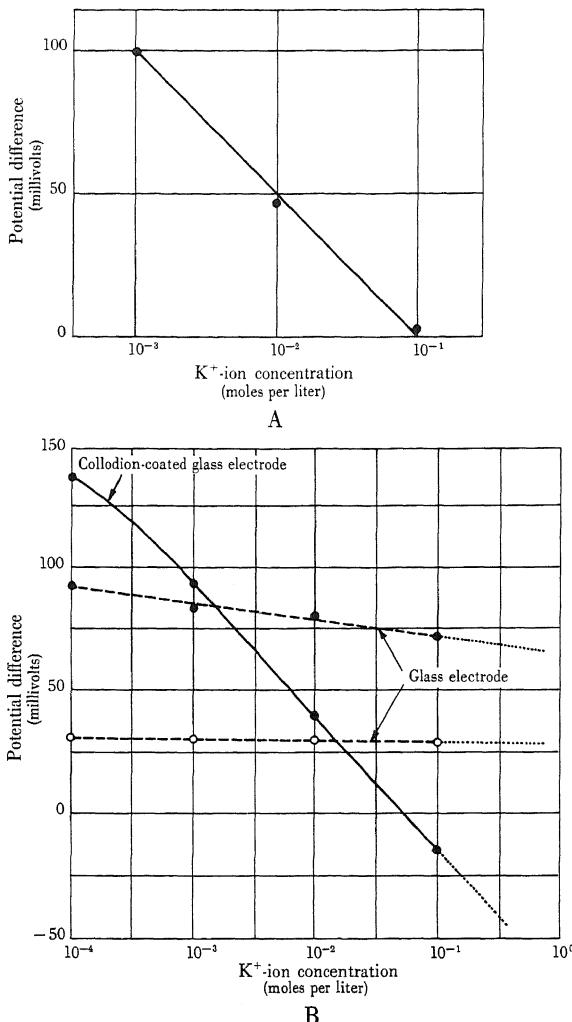
$$\psi = \frac{RT}{\sigma} \ln \frac{a_{in}^H}{a_{out}^H} \quad (10-21)$$

where  $a_{in}^H$  refers to the  $H^+$ -ion activity inside the glass bulb, usually that of 0.1*N* HCl, and  $a_{out}^H$  is the activity of the  $H^+$  ion in the outside solution. This is the conventional view, but not all investigators in this field agree on this concept. Horovitz (1923) and Haugaard (1941), for example (see also Freundlich, 1926, Chapter 5), have offered convincing evidence that the potential measured above is really an algebraic sum of two surface potentials. An equally functional  $H^+$ -ion-measuring glass electrode is obtained even when the inside of the bulb is filled, not with 0.1*N* HCl, but with mercury, or simply coated with a layer of metal (M. R. Thompson, 1932; Hubbard and Rynders, 1948). Here there is no membrane equilibrium.

A second long-accepted model of the living cell membrane is dried collodion membrane. Michaelis and co-workers demonstrated that such a membrane is capable of giving a nearly ideal potential when it separates two aqueous solutions containing different  $K^+$ -ion concentrations (Figure 10.7A.). In other experiments Michaelis was able to demonstrate selective permeability for ions such as  $K^+$  and  $Na^+$  through dried collodion membranes. These experiments provided the foundation for the membrane-potential theory (Michaelis and Fujita, 1925; Michaelis and Perlzweig, 1927).

Our knowledge of the glass electrode may lead one to wonder whether the potential measured in the collodion membrane simply represents the algebraic sum of two surface potentials. If this were so, selective ionic permeability would be related to the potential, but not—as the membrane theory suggests—the cause of it. To test the hypothesis, a soft glass bulb normally sensitive to the  $H^+$  ion but not to the  $K^+$  ion was coated with a 3 $\mu$  layer of dried collodion, and the potential measured against varying external  $K^+$ -ion concentrations. The results (Figure 10.7B) show that, within the range of  $K^+$ -ion concentrations 1m*M* to 100m*M*, a potential change of 54mv can be measured for each tenfold change of [K]; this change is identical with the change measured on a simple dried collodion-membrane electrode (Figure 10.7A.).

As mentioned earlier, the glass electrode is totally insensitive to the  $K^+$  ion. According to the membrane theory, this proves the impermeability of the glass bulb to the  $K^+$  ion. Similarly, in terms of the membrane theory, one must interpret the demonstrated  $K^+$ -ion sensitivity of dried or wet collodion as a consequence of high  $K^+$ -ion permeability. In the combination membrane used, the glass component is a great deal thicker than the collodion component; thus the over-all permeability to the  $K^+$  ion must also be nil. Yet the  $K^+$ -ion sensitivity demonstrated by this combination membrane is equal to that demonstrated by the simple collodion membrane. It has long been known that pure collodion does



**Figure 10.7. THE EFFECT OF VARIATION OF K<sup>+</sup>-ION CONCENTRATION ON ELECTRODE POTENTIAL.** A, the effect of varying the external K<sup>+</sup>-ion concentration on the potential across a simple dried collodion membrane separating one aqueous phase containing a KCl solution of constant concentration and another phase in which the K<sup>+</sup>-ion concentration is varied. (Michaelis and Fujita, 1925.) B, the potential differences between the inside and outside of a soft glass bulb were measured when the solution either inside the bulb (broken line, empty circles), or outside the bulb (broken line, solid circles) contained a varying K<sup>+</sup>-ion concentration; the other phase contained 0.1*N* HCl. Both curves show an essential insensitivity to variation of the K<sup>+</sup>-ion concentration. The solid line represents the response of the bulb after it had received a thin coating (3μ) of dried collodion. The K<sup>+</sup>-ion sensitivity is identical with that of the pure collodion membrane electrode, A. Note that the convention adopted for the sign of glass electrode potentials is opposite to that adopted for living-cell potentials. In these and subsequent data on glass electrodes, the living-cell potential convention is used. (Ling and Kushnir, see Ling, 1960.)

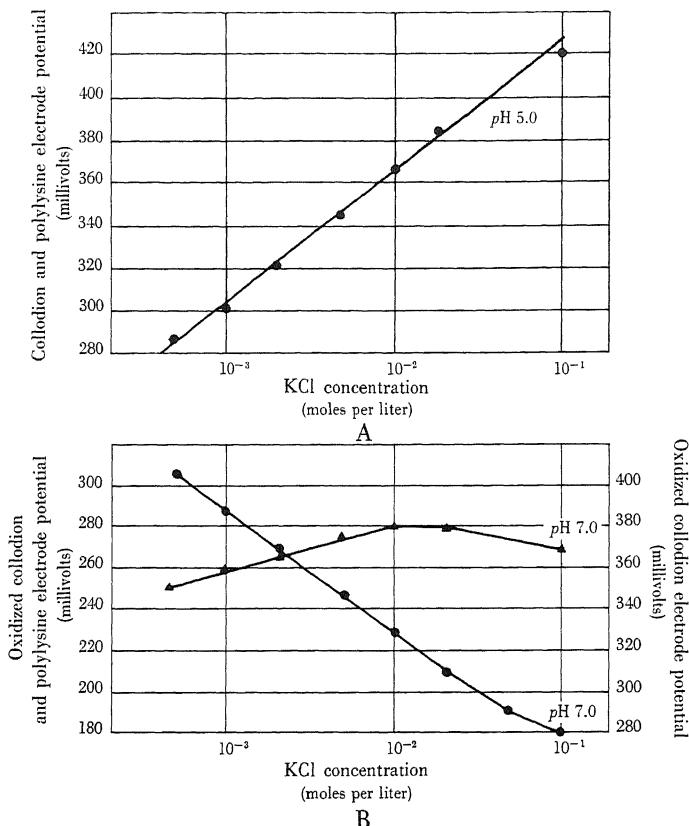
not engender potentials and that it develops electrode behavior only after it has acquired fixed carboxyl groups as a result of incidental or deliberate oxidation (Sollner *et al.*, 1941a,b). The conclusion is clear: The potential is determined only by the nature and density of the fixed ions on and near the surface of the electrode;\* it bears only an indirect relation to the gross permeability of ions to the membrane separating two solutions.

Another series of experiments, recently performed by L. D. Kushnir and the author, offers further support for the present theory of electrode potentials. When a glass electrode coated with wet collodion is soaked in a dilute solution of poly-L-lysine for about 30 minutes, the positively charged molecules of this peptide should be expected to permeate the collodion coating to a small depth. If the electrode is then dried, the poly-L-lysine molecules should become more or less permanently fixed in their positions. Such an electrode must possess both some fixed anions (the sparse carboxyl groups of the original collodion) and some fixed cations (the  $\epsilon$ -amino groups of the now fixed poly-L-lysine). Consider a similar electrode which, prior to treatment with poly-L-lysine, has been oxidized to increase the number of fixed carboxyl groups (Sollner *et al.*, 1941a,b). Such an amphoteric surface-fixed-charge system should behave as if it were a mixed cation-anion-sensitive electrode at *pH* values where both the carboxyl and  $\epsilon$ -amino groups exist as charged ions. At a lower *pH*, after the carboxyl group has become deionized leaving the  $\epsilon$ -amino groups as the only fixed ions, the electrode should behave as a typical anion electrode. All of these anticipated results have been verified. In KCl solution at *pH* 7 or higher, a poly-L-lysine-treated, oxidized, collodion-coated glass electrode responds to both cations and anions. In KCl solutions at *pH* 5, on the other hand, the electrode behaves as if it were an ideal anion electrode (see Figure 10.8). An unoxidized poly-L-lysine-treated electrode has fewer fixed carboxyl groups; in accordance with expectation, it behaves as a more nearly ideal anion electrode.

Since our conclusions about surface potentials are directly applicable only to the models we have discussed, we shall next present an experiment using the living cell itself. The results of this experiment are also incompatible with the membrane theory of cellular potential and they strongly support the explanation offered by the present hypothesis.

According to the association-induction hypothesis, the resting potential of a frog muscle fiber, like that of other cells, is an expression of an equilibrium state

\* It is legitimate to ask whether a potential arising from a diffusion front of, say,  $K^+$  and  $Cl^-$  ions *within* the collodion or glass phase contributes significantly to the measured potential. Were this the case, all *pH* measurements made with glass electrodes would be fallacious, for the readings taken would reflect significantly the acidity of the solution to which the glass electrode had *previously* been exposed in addition to the acidity of the solution whose *pH* was presently being measured. Since this clearly does not occur, we must conclude that such a diffusion front, if it does give rise to a potential within the glass phase, is of trivial importance (see also pages 265 and 266).

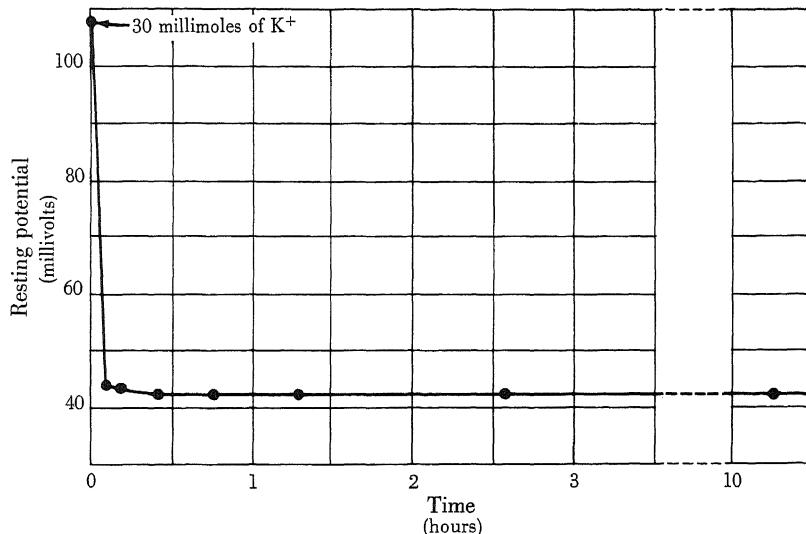


**Figure 10.8. ANION SENSITIVITY OF POLY-L-LYSINE-TREATED, COLLODION-COATED GLASS ELECTRODE.** Three glass electrodes were coated with a thin layer of collodion and treated as follows: A, a nonoxidized, wet electrode was soaked for three hours in an aqueous solution containing 3 mg/ml poly-L-lysine (Mann Biochemicals); B, two electrodes were oxidized by dipping in a 1*N* NaOH solution for 10 minutes; the electrode represented by circles was washed in distilled water; the electrode represented by triangles was washed and treated as for A. All electrodes were dried in a humidity chamber. The KCl solution at pH 5 was not buffered; that at pH 7 was buffered with HCl and 0.005*M* tris buffer. These electrodes have considerable stability. Kept in distilled water they show the behavior illustrated for several weeks. In these figures, the potential is considered positive if the outside solution is positive with respect to the collodion phase. The unoxidized collodion, poly-L-lysine-treated electrode shows ideal anion sensitivity; the oxidized collodion electrode shows ideal K<sup>+</sup>-ion sensitivity; and the oxidized collodion, poly-L-lysine-coated electrode shows mixed sensitivity. For sign of potential, see legend of Figure 10.7. (Ling and Kushnir, unpublished.)

existing between the K<sup>+</sup> ions in the external solution and the K<sup>+</sup> ions adsorbed on the fixed anions in a superficial surface layer of the cell. Thus a variation in the external K<sup>+</sup>-ion concentration should lead to a nearly instantaneous readjustment of the equilibrium at this surface. Concurrently, there should be an almost

instantaneous change to a new, stable, and unvarying resting potential. Thus, the time course of change of the resting potential is expected to be independent of the change of gross intracellular  $K^+$ -ion concentration while the membrane theory demands that they run roughly parallel courses.

Figure 10.9 shows the results of an experiment in which a frog *sartorius* muscle, resting in a Ringer's solution containing 2.5 millimoles of  $K^+$  ion per liter is suddenly plunged into a Ringer's solution containing 30 millimoles of  $K^+$  ion per liter and a normal amount of  $Na^+$  ion ( $pH\ 7.4$ ). Within the time required



**Figure 10.9. THE EFFECT OF INCREASED EXTERNAL  $K^+$ -ION CONCENTRATION ON THE RESTING POTENTIAL OF THE FROG *SARTORIUS* MUSCLE** (Room temperature). Ringer's solution containing 30 millimoles of  $K^+$  ion per liter was added at zero time and the potential followed with a microelectrode. (Ling, 1960.)

to make the first measurement, the resting potential dropped to a new equilibrium value from which there was no significant deviation during the ensuing 10 hours. However, it is well established that in such a high  $K^+$ -ion concentration, the  $[K]_{in}$  of frog *sartorius* muscle will steadily rise, approaching an equilibrium in six to 10 hours (room temperature) with a half time of equilibration of one-half hour to two hours (Fenn and Cobb, 1934; E. J. Conway, 1946; E. J. Harris, 1952). Equations (10-2) and (10-4) predict a gradual and continual rise of the measured potential within this period of time until the new equilibrium  $[K]_{in}$  is reached. That the total potential change is achieved immediately and that, once established it does not change further, demonstrate the inadequacy of the membrane theory. The same experiment eliminates the possibility of a sizable diffusion potential at the diffusion front of the inward-migrating excess  $K^+$  ion; if such

a diffusion potential had existed, it would have died out after about 10 hours when the new equilibrium,  $[K]_{in}$ , was reached (see also pages 265 and 266). Thus, as predicted by equation (10-12), the surface\* of the living cell is the only site at which the potential can possibly be generated.

#### 10.4. The Surface Ionic Sites of Living Cells

An important assumption underlying the development of equation (10-12) is that the surface sites of muscle and nerve cells are predominantly, if not exclusively, anionic.<sup>†</sup> We have made this assumption in spite of the evidence presented in other chapters that the bulk-phase fixed-charge system is essentially amphoteric. Let us now examine experimental evidence which justifies this assumption.

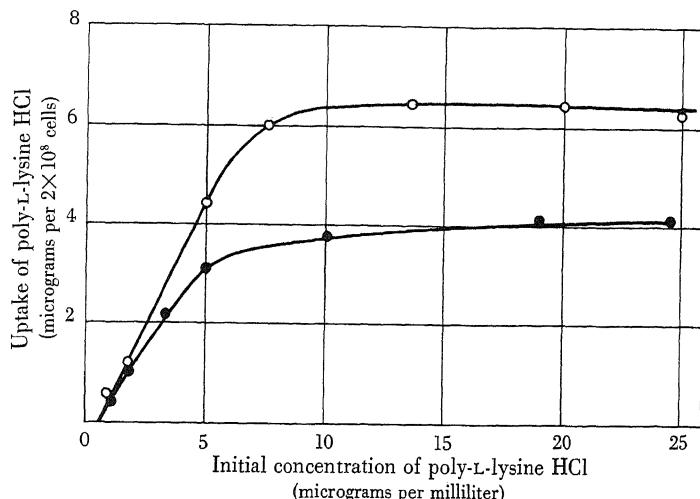
Katchalski and co-workers (1953) studied the action of various polyamino acids on bacteria. Neutral polyamino acids, anionic polyamino<sup>‡</sup> acids, and monomeric lysine had no effect on bacterial growth. Yet all cationic polyamino acids, whatever the exact nature of the amino-acid monomers, inhibited bacterial growth. Cationic polyamino acids like poly-L-lysine were actually adsorbed from the medium by the bacterial cell (Figure 10.10). This adsorption leads to a change of the electrophoretic mobility of the cells so that they tend to move in the direction opposite (toward the cathode) to their usual direction of motion in an electric field. This effect, which cannot be reversed by monomeric dicarboxylic amino acids, can be quantitatively reversed by the addition of anionic polyamino acids, like polyaspartic acid (Figure 10.11)<sup>§</sup> and is strongly influenced by the ionic strength of the solution. Table 10.2 presents similar results obtained from experiments with red blood cells; cationic polyamino acids have been found to agglutinate and hemolyze these cells while neutral or anionic polyamino acids have no

\* In measuring muscle-cell resting potentials it has been found that, with the best electrodes, the maximum potential is measured as soon as the electrode tip touches the cell surface. There has been no evidence that a potential gradient can be measured as the microelectrode proceeds toward the center of the fiber. Since many of the electrode tips have diameters of about 1000 Å (see Figure 4 of J. T. Alexander and Nastuk, 1953, and Figure 1 of Ottoson and Svaetichin, 1952), the inability to measure any gradient adds weight to the conclusion that the potential gradient is very sharp and confined to a shallow surface depth (see Figure 10.6). For this reason the *c*-value of the anionic surface sites must determine the resting and action potentials.

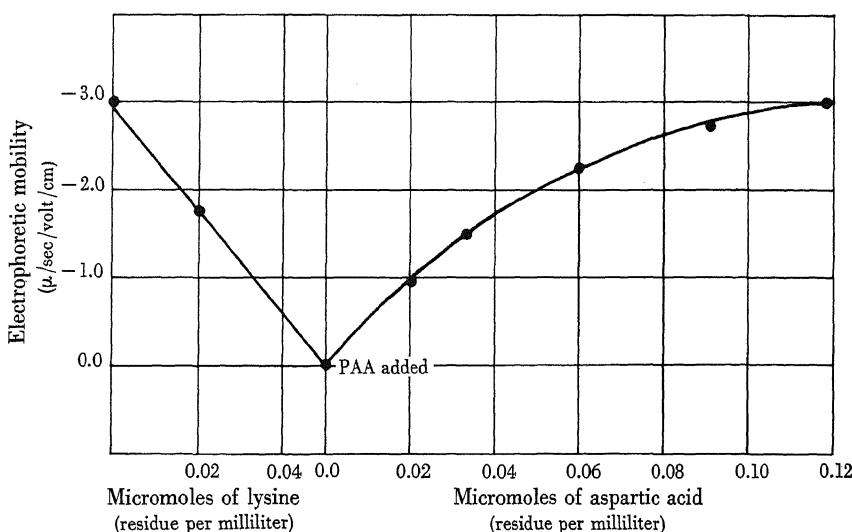
† This is not to say that the surfaces of all cells must be predominantly anionic; although this is seemingly the case for red blood cells and bacteria as well as muscle and nerve cells, there is evidence that the surface potentials of activated eggs show a sensitivity to free anions in the external solution (Maéno, 1959), indicating a predominantly cationic surface.

‡ The neutral polyamino acid tested was poly-DL-alanine. Anionic polyamino acids tested included poly-D-glutamic acid and poly-L-aspartic acid. The cationic polyamino acids studied were poly-L-lysine, poly-DL-lysine, poly-DL-ornithine, and poly-DL-arginine.

§ This fact alone offers considerable support for the interpretation of the increased degree of association of polypeptides as semifixed-charge systems expounded in Section 7.3A. The same interpretation, based on entropic considerations, can be applied to many other polypeptides which are physiologically active at very low concentrations (see, for instance, Gaddum, 1955).



**Figure 10.10.** UPTAKE OF POLY-L-LYSINE BY BACTERIA. Adsorption experiments were carried out with  $2 \times 10^8$  cells per milliliter in  $0.001M$  phosphate buffer, pH 7.0 at  $20^\circ\text{C}$ . Open circles indicate uptake by the gram-negative bacteria *Escherichia coli*; solid circles indicate uptake by the gram-positive bacteria *Staphylococcus aureus*. The leveling off of the curve indicates a typical adsorption phenomenon. (After Katchalski *et al.*, 1953.)



**Figure 10.11.** RESTORATION OF ELECTROPHORETIC MOBILITY OF *ESCHERICHIA COLI*. The electrophoretic mobility of the bacteria could be decreased (or made to reverse its sign) quantitatively by an increasing concentration of polylysine. The normal mobility can be restored by addition of equivalent or larger amounts of polyaspartic acid (PAA). The experiment was performed in  $0.001M$  phosphate buffer at pH 7.0. (Figure after Katchalski *et al.*, 1953.)

effect (Nevo *et al.*, 1955; Katchalsky *et al.*, 1959). These studies led Nevo and his co-workers to the conclusion that polylysine is adsorbed only on the cell surface of red blood cells.

Preliminary experiments by Mrs. Margaret Neville in this laboratory suggest that polyamino acids have similar effects on muscle cells. Thus, poly-L-aspartic

	Degree of polymerization, D.P.	Minimal concentration, $\mu\text{g}/\text{ml}$
<b>Polybases</b>		
Polylysine hydrobromide	30-36	1-2
Polyornithine hydrochloride	27	1-2
Polyarginine sulfate	27	1-2
Polyvinyl amine hydrobromide	100	1
Polyvinyl piperidine hydrochloride	90	100
Protamine sulfate	19	60
<b>Polyacids</b>		
Polyaspartic acid—sodium salt	40	
Polyglutamic acid—sodium salt	200	
Polycysteic acid—sodium salt	8	
Polyvinyl sulfonic acid—sodium salt	$\sim 1000$	
Poly-orthophosphate—sodium salt	16, 30, 150	
Deoxyribonucleate—sodium salt		no agglutination occurred at all concentrations tested up to 5 mg/ml
Neutral polymers		
Polyalanine	$\sim 100$	

**Table 10.2. THE AGGLUTINATION EFFECT OF POLYELECTROLYTES.** Minimal concentration refers to the concentration causing agglutination of a suspension containing  $10^6$  erythrocytes per milliliter of suspension. Degree of polymerization refers to the average number of monomers in each polymer. (Table from Katchalsky *et al.*, 1959.)

acid has no appreciable effect on the resting potential of the frog *sartorius* muscle, whereas poly-L-lysine has a profound effect at a concentration as low as  $2 \mu\text{g}/\text{ml}$ .

Tasaki *et al.* (1961) have collected further evidence pointing to the same conclusion. These investigators injected radioactive tracers into squid axons and analyzed the time course of the loss of these isotopes from the axons. The outward movements of anions like chloride, sulfate, and phosphate, measured in terms of time constants of loss, are one order of magnitude slower than the monovalent cations,  $\text{Na}^+$  and  $\text{K}^+$ , studied. From these results they concluded that "the nerve membrane has properties of a 'charged membrane' in which negatively charged radicals are relatively immobile" (also see footnote on page 214).

We accept these findings of Katchalsky *et al.*, Neville, and Tasaki *et al.* as evidence that the surface fixed sites of the muscle cells discussed are predominantly

anionic. This conclusion both confirms the fundamental assumption implicit in equation (10-12) and offers explanations for several striking experimental observations.

#### A. CHANGES OF THE ELECTRICAL PROPERTIES OF NERVE AND MUSCLE SURFACES FOLLOWING TREATMENT WITH PHOSPHOLIPASES

Tobias (1955, 1958) showed that, applied from the outside, proteolytic enzymes\* do not destroy the function of nerve axons and that specific phospholipases such as lecithinases A and C do have this effect. Tobias interpreted these experiments as indications that the cell surface is composed of a lipoid (probably lecithin) membrane† which is affected enzymatically by phospholipase. The work of Katchalsky *et al.* (1959) on red blood cells and Katchalski *et al.* (1953) on bacteria indicates the fundamental anionic nature of the surfaces of these cells. Also, preliminary studies done in our laboratory indicate that these conclusions apply to muscle. Thus, we suggest an alternative to the explanation offered by Tobias.

Specifically, Tobias found that phospholipase A (from cobra venom) and phospholipase C (from tetanus toxin) produce similar depolarizations on lobster giant axon and on frog *sartorius* muscle. He also found that erythrocytes are hemolyzed by a lower concentration of phospholipase A. Phospholipase C, isolated from the tetanus bacillus, *Clostridium welchii*, had been studied by McCrea (1947) working in Burnett's laboratory. He found that phospholipase C causes erythrocytes to lose their ability to agglutinate with a certain influenza virus. McCrea, however, concluded that this action of phospholipase C was not a result of phospholipase enzyme activity because there were large differences among parameters such as optimum pH and heat stability between the two actions‡ (Figure 10.12).

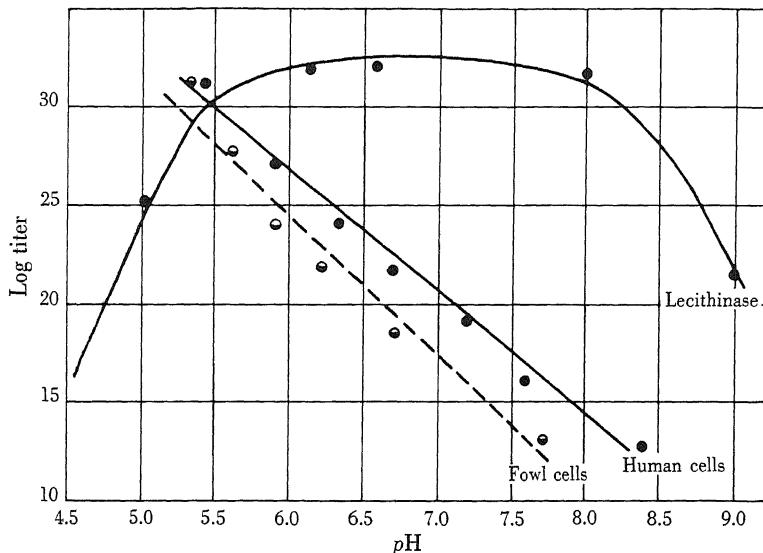
We suggest that the above effects of the phospholipases on the surface properties of cells arise from the fact that phospholipases are polypeptides or proteins bearing large numbers of cationic groups. The work of McCrea and of Katchalsky supports this view. McCrea found that phospholipase C from tetanus toxin rapidly adsorbs onto erythrocytes. Since Katchalsky *et al.* have shown that only cationic polyamino acids or proteins interact with the erythrocyte surface, phospholipase C is probably a cationic protein or polypeptide. As such, by virtue of its cationic polypeptide nature, it should produce effects on cells similar to the effects

\* See Appendix E for explanation of lack of action of proteolytic enzymes.

† It has often been hypothesized that the "plasma membrane" of the cell (and therefore the cell surface) is composed primarily of phospholipids. The nature of the surface ionic sites will not be discussed here; for a review of the scant evidence supporting their lipid nature, see Appendix E.

‡ It has come to the author's attention that very recently, a considerable amount of work has been done in fractionating the protein components of cobra venoms. All the data showed separation of lecithinase A and other enzyme activities from the neurotoxic component. See, for example, Sasaki, 1960; Carey and Wright, 1959. (This footnote was added in proof February, 1962.)

demonstrated by Katchalsky and co-workers for other nonenzymatic cationic polyamino acids (for hemolytic effect of poly-L-lysine on erythrocytes see Nevo *et al.*, 1955).



**Figure 10.12. THE EFFECT OF pH ON THE ACTIVITY OF LECITHINASE C AND ON ITS EFFECT ON THE RED-BLOOD-CELL SURFACE.** The  $\alpha$ -toxin of *Clostridium welchii* shows both lecithinase activity (lecithinase C) and an effect which alters the surface properties of the red blood cell. The upper curve shows the effect of pH on the lecithinase activity while the lower curves show the effect of pH on the effect of the enzyme on the surfaces of human and fowl erythrocytes. This experiment is one of many demonstrating that lecithinase does not affect the surface properties of cells through its enzymatic action. (Figure after McCrea, 1947.)

## B. OBSERVATIONS ON MUSCLE RESTING POTENTIALS

The predominantly anionic nature of the fixed sites indicates that free anions like the  $\text{Cl}^-$  ion do not participate directly in determining the magnitude of the equilibrium resting potential. This prediction is borne out by the long-established lack of lasting change in the resting potential after the  $\text{Cl}^-$  ion is substituted by other anions like thiocyanate, sulfate, and so on (Ling, unpublished; Hodgkin and Horowicz, 1959). The importance of the cationic components of the bathing solution in determining the magnitude of the resting and action potentials has already

been discussed in detail. The prediction is also supported by the finding that anions injected into squid nerve axon do not alter their rates of outward migration in response to stimulation of the nerve, while similarly injected  $\text{Na}^+$  and  $\text{K}^+$  ions sharply increased their outward flux rates in response to the same stimuli (Tasaki *et al.*, 1961).

Similarly, the predominantly anionic nature of the surface sites provides a basis for understanding the experimental findings of Koketsu and Kimura (1960) and of Tobias (1950) pointed out in Section 10.1. Removal of countercations from the primarily anionic surface sites is much more difficult than removal of the countercations from the fixed anionic sites within the bulk phase where immediately adjacent fixed cationic sites exist. To remove a free countercation (say,  $\text{K}^+$  ion) from a system containing only anionic fixed polar groups, the work needed is related to  $\Delta E_K + \psi\mathfrak{F}$ , and also depends on the inductive and direct electrostatic effects discussed in Chapter 2. To remove a  $\text{K}^+$  ion from the amphoteric fixed-charge system, the work needed is related to  $\Delta E_K - \Delta E_{f+} + \psi\mathfrak{F}$ ; since to preserve macroscopic electroneutrality the loss of the  $\text{K}^+$  ion must be accompanied by the loss of an equivalent anion, the inductive and direct effects may be much smaller. Since the magnitude of  $\Delta E_K + \psi\mathfrak{F}$  is always greater than that of  $\Delta E_K - \Delta E_{f+} + \psi\mathfrak{F}$ , in 0.112M sucrose or distilled water, the  $\text{K}^+$  ion in the interior amphoteric fixed-charge system should be lost before that on the surface anionic fixed sites. Since the surface sites determine the potential, it is not surprising that, even after a loss of 80 to 90 per cent of the total gross  $\text{K}^+$ -ion content of the cell, it retains a resting potential which is nearly normal (Table 10.1).

### 10.5. Summary

In Chapter 2, we pointed out that a typical cell is a complex fixed-charge system even though the nature, electrical polarity, density, and other characteristics of fixed ionic sites in different regions of the cell can be quite dissimilar. The discussion of cellular electrical potentials has offered a vantage point from which to view the particular importance of the characteristics of the fixed polar groups on the cell surface. Thus, we have presented evidence indicating that the surface sites of muscle and nerve consist primarily of fixed anions and that the fixed sites in the bulk phase are amphoteric. The cell surface about which we have been speaking corresponds roughly with what conventionally has been regarded as the "cell membrane" and, indeed, if one so chooses, there is no reason not to refer to this cell surface as the "membrane." Only one must keep in mind that this "membrane" is more like, say, the skin of an apple which itself constitutes a phase similar to the bulk phase it encloses than the "plasma membrane" in the conventional sense which separates two essentially similar aqueous phases.

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# 11

## IONIC PERMEABILITY AND DIFFUSION

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Before the advent of radioactive tracers, studies of permeability were very much restricted by the experimental methods available. One technique commonly employed was the observation of volume changes in cells bathed in a concentrated solution containing as its major solute the ion or nonelectrolyte under study (see Chapter 12). A cell which became swollen in such a solution was considered permeable to the substance in question; cells which did not swell were considered impermeable. Many of the results of such studies were consistent with the idea of a sievelike cell membrane (see Boyle and Conway, 1941). However, radioactive-tracer techniques facilitated the discovery of many substances (of which the Na<sup>+</sup> ion is a prime example; Table 9.1) which, although they permeate cells, do not cause cellular swelling. This work dramatically demonstrated the lack of a causal relationship between permeability and accumulation of substances within cells. In Chapter 10, we presented experimental evidence which disproves the existence of causal relationship between permeability and cellular potentials. However, although permeability is not causally related to either of these physiological activities, ionic permeability *per se* has great significance for the full understanding of the living state.

According to the association-induction hypothesis, ionic permeability\* is linked with another phenomenon that has received relatively little attention from cell biologists. This is the diffusion of ions within the bulk-phase cytoplasm of the cells. For this reason, the problems of permeability into and diffusion

\* Note that conventionally the terms permeation and permeability refer to the passage of a substance from one phase through a second phase different from the first to emerge into a third phase similar to the first. This is the significance of permeability in terms of the membrane concept. Permeation and permeability, as discussed in this chapter, have somewhat different connotations. As presently used, the terms refer to the passage of a substance through the surface layer of a fixed-charge system, whether or not this surface layer is similar to the bulk phase of the fixed-charge system.

within cells are examined together in a chapter separate from those on ionic accumulation and on cellular potentials.

The importance of permeability in the membrane hypothesis led to the accumulation of a large body of descriptive data on the permeability of many types of cells to a variety of substances. The early "sieve" ideas were conceptually clear but basically erroneous. Since these ideas were disproved, neither a workable mechanism for cellular permeability nor a theoretical derivation of the permeability constants has been advanced. Few workers have paid attention to the fact that permeability *constants* are not constants at all, but are complex functions which depend upon temperature, the nature and concentrations of other ions present, and other factors. The present hypothesis allows the construction of workable theoretical models for ionic permeability into and out of, and for diffusion within the fixed-charge system. These models resolve the apparent paradox of the accumulation of  $K^+$  ion by adsorption onto fixed ionic sites as proposed by the present theory and the evidence that the diffusion coefficient for part of the intracellular  $K^+$  ions at least is of the same order of magnitude as the diffusion coefficient for  $K^+$  ions in a dilute salt solution (Hodgkin and Keynes, 1953). In addition, the experimental facts presented in this chapter are predictable on the basis of the association-induction hypothesis although many of them cannot be explained in terms of the membrane-pump hypothesis. We believe that the agreement of these facts with our hypothesis can leave little doubt that protoplasm, whether in the form of a cell surface or of cytoplasm, is a fixed-charge system.

### 11.1. Surface-Limited and Bulk-Phase-Limited Ion Exchange

The primary purpose of this chapter is to examine diffusion processes into and within the cell. This examination is made possible by radioactive tracers, which, as we mentioned, led to the first understanding of the failure of the classical membrane theory. Radioactive tracers enable us to study the rate of exchange of, say,  $K^+$  or  $Na^+$  ions between the intracellular and extracellular phases without disturbing the normal physiological environment of the cell. For example, by allowing the cell to incorporate radioactive  $K^{42}$  and then washing the cell in non-radioactive Ringer's solution, we can follow the time course of the loss of  $K^{42}$  from the cell. Such experiments serve to determine the rate at which a normal cell exchanges its  $K^+$  ion. As in all rate processes, this exchange rate measures the velocity with which the  $K^+$  ion passes through a rate-limiting step. In the classical membrane theory, the intracellular  $K^+$  ion as well as the  $Na^+$  ion is assumed to be free in a dilute solution. Since the rate of intracellular diffusion must then be rapid, the rate-limiting factor in the migration of the  $K^+$  or  $Na^+$  ion in and out of the cell must be the slowest step in its journey through the cell membrane. The association-induction hypothesis, however, maintains that,

although, for some ions under certain conditions (Section 11.1D), the exchange of an ion on the surface of the fixed-charge system with a similar ion in the external free solution may be the rate-limiting step, under a different set of conditions, the rate-limiting step can occur during migration within the bulk phase of the intracellular fixed-charge system. Under other conditions, the original desorption of the ion from fixed ionic sites within the cell may be the rate-limiting step. The diffusion experiments discussed below demonstrate the inadequacy of the membrane concept and seem predictable only within the framework of the association-induction hypothesis.

#### A. SURFACE-LIMITED EXCHANGE

The instantaneous inward-flux rate for the migration of ions of species  $j$  into the intracellular phase of a single cell or population of cells is given by the equation:

$$\frac{dC_j^{\text{in}}}{dt} = \frac{A}{V} P_j C_j^{\text{ex}} \quad (11-1)$$

where  $C_j^{\text{in}}$  and  $C_j^{\text{ex}}$  are the intracellular and extracellular concentrations of the  $j$ th ion, respectively;  $A$  is the sum of the surface areas of the cells;  $V$  is the total volume of the cells; and  $P_j$ , the surface permeability, is a coefficient with the dimensions, cm/sec. The outward flux in a surface-limited exchange follows an analogous relationship except for the existence of a surface potential  $\psi$ . Thus, if the  $j$ th ion is a monovalent cation and the potential is such that the intracellular phase is negative,

$$-\frac{dC_j^{\text{in}}}{dt} = \frac{A}{V} P_j C_j^{\text{in}} \exp\left(\frac{-\psi\mathfrak{F}}{RT}\right). \quad (11-2)$$

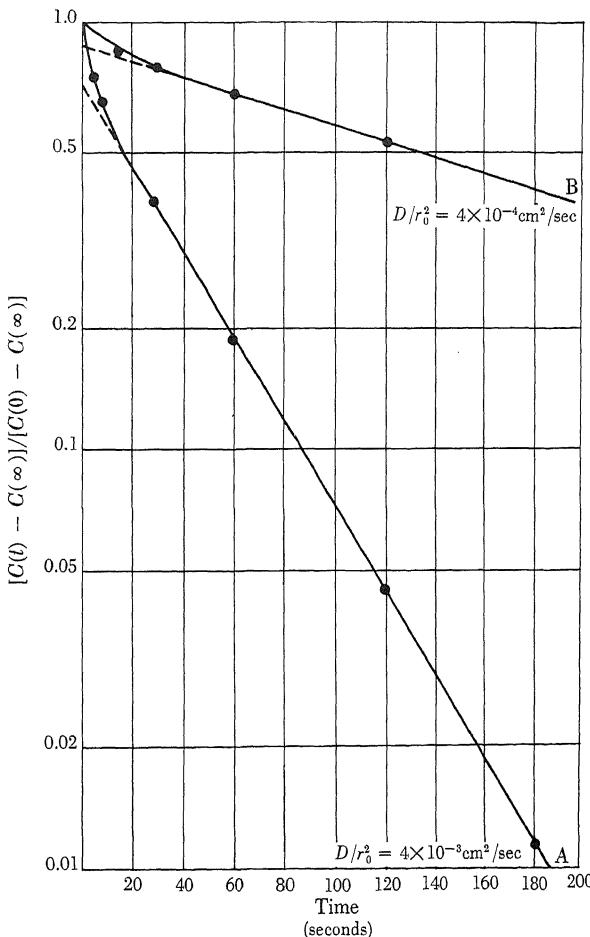
Integrating equation (11-2), we obtain the equation

$$\ln C_j^{\text{in}}(t) = \frac{-A}{V} P_j t \exp\left(\frac{-\psi\mathfrak{F}}{RT}\right) + \ln C_0 \quad (11-3)$$

where  $C_j^{\text{in}}(t)$  is  $C_j^{\text{in}}$  at time  $t$ , and  $\ln C_0$  is an integration constant. This shows that for a single cell or a population of cells with uniform shape, a plot of the logarithm of the concentration of a radioactive agent against  $t$  for a surface-limited exchange should give a straight line.

#### B. BULK-PHASE-LIMITED EXCHANGE

According to the association-induction hypothesis, the bulk phase of the cell is a fixed-charge system. Accepting this view, we can predict that under certain conditions, the surface exchange rate will be faster than the bulk-phase diffusion rate. If the rate-limiting step is removed from the surface, ion exchange will no longer follow the relation developed in the last section. Bulk-phase diffusion processes are determined both by the rate of diffusion within the bulk phase,



**Figure 11.1. THE TIME COURSE OF BULK-PHASE-LIMITED DIFFUSION FROM CYLINDERS.** Theoretical curves are plotted from equation (11-4) with two different values of  $D/r_0^2$ . The dotted lines represent the time course if the entire curves are plotted from equation (11-6).

which is defined by a diffusion coefficient  $D$  whose dimensions are  $\text{cm}^2/\text{sec}$ , and by the geometry of the system. Fortunately, most cells suitable for *in vitro* studies are either cylindrical or spherical; we can directly apply the solutions for the diffusion equations given by Dünwald and Wagner (1934) to the cylindrical cells. If the cylinder is considerably longer than it is wide, as most muscle and nerve fibers are, the length of the cylinder can be considered infinite. Representing the radius of the cylinder by  $r_0$ , the initial uniform ion concentration by  $C(0)$ , the final concentration by  $C(\infty)$ , and the average concentration at any

time  $t$  by  $C(t)$ , we write

$$\frac{C(t) - C(\infty)}{C(0) - C(\infty)} = \sum_{n=1}^{\infty} \frac{4}{\xi_n^2} \exp\left(-\frac{\xi_n^2 D t}{r_0^2}\right) \quad (11-4)$$

where  $\xi_n$  is the  $n$ th root of the equation,  $J_0(\xi) = 0$ , in which  $J_0(\xi)$  is the Bessel function of order zero. These roots are  $\xi = 2.405, 5.520, 8.654, 11.792, 14.931, 18.071, \dots$ . When  $t$  is sufficiently large, we can neglect all terms but the first and equation (11-4) reduces to

$$\frac{C(t) - C(\infty)}{C(0) - C(\infty)} = \frac{4}{(2.405)^2} \exp\left[-\frac{(2.405)^2 D}{r_0^2} t\right] \quad (11-5)$$

or

$$\log \frac{C(t) - C(\infty)}{C(0) - C(\infty)} = -0.1604 - 2.512 \left(\frac{D}{r_0^2} t\right). \quad (11-6)$$

Equation (11-4) is plotted in Figure 11.1 for two different values of  $D/r_0^2$ . The curve flattens [that is, it follows equation (11-6)] when  $t$  is large, and its slope gives the value of  $D/r_0^2$ ; when the radius of the cylinder can be measured, the diffusion coefficient  $D$  can be estimated.

### C. ION EXCHANGE IN LIVING CELLS

If one equilibrates a tissue in a solution containing a radioisotope which enters the cell and one then washes the preparation continuously in a stream of non-radioactive solution, a plot of the logarithm of the radioactivity remaining in the cell against time should give a straight line if outward migration is surface limited (as demanded by the membrane theory). If outward migration is bulk-phase limited, such a plot should give a nonrectilinear curve like that shown in Figure 11.1. In reality, this clear theoretical distinction becomes obscured by errors from three possible sources. (1) Adhering radioactive intercellular fluids may contribute a rapidly exchanging fraction which makes the first portion of the curve bend. (2) Heterogeneity in the cell diameters is often present even when the permeability per unit surface area is the same for all fibers. Since the slope of the  $\ln C$ -versus- $t$  plot for surface-limited exchange depends on  $A/V$  (the average surface area per unit volume of a cell, which varies with cells of different sizes), fibers having different diameters will give different slopes. Equation (11-3) can be written in exponential form:

$$C_j(t) = C_0 \exp\left(-\frac{A}{V} P_j t \exp \frac{-\psi \mathcal{F}}{RT}\right). \quad (11-7)$$

If the heterogeneous fiber population can be represented as consisting of  $n_1$  fibers with the ratio  $(A/V)_1$ ,  $n_2$  fibers with the ratio  $(A/V)_2$ , and so on and the total

number of fibers is  $n$ , the average concentration of the  $j$ th ion at time  $t$  will be:

$$C_j(t) = \frac{\sum_i n_i V_i C_j^i(t)}{\sum_i n_i V_i}$$

or

$$C_j(t) = C_0 \frac{\sum n_i V_i \exp \left[ -\left( \frac{A}{V} \right)_i P_j t \exp \frac{-\psi \mathcal{F}}{RT} \right]}{\sum n_i V_i}. \quad (11-8)$$

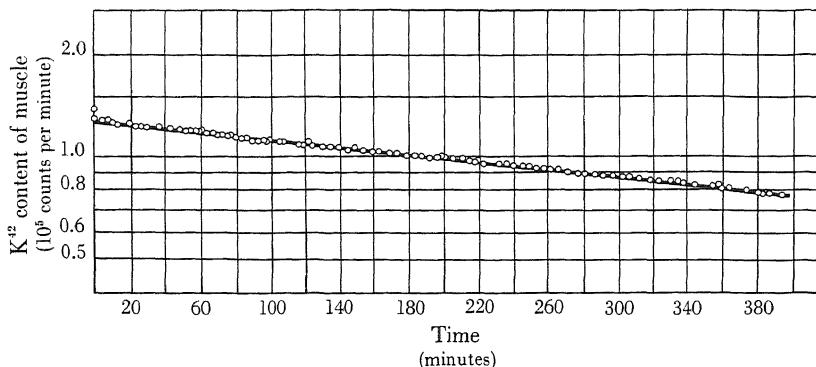
When  $C_j(t)$  is plotted semilogarithmically against time, the curve is not a straight line, as for a homogeneous population, but a curved one. (Such a curve, constructed from a set of straight lines, is shown in Figure 11.28.) However, the curvature which arises from the heterogeneity of fiber diameters should remain essentially the same for the diffusion of all species of ions from the tissue. (3) There may be a difference in the surface permeability constant ( $P_j$ ) for different cells.

Each of these three causes for deviation from the ideal has been used to explain away curvatures in  $\ln C$ -versus- $t$  plots by investigators who expected a linear function. Fortunately, there are several ways to distinguish these errors from the "true" curves arising from the process of diffusion whether it is surface or bulk-phase limited. A fourth source of error (not usually mentioned) which can affect the earlier phase of the exchange of  $\text{Na}^+$ , but not that of  $\text{K}^+$ , arises from the adsorption of the  $\text{Na}^+$  ion onto connective-tissue elements and elements associated with small blood vessels and nerves. The details of the method for the correction of this error have been given under Table 8.7.

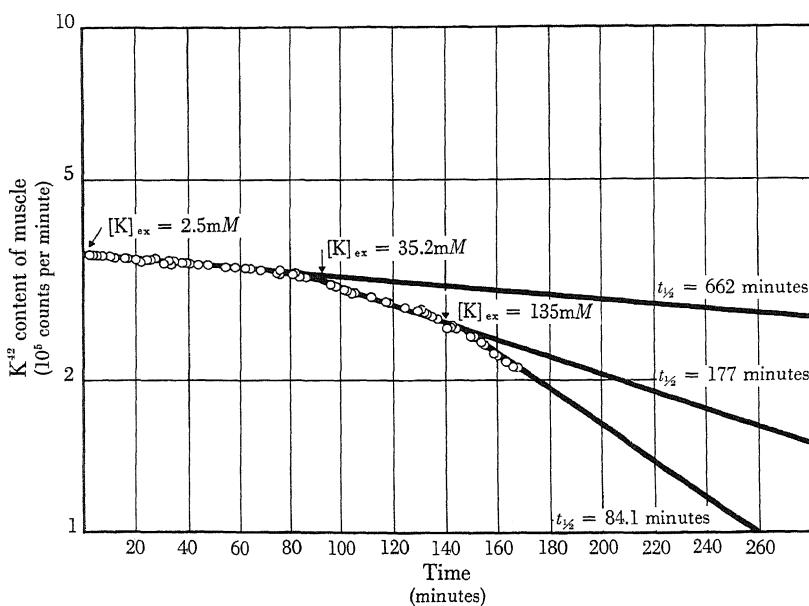
#### D. SURFACE-LIMITED EXCHANGE OF $\text{K}^+$ ION IN THE FROG *SARTORIUS* MUSCLE

If exchange of an ion, say  $\text{K}^+$ , is surface limited, we expect to find a linear relationship between  $\ln C$  and  $t$ . This expectation is confirmed by an experiment in which a frog *sartorius* muscle, equilibrated with  $\text{K}^{42}$ , was washed continuously in a non-radioactive Ringer's solution for six hours. That the  $\ln C$ -versus- $t$  plot is a straight line seems obvious from Figure 11.2. This shows that the three possible sources of error, adhering extracellular fluid, heterogeneous  $A/V$  values, and heterogeneous  $P_{\text{K}}$  values (permeability constants for  $\text{K}^+$  ions), are insignificant for frog *sartorius* muscle, at  $25^\circ\text{C}$ .

A further demonstration that the exchange of  $\text{K}^+$  ion is surface limited is provided by its dependence on the surface potential  $\psi$  [equation (11-3)]. From Chapter 10, we know that  $\psi$  varies inversely with the logarithm of  $[\text{K}]_{\text{ex}}$  (the external  $\text{K}^+$ -ion concentration). A change of the slope but not in the rectilinearity of the plot of  $[C(t) - C(\infty)]/[C(0) - C(\infty)]$  versus  $t$  should follow variations of  $[\text{K}]_{\text{ex}}$ ;



**Figure 11.2. THE TIME COURSE OF  $K^+$ -ION EXCHANGE IN FROG SARTORIUS MUSCLE ( $25^\circ C$ ).** A muscle (75.2mg) was equilibrated with Ringer's solution containing  $K^{42}$  for 68 hours at  $2^\circ C$ . The amount of radioactivity remaining in the muscle was counted as the muscle was continuously washed in Ringer's solution containing no radioactive  $K^+$  ion. A diagram of the apparatus is provided in Figure 8.5 (see also the control experiment shown in Figure 11.24). (Ling and Ochsenfeld, unpublished.)

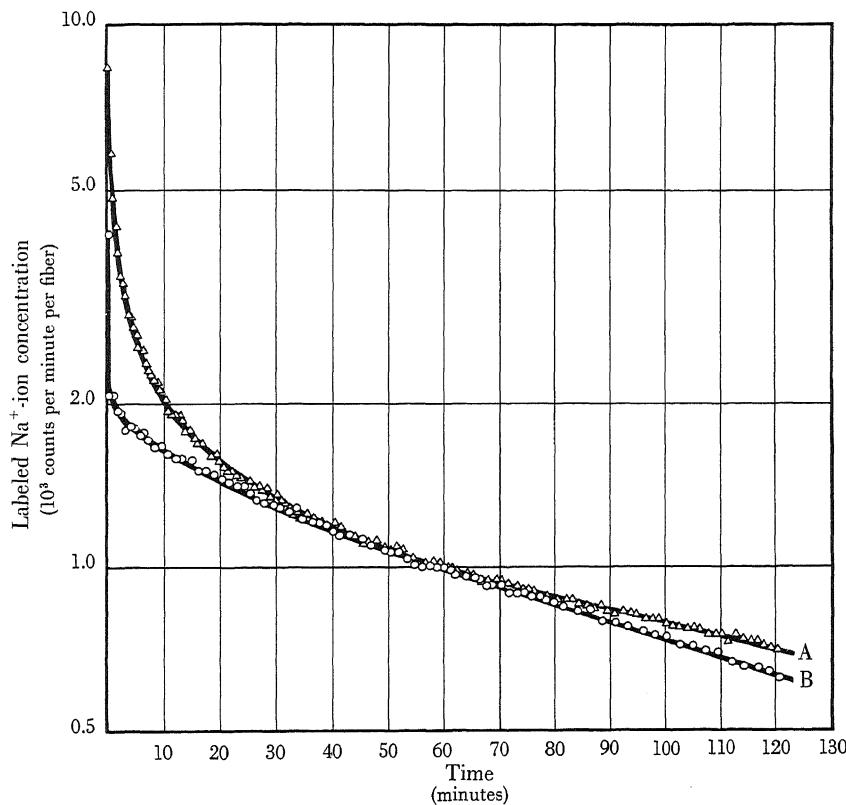


**Figure 11.3. THE EFFECT OF VARIATION OF THE EXTERNAL  $K^+$ -ION CONCENTRATION ON THE RATE OF EXCHANGE OF THE  $K^{42}$  ION.** The slope of the lines extrapolated from the graph gives the half time of exchange  $t_{1/2}$  for  $K^{42}$  in the particular external  $K^+$ -ion concentration;  $t_{1/2}$  is related to  $\psi$  by the relation,  $t_{1/2} = \ln 2 / [(A/V)P_K \exp (-\psi \mathcal{F}/RT)]$ .

this can be demonstrated experimentally (Figure 11.3). However, when the flux rate of  $\text{Na}^{+}$  ion is studied in an analogous experiment on *sartorius* muscle, the result is entirely different; the curve obtained here could not possibly represent a straight line (Figure 11.25). Thus, the exchange mechanisms must be fundamentally different.

#### E. EXCHANGE OF $\text{Na}^{22}$ IN A SINGLE-MUSCLE-FIBER PREPARATION

The three sources of error mentioned above can all be eliminated by studying the rate of exchange of an ion in an isolated muscle fiber. Since the fiber is not surrounded by any other fibers, the extracellular radioactivity is instantly washed



**Figure 11.4. THE TIME COURSE OF  $\text{Na}^{22}$ -ION EXCHANGE IN SINGLE MUSCLE FIBERS DISSECTED FROM THE SEMITENDINOSUS MUSCLE OF A BULLFROG.** A muscle fiber was dissected from the *semitendinosus* muscle of a bullfrog, placed on a strip of lucite for support, and washed as in the experiment described under Figure 8.5. Experiments in which the muscle fiber appeared abnormal after the completion of the experiment were excluded. Fiber A was soaked in  $\text{Na}^{22}$ -Ringer's solution for ten minutes at  $0^\circ\text{C}$  before washing began; fiber B, for 70 minutes at  $4^\circ\text{C}$  (also see Figure 11.29).

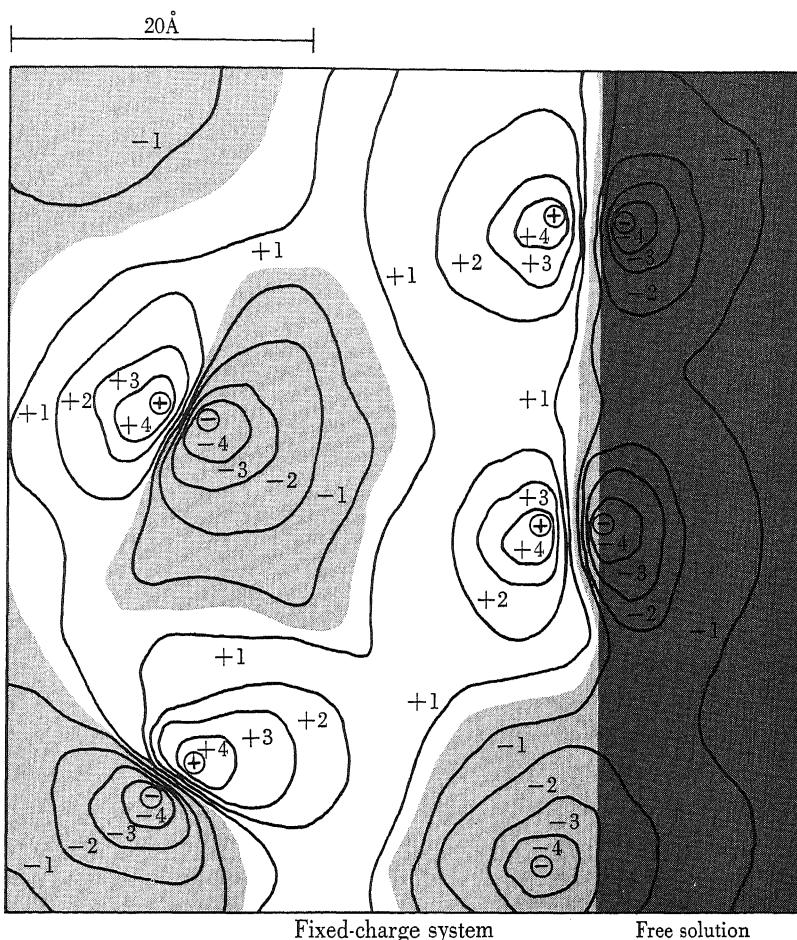
away. The surface permeability of a single fiber must be considered uniform, otherwise a constant electrical potential would exist between one part of the fiber and another; this has not been found (see Ling and Gerard, 1949a). Being a single entity, the muscle fiber has a unique  $A/V$  value. Thus, if the  $\text{Na}^+$ -ion exchange is surface limited, the  $\ln C$ -versus- $t$  plot of a single fiber should be a straight line. Figure 11.4 shows that it is not. Its shape, in fact, suggests a bulk-phase-limited exchange (see Figure 11.1).

The process of bulk-phase-limited exchange is analyzed in the last section of this chapter. It has been demonstrated that for  $\text{K}^+$ , a strongly adsorbed ion, the rate-limiting step in exchange between the cell and the external milieu occurs at the cell surface. For  $\text{Na}^+$ , a weakly adsorbed ion, the rate-limiting step is the bulk phase. Following convention, we shall refer to the surface-limited step as permeation, and the bulk-phase-limited step as diffusion. According to the present hypothesis, the two processes are similar. The chief difference between them lies in the fact that permeation involves migration of particles between an external free-solution phase and the fixed-charge phase, while diffusion involves migration from one locus within the fixed-charge phase to another locus in the same phase. We shall discuss these phenomena separately, pointing out the basic similarity in the mechanism of the two processes for the purpose of illustrating the unifying nature of the association-induction hypothesis.

## 11.2. Ionic Permeation

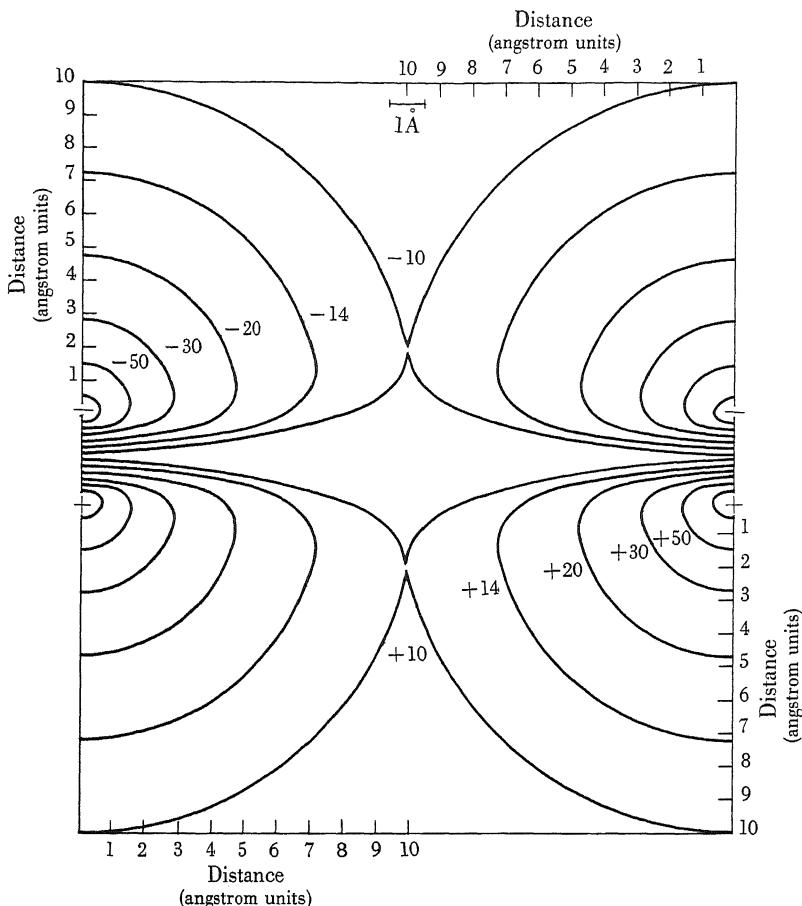
It is a familiar fact to most biologists that a wad of sterilized cotton can bar the entry of bacteria much smaller than the meshes of the cotton wad into culture tubes. The grid in a triode electronic tube operates on the same principle. This grid, which has meshes many times the size of electrons, bars their passage only when it is charged by the application of a grid voltage. These phenomena are due to the electrostatic charges carried by the bacteria and the cotton, by the electrons and the grid; particles which pass through an electrically neutral mesh with no difficulty are prevented from passing when the mesh is charged. In living cells, even though the fixed ions are fairly far apart and the meshes fairly large, the surface of the fixed-charge system serves as a similar barrier to charge-bearing particles, ions.

Let us consider an anion, fixed rigidly in space, accompanied by a cation which is free to move tangentially within a spherical shell around the fixed anion. We assume that the  $c$ -value of the fixed anion and the nature of the countercation necessitate the preference of configuration 0. In this case, the adsorption energy approximates the Coulombic interaction energy and is proportional to  $1/r$ , where  $r$  is the equilibrium distance between the charges. Next, we can calculate the Coulombic energy exerted by this ion pair on a second counterion at various loca-



**Figure 11.5. THE ELECTRIC POTENTIAL FIELDS NEAR THE SURFACE OF A FIXED-CHARGE SYSTEM.** Each line represents a more or less permanent isopotential line. Positive and negative numbers refer to the magnitude of repulsive and attractive electric fields. The diagram demonstrates the continuous energy barriers to the entry of cations and anions through the "meshes" and the sloping valleys favoring the formation of triplets and doublets. (Ling, 1960.)

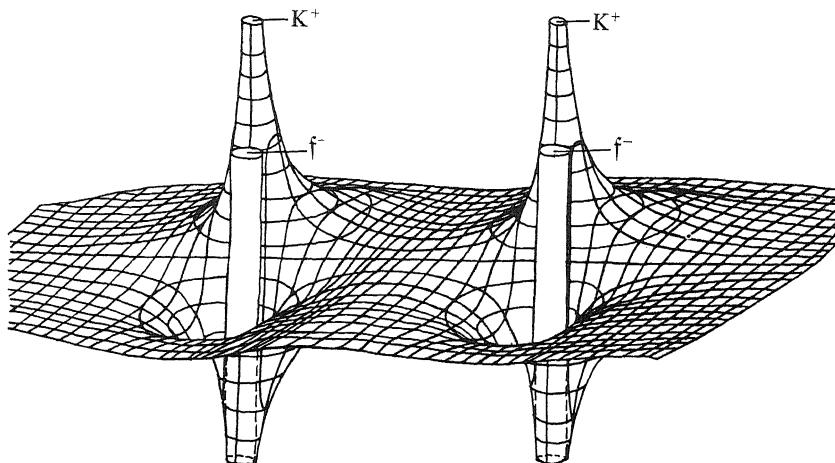
tions in the vicinity, assuming that the original ion pair is not affected by the approaching counterion. Remembering the effect of dielectric saturation discussed in Chapter 2, we can estimate the potential field in a fixed-charge system with a 20 Å microcell diameter as shown in Figures 11.5 and 11.6. For better visualization, a qualitative picture of the space-energy relation is presented in Figure 11.7. Superimposed on the space-energy relation is an entropy relation arising from the configurational and rotational entropies at adsorption sites as well



**Figure 11.6. THE ELECTROSTATIC FIELD OF A FIXED-CHARGE COUNTERION SYSTEM AS SEEN BY AN APPROACHING UNIVALENT FREE CATION.** This is a theoretical diagram. It is assumed that the presence of the approaching free cation does not affect the positions of the associated counterions. Only the Coulombic term, proportional to  $1/r$ , where  $r$  is the distance between the charged particles, has been taken into account in this diagram.

as at interstitial sites. The energy and entropy, considered together in their spatial distribution, give the fixed-charge system certain unique properties.

Since it is both energetically and entropically unfavorable for ions to pass from free solution through the interstices in the surface, these interstices between the fixed charges present barriers to the entry of most ions. On the other hand, the portion of the surface occupied by fixed ions may be divided into two categories according to whether or not the fixed ionic site is coupled with a counterion. Those which are coupled with a counterion can be divided into two categories: those in a



**Figure 11.7. THE ELECTROSTATIC FIELD NEAR A PAIR OF FIXED ANIONS AND THEIR COUNTER-CATIONS.** The height or depth of the isopotential line at any locus qualitatively represents the sum of the repulsive and attractive forces acting on a cation. Arrows indicate location of fixed anion  $f^-$  and of countercation  $K^+$ .

momentary configuration with the counterion facing the outside, and those with the counterion facing the inside. Since the fixed-charge system under discussion is anionic, the fraction of ion pairs with the countercation directed inward will repel an approaching cation. The vacant fixed anions as well as the occupied fixed ion-counterion pair with the anionic element directed inward present contours of decreasing energy which form a less effective barrier to the approach and eventual association of a cation from without. This association and the eventual dissociation of the counterion from the fixed anion are, of course, microscopic facets of an equilibrium.

#### A. ADSORPTION-DESORPTION MIGRATION

There is an equilibrium between the adsorbed ions and the free ions in both the external free-solution phase and the fixed-charge phase. Thus, if the total number of fixed sites per unit surface area of living cell is  $\{f\}_{surf}$ , then the number of sites per unit surface area occupied by the external  $i$ th ion,  $\{p_i^{ex} \cdot f\}_{surf}$ , will be

$$\{p_i^{ex} \cdot f\}_{surf} = \frac{\{f\}_{surf} [p_i]_{ex} \frac{(p.f.)_i^{ads}}{(p.f.)_i^{fr}} \exp\left(\frac{-\Delta E_i}{RT}\right)}{1 + \sum_i [p_i]_{ex} \frac{(p.f.)_i^{ads}}{(p.f.)_i^{fr}} \exp\left(\frac{-\Delta E_i}{RT}\right) + \sum_i [p_i]_{ins} \frac{(p.f.)_i^{ads}}{(p.f.)_i^{ins}} \exp\left(\frac{-\Delta E_i}{RT}\right)}$$
(11-9)

where  $[p_i]_{\text{ex}}$  and  $[p_i]_{\text{ins}}$  refer to the concentrations of the  $i$ th ion in the external free solution and in the interstitial spaces of the fixed-charge system, respectively;  $(\text{p.f.})_i^{\text{ads}}$ ,  $(\text{p.f.})_i^{\text{fr}}$ , and  $(\text{p.f.})_i^{\text{ins}}$  are the partition functions for the  $i$ th ion in the associated state, in the free state, and in the interstitial portion;  $\Delta E_i$  is the adsorption energy of the  $i$ th ion.

An external  $i$ th ion occupying a site on the surface of the fixed-charge system may return to the external phase. In this case the event is of no significance to our discussion. On the other hand, it may desorb and enter into the fixed-charge phase; this constitutes an adsorption-desorption permeation. There are two ways in which this may be achieved, depending upon whether or not a third ion is involved; we shall refer to these phenomena as *doublet* and *triplet* migration.

### (1) Doublet migration

An associated ion may gain sufficient kinetic energy to jump out of its adsorption site. Whether such a desorbed ion returns to the external solution or enters the fixed-charge phase depends upon the spatial orientation of the doublet:  $-+$  or  $+-$ . In Chapter 10, we demonstrated that if the surface of a fixed-charge system is in equilibrium with an external free solution, a surface potential  $\psi$  exists. (In living cells,  $\psi$  represents the resting potential.) If  $\psi$  is oriented so that the potential inside the anionic fixed-charge system is negative with respect to the external phase, the countercation must be oriented so that entry into the fixed-charge phase is more probable by the factor  $\exp(\psi\mathcal{F}/RT)$  than exit to the free solution. The doublet-migration rate at which ions of species  $p_i$  enter the fixed-charge phase is, therefore, according to the theory of absolute reaction rate (Glasstone *et al.*, 1941):

$$(V_i)_{\text{dou}}^{\text{inward}} = \frac{kT}{h} \{p_i^{\text{ex}} \cdot f\}_{\text{surf}}^{\ddagger} \quad (11-10)$$

where

$$\begin{aligned} \{p_i^{\text{ex}} \cdot f\}_{\text{surf}}^{\ddagger} &= \\ &\frac{\exp(\psi\mathcal{F}/RT)}{1 + \exp(\psi\mathcal{F}/RT)} \{p_i^{\text{ex}} \cdot f\}_{\text{surf}} \exp\left[\frac{-(-\Delta E_i + \varepsilon_{\text{di}}^{\ddagger})}{RT}\right] = \frac{\exp(\psi\mathcal{F}/RT)}{1 + \exp(\psi\mathcal{F}/RT)} \times \\ &\times \frac{\{f\}_{\text{surf}} [p_i]_{\text{ex}} \frac{(\text{p.f.})_i^{\text{ads}}}{(\text{p.f.})_i^{\text{fr}}} \exp\left(\frac{-\Delta E_i}{RT}\right) \exp\left(\frac{\Delta E_i - \varepsilon_{\text{di}}^{\ddagger}}{RT}\right)}{1 + \sum_i [p_i]_{\text{ex}} \frac{(\text{p.f.})_i^{\text{ads}}}{(\text{p.f.})_i^{\text{fr}}} \exp\left(\frac{-\Delta E_i}{RT}\right) + \sum_i [p_i]_{\text{ins}} \frac{(\text{p.f.})_i^{\text{ads}}}{(\text{p.f.})_i^{\text{ins}}} \exp\left(\frac{-\Delta E_i}{RT}\right)} \quad (11-11) \end{aligned}$$

where  $-\Delta E_i + \varepsilon_{\text{di}}^{\ddagger}$  is the total activation energy for the doublet dissociation. In equation (11-11), the term  $\exp(-\Delta E_i/RT)$  and the term  $\exp(\Delta E_i/RT)$  cancel, and the surface concentration of the activated complex,  $\{p_i^{\text{ex}} \cdot f\}_{\text{surf}}^{\ddagger}$ , is determined by

$\varepsilon_{di}^t$ , the activation energy. It is the value of  $\varepsilon_{di}^t$  that deserves special discussion. If the adsorbed ion and the fixed charge existed in a vacuum,  $\varepsilon_{di}^t$  would have a small, probably negligible, value. Any collision that gave the adsorbed ion energy in excess of  $\Delta E_i$ , the total adsorption energy, would be sufficient to start it on a frictionless journey out of the energy well.

Actually, the fixed-charge system is embedded in a dense crowd of water molecules. The mean free path of a particle in such a condensed system is usually very short. The adsorption energy, due primarily to the Coulombic term and proportional to  $1/r$ , is a long-range force extending over distances far greater than the mean free path. Consequently, the  $i$ th ion, even when it has received energy that would be sufficient to liberate it from its site if the event occurred in a vacuum,\* still has a great probability of returning to the adsorbed position because of collisions with surrounding water molecules. The result is that the average activation energy must be considerably greater than that needed for dissociation in a vacuum; thus, in the fixed-charge system,  $\varepsilon_{di}^t$  has an appreciable value.

## (2) Triplet migration

The great quantity of energy necessary for doublet dissociation increases the importance of a second mode of adsorption-desorption entry. This is triplet migration. Viewed from a distance, a fixed anion-cation pair is neutral; viewed from the position of a third ion in its vicinity, however, this is not the case. On the contrary, a second counterion will be attracted or repulsed, depending upon which end of the dipolar pair is being approached. If the momentary configuration of the pair is  $+ - \leftarrow$ . the arrow indicates the minimum energy path along which a second counterion can approach the doublet until it is associated closely enough to form a triplet.<sup>†</sup> As the second counterion approaches the ion pair, it affects the pair. The effect on the interaction between the original counterion and the fixed anion is analogous to the effect of a gradual decline of the  $c$ -value of the anion. From the theoretical calculations given in Section 4.3, such a  $c$ -value decline will be accompanied by a change to a higher configuration wherein more water molecules are interspersed between the fixed anions and the original counterions (Figures 4.3 and 4.6). Finally, an equilibrium state is reached when the two counterions have both assumed relatively high configurations.

The triplet activation energy  $\varepsilon_{ti}^t$  corresponds to the doublet activation energy  $\varepsilon_{di}^t$  mentioned above. For most ionic entry the term  $\Delta E_i$  cancels out as in the

\* This is a hypothetical condition in which ions remain hydrated in an environment essentially free of densely packed water molecules.

† Triplet formation in a fixed-charge system is a very likely event; its rare occurrence in dilute solutions is primarily a consequence of the entropy loss in such complex associations. Even in free solutions, triplet formation increases with ionic concentration (Kraus, 1954).

case of the doublet; thus the term  $\varepsilon_{ti}^{\ddagger}$  plays the dominant role in determining the rate of ionic entry.

We have stated that the doublet activation energy  $\varepsilon_{di}^{\ddagger}$  is large because the Coulombic interaction energy between a cation and an anion varies with  $1/r$ . However, the dissociation energy of an ion of species  $i$  from a triplet,  $p_i \cdot f \cdot p_j$ , is the interaction energy of the  $i$ th ion  $p_i$  with the dipolar pair,  $f \cdot p_j$ . The interaction of an ion with a dipole is proportional to  $1/r^2$ ; thus the effective range of triplet interaction is much shorter than that of doublet interaction. The probability of the return of an activated ion must be much smaller than it is in the doublet migration. For this reason the triplet activation energy  $\varepsilon_{ti}^{\ddagger}$  must be considerably smaller than the doublet activation energy  $\varepsilon_{di}^{\ddagger}$ .

The triplet route is more probable than the doublet route because of the presence of lower energy barriers. It is also the truly discriminatory route because the rate of triplet formation is directly proportional to the number of  $i$ th ions occupying fixed ionic sites; this number is directly related to the adsorption energy of the ion. We recall that the doublet route is not directly dependent on the adsorption-energy term because these more strongly adsorbed ions are desorbed less easily. The terms,  $\exp(-\Delta E_i/RT)$  and  $\exp(\Delta E_i/RT)$ , cancel out and consequently external cations of various coexisting species enter by the doublet route at the same rate.

Now if we call the ion of species  $p_j$  the second counterion, and if the surface concentration of the triplet complex is  $\{p_i^{\text{ex}} \cdot f \cdot p_j^{\text{ex}}\}_{\text{surf}}^{\ddagger}$ , we can define the rate of triplet migration,

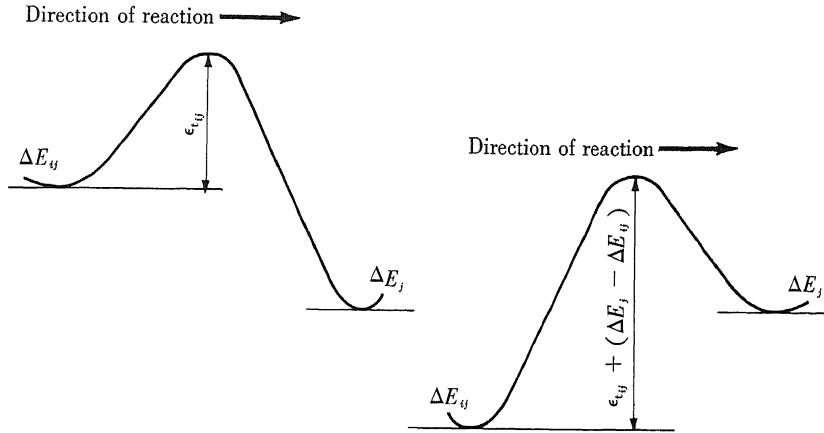
$$(V_i)_{\text{tri}}^{\text{inward}} = \frac{kT}{h} \{p_i^{\text{ex}} \cdot f \cdot p_j^{\text{ex}}\}_{\text{surf}}^{\ddagger} \quad (11-12)$$

For the  $i$ th ion to enter the fixed-charge phase, it must be oriented inward and the  $j$ th ion must therefore come from the external free-solution phase. The surface concentration of the triplet complex is given by the equation

$$\begin{aligned} \{p_i^{\text{ex}} \cdot f \cdot p_j^{\text{ex}}\}_{\text{surf}}^{\ddagger} &= \frac{\exp(\psi\varphi/RT)}{1 + \exp(\psi\varphi/RT)} \{p_i^{\text{ex}} \cdot f\}_{\text{surf}} \times \\ &\times \frac{[p_j]_{\text{ex}} \frac{(\text{p.f.})_{ij}^{\text{tri}}}{(\text{p.f.})_j^{\text{fr}}} \exp\left[\frac{-(\Delta E_{ij} + \Delta E_i)}{RT}\right] \exp\left[\frac{\pm[\Delta E_{ij} - \Delta E_j] - \varepsilon_{ti}^{\ddagger}}{RT}\right]}{1 + \left\{ \sum_j [p_j]_{\text{ex}} \frac{(\text{p.f.})_{ij}^{\text{tri}}}{(\text{p.f.})_j^{\text{fr}}} + \sum_j [p_j]_{\text{ins}} \frac{(\text{p.f.})_{ij}^{\text{tri}}}{(\text{p.f.})_j^{\text{ins}}} \right\} \exp\left[\frac{-(\Delta E_{ij} + \Delta E_i)}{RT}\right]} \end{aligned} \quad (11-13)$$

where  $(\text{p.f.})_{ij}^{\text{tri}}$  is the partition function of the triplet, and  $[p_j]_{\text{ex}}$  and  $(\text{p.f.})_j^{\text{fr}}$  refer to the external  $j$ th ion concentration and its partition function, respectively.

Referring to Figure 11.8, if  $\Delta E_{ij}$  is higher than  $\Delta E_j$ , then the effective activation energy is  $\epsilon_{ij}^+$ . However, if  $\Delta E_j$  is higher than  $\Delta E_{ij}$ , then the effective activation



**Figure 11.8. THE ACTIVATION ENERGY FOR TRIPLET ASSOCIATION-DISSOCIATION MIGRATION.** The long arrow indicates the direction of the chemical reaction.

energy is  $\Delta E_j - \Delta E_{ij} + \epsilon_{ij}^+$ . Thus, we have defined the function  $s_{\pm}(\Delta E_{ij} - \Delta E_j)$  in equation (11-13) so that

$$s_{\pm}(\Delta E_{ij} - \Delta E_j) = \Delta E_{ij} - \Delta E_j \quad \text{if } \Delta E_{ij} < \Delta E_j \\ s_{\pm}(\Delta E_{ij} - \Delta E_j) = 0 \quad \text{if } \Delta E_{ij} > \Delta E_j. \quad (11-14)$$

## B. INTERSTITIAL BARRIERS AND SALTATORY MIGRATION

There are no holes between the ion pairs. The energy barriers (represented in Figures 11.5 to 11.7 as continuous mountain ranges of high energy surrounded by low-energy moats with sloping bottoms) effectively prevent most ions, because they have insufficient kinetic energy, from entering the fixed-charge system by crossing the interstices; this situation is not at all unlike that in the electron-tube grid and in the cotton wad. A second factor, particularly important in the entry of neutral particles, is the entropy barrier which originates in the lower partition function for an ion or particle in free solution. This low interstitial partition function is largely due to the restricted rotational movements discussed in Section 2.4. This barrier also discourages the passage of ions into the fixed-charge system through the interstices.

Although the combined energy and entropy barriers effectively prevent most external ions from entering the system, a few ions do have sufficient kinetic energy to enter through the meshes. We shall refer to this mode of migration as *saltatory* migration.\*

The rate of saltatory permeation is related to  $[p_i]$ , the concentration of the entrant ion, but is independent of the concentration of the other entrant ions. The symbol  $\{\iota\}$  represents the number of interstices per unit surface area of a fixed-charge system, and the interstices have an average energy barrier equal to  $\varphi$ ; we suppose that  $(V_i)_{\text{sal}}^{\text{inward}}$ , the inward rate of saltatory migration of the  $p_i$  ion is related to  $[p_i^{\text{ex}}]_{\text{sal}}^{\ddagger}$ , the equilibrium concentration of the external  $i$ th ion, at the energy barrier and express this relationship with the equation

$$(V_i)_{\text{sal}}^{\text{inward}} = \frac{kT}{h} [p_i^{\text{ex}}]_{\text{sal}}^{\ddagger} \quad (11-15)$$

where

$$[p_i^{\text{ex}}]_{\text{sal}}^{\ddagger} = \{\iota\} [p_i]_{\text{ex}} \frac{(p.f.)_i^{\text{ins}}}{(p.f.)_i^{\text{fr}}} \exp\left(\frac{-\varphi - \varepsilon_s^{\ddagger}}{RT}\right). \quad (11-16)$$

Thus  $[p_i^{\text{ex}}]_{\text{sal}}^{\ddagger}$  is equivalent to the concentration of "activated complex" (see Glasstone *et al.*, 1941), while  $\varepsilon_s^{\ddagger}$  is the corresponding activation-energy term. Although adsorption-desorption migration clearly depends on competing ions of the same charge present in the medium, saltatory migration is essentially independent of such ions.

From the basic assumptions of the present hypothesis, we have derived three equations describing the rate of ionic permeation into a fixed-charge system from an external free solution; each equation gives the rate for a different mode of entry. We shall now demonstrate that these equations predict certain phenomena of ionic entry that can readily be put to experimental test.

### C. COMPETITIVE ENTRY OF STRONGLY ADSORBED IONS

Both doublet and triplet adsorption-desorption migration for the  $i$ th external ion depend fundamentally on the mole fraction of fixed ionic sites occupied by the  $i$ th ion [equation (11-9)]. There is only a finite number of such fixed ionic sites; clearly the mole fraction of  $i$ th-ion-fixed-ion doublets depends on the  $i$ th-ion adsorption energy, and on the adsorption energy and concentration of

\* We have presented saltatory migration as totally independent of adsorbed ions and fixed sites to emphasize the lack of direct competition for these sites. In reality, an ion in the process of saltatory migration occupies a position similar to the third ion in a triplet. This is true for interstitial ions in general, whether they are on the surface or within the bulk of the fixed-charge system. Since the  $\text{Na}^+$  ion prefers high configurations, this also partially accounts for the high percentage of  $\text{Na}^+$  ions existing as interstitial ions in the bulk phase and on the surface (see Sections 11.2A and 11.3C).

counterions competing for the same sites. We define  $\mathcal{K}_i$ , the dissociation constant for  $i$ th-ion adsorption,

$$\mathcal{K}_i = \frac{(\text{p.f.})_i^{\text{fr}}}{(\text{p.f.})_i^{\text{ads}}} \exp\left(\frac{\Delta E_i}{RT}\right). \quad (11-17)$$

Note that the dissociation constant  $\mathcal{K}_i$  is the reciprocal of the equilibrium constant  $K_i$ . Let us assume that there are only two major external ions,  $p_i$  and  $p_j$ , competing for the sites and neglect all intracellular ions. At equilibrium, of  $\{\mathfrak{f}\}_0$ , the total number of fixed ions per unit surface area,  $\{\mathfrak{f}\}_{\text{vac}}$  are vacant. Thus we can write,

$$\{\mathfrak{f}\}_0 = \{\mathfrak{f}\}_{\text{vac}} + \{p_i^{\text{ex}} \cdot \mathfrak{f}\} + \{p_j^{\text{ex}} \cdot \mathfrak{f}\}. \quad (11-18)$$

However,

$$\{p_i^{\text{ex}} \cdot \mathfrak{f}\} = [p_i]_{\text{ex}} \{\mathfrak{f}\}_{\text{vac}} \frac{1}{\mathcal{K}_i} \quad (11-19)$$

$$\{p_j^{\text{ex}} \cdot \mathfrak{f}\} = [p_j]_{\text{ex}} \{\mathfrak{f}\}_{\text{vac}} \frac{1}{\mathcal{K}_j}. \quad (11-20)$$

Since the rate of  $i$ th-ion permeation is proportional to the number of  $i$ th-ion-fixed-site pairs, one may expect that a theoretical maximum rate of entry,  $(V_i^{\text{inward}})_{\text{max}}$ , should be reached when  $\{p_i^{\text{ex}} \cdot \mathfrak{f}\} = \{\mathfrak{f}\}_0$  and that the actual rate of  $i$ th-ion permeation,  $V_i^{\text{inward}}$ , should be related to  $(V_i^{\text{inward}})_{\text{max}}$  in such a way that

$$\frac{(V_i^{\text{inward}})_{\text{max}}}{V_i^{\text{inward}}} = \frac{\{\mathfrak{f}\}_0}{\{p_i^{\text{ex}} \cdot \mathfrak{f}\}}. \quad (11-21)$$

Substitution of equations (11-19) and (11-20) into equation (11-18) yields

$$\begin{aligned} \{\mathfrak{f}\}_0 &= \{\mathfrak{f}\}_{\text{vac}} + \{\mathfrak{f}\}_{\text{vac}} \frac{1}{\mathcal{K}_i} [p_i]_{\text{ex}} + \{\mathfrak{f}\}_{\text{vac}} \frac{1}{\mathcal{K}_j} [p_j]_{\text{ex}} \\ &= \{\mathfrak{f}\}_{\text{vac}} \left( 1 + \frac{[p_i]_{\text{ex}}}{\mathcal{K}_i} + \frac{[p_j]_{\text{ex}}}{\mathcal{K}_j} \right). \end{aligned} \quad (11-22)$$

Then,

$$\frac{(V_i^{\text{inward}})_{\text{max}}}{V_i^{\text{inward}}} = \frac{\mathcal{K}_i}{[p_i]_{\text{ex}}} \left( 1 + \frac{[p_i]_{\text{ex}}}{\mathcal{K}_i} + \frac{[p_j]_{\text{ex}}}{\mathcal{K}_j} \right) \quad (11-23)$$

and

$$\frac{1}{V_i^{\text{inward}}} = \frac{1}{(V_i^{\text{inward}})_{\text{max}}} \left( \mathcal{K}_i + \frac{\mathcal{K}_i [p_j]_{\text{ex}}}{\mathcal{K}_j} \right) \frac{1}{[p_i]_{\text{ex}}} + \frac{1}{(V_i^{\text{inward}})_{\text{max}}}. \quad (11-24)$$

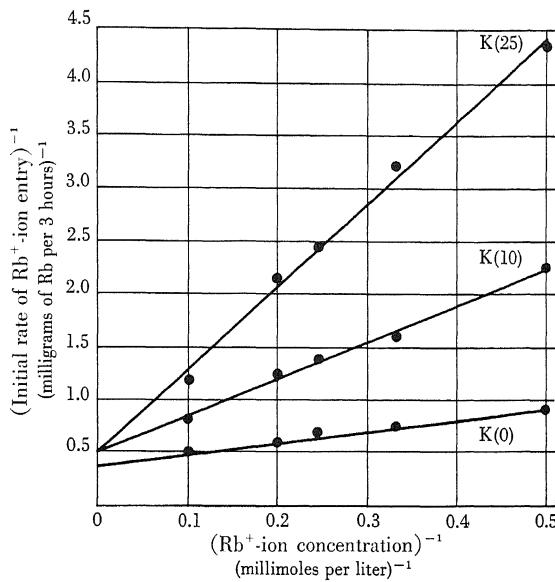
This equation predicts that if, for the  $i$ th ion, the major route of entry is either doublet or triplet dissociation, a plot of the reciprocal of  $V_i^{\text{inward}}$ , the initial rate of entry of the  $i$ th ion, against the reciprocal of  $[p_i]_{\text{ex}}$ , the  $i$ th-ion concentration,

should give a straight line.\* Further, if a family of straight lines is obtained from plots of equation (11-24) at different concentrations of the  $j$ th interfering ion, these should converge to the same locus on the ordinate when  $[p_j]_{\text{ex}}$  approaches infinity. If, for a particular pair of ions, experimental data give these results, the correctness of the basic assumption is proven.

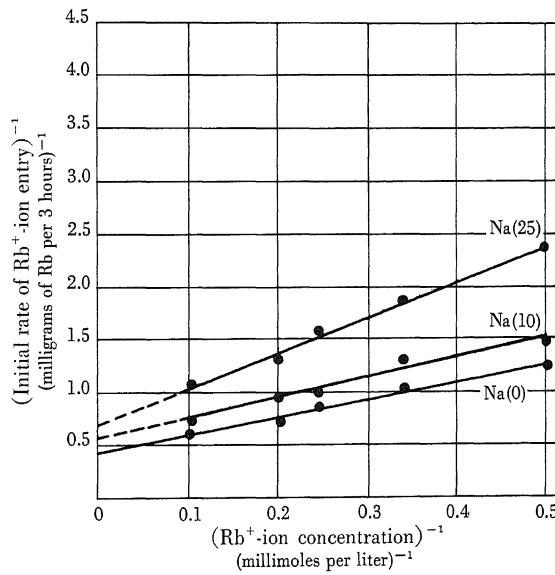
Epstein and Hagen (1952) performed a formal kinetic analysis of this nature for cation permeability. It demonstrates competitive inhibition among  $\text{K}^+$ ,  $\text{Rb}^+$ , and  $\text{Cs}^+$  ions for entry into barley roots, and a lack of such direct competition between  $\text{Na}^+$  and  $\text{Rb}^+$  ions. A part of this data, plotted according to equation (11-24) is reproduced in Figure 11.9. The present author found similar competition in the permeation of radioactive alkali-metal ions,  $\text{K}^+$ ,  $\text{Rb}^+$ , and  $\text{Cs}^+$ , into muscle cells and interpreted his results in terms of the fixed-charge hypothesis† as evidence that strongly adsorbed ions enter the living-cell fixed-charge system primarily by a process of association with, followed by dissociation from, fixed ionic sites and that in this process there is competition for the fixed sites as suggested by Figure 11.10 (see Ling, 1960; for abstracts, see Ling, 1953, 1955b; Ling and Schmolinske, 1954). E. J. Conway and Duggan (1958), working with yeast, arrived at the same competitive relationship. Epstein and Hagen, and Conway and Duggan interpreted their results in terms of a "carrier" mechanism (see also Section 9.3C on the entry of amino acids). A carrier molecule is thought to shuttle back and forth within the cell membrane transporting its ionic passengers between the intracellular and extracellular phases. Such a mechanism requires energy in quantities that are not consistent with the experimental observations presented in Chapter 8. There is no known model system which actually demonstrates such competitive ionic entry on the basis of a carrier system. On the other hand, numerous fixed-charge systems exist; the present hypothesis demands that competitive ionic entry be demonstrable in all of these systems, whether alive or not. To test this hypothesis, we have chosen three well-established fixed-charge systems (Ling and Kushnir, unpublished), a sulfonate exchange resin of the polymerized polystyrene-divinylbenzene type bearing fixed sulfonate groups; an oxidized dried collodion membrane long known to contain a large number of fixed carboxyl groups (Sollner *et al.*, 1941a,b); wool, a pure protein whose glutamic-acid and aspartic-acid residues (at a concentration of about 800 mM/kg, see Alexander and Hudson, 1954) should provide a large number of fixed anionic sites.

\* The derivation of this equation is analogous to the Michaelis-Menten derivation of the theory of the rate of enzyme activity. The present application, however, does not involve chemical changes like those which underlie all enzyme activities, the adsorption-desorption process leaves the ions entirely unchanged although the kinetics followed are nearly identical. This method of plotting the reciprocal of the rate of reaction against the reciprocal of the concentration of the adsorbent studied was first suggested by Hitchcock (1931) who advocated its use in testing the Langmuir adsorption equation. Such a plot is commonly known as a Line-weaver and Burk plot.

† For the abandonment of this older title, the fixed-charge hypothesis, and the introduction of the new title, the association-induction hypothesis, see footnote on page 214.

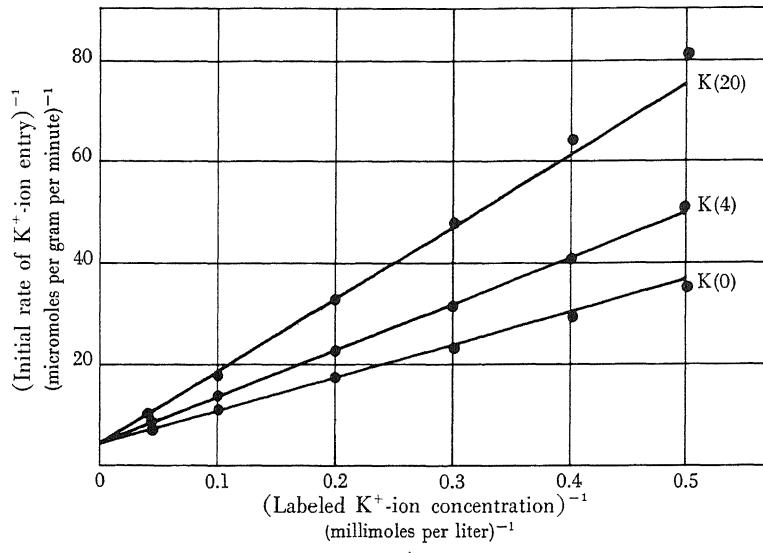


A

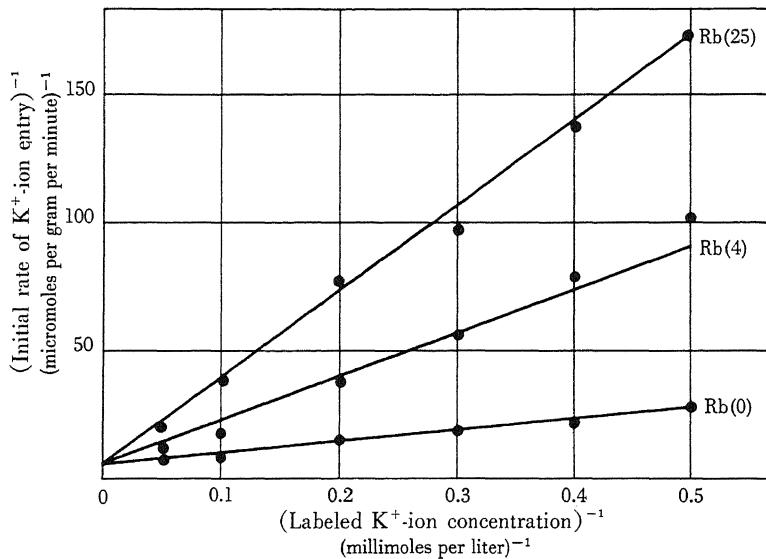


B

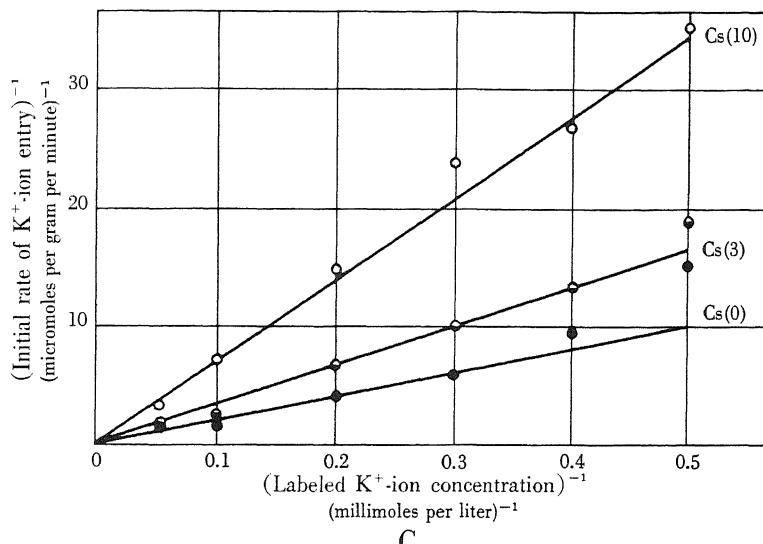
**Figure 11.9. INHIBITION BY  $\text{K}^+$  AND  $\text{Na}^+$  OF THE INITIAL RATE OF RADIOACTIVE  $\text{Rb}^+$ -ION ACCUMULATION IN BARLEY ROOTS (24°C).** A, competitive inhibition by  $\text{K}^+$  ion (0, 10, 25mM); B,  $\text{Na}^+$  ion (0, 10, 25mM) does not show the same effect. (Figure after Epstein and Hagen, 1952.)



A



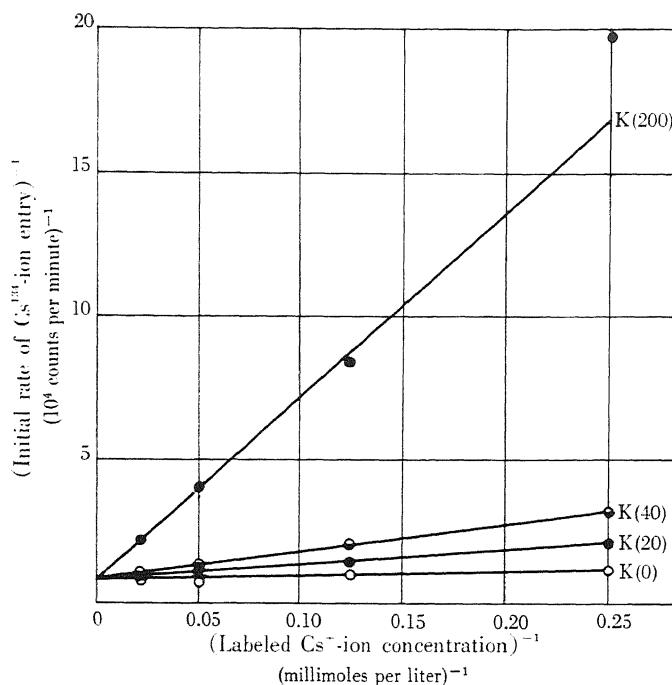
B



**Figure 11.10.** THE EFFECT OF VARYING THE EXTERNAL  $K^+$ -,  $Rb^+$ -, AND  $Cs^+$ -ION CONCENTRATIONS UPON THE INITIAL RATE OF ENTRY OF  $K^+$  ION INTO FROG MUSCLES ( $0^\circ C$ ). A, the entry of  $K^{42}$  into frog *sartorius*, *semitendinosus*, *tibialis anticus longus*, and *iliofibularis* in the presence of 0, 4, and  $20\text{mM}$   $K^+$  ion, pH 7.4. B, the entry of  $K^{42}$  into the above muscles in the presence of 0, 4, and  $25\text{mM}$   $Rb^+$  ion, pH 7.4 (Ling, 1960). C, the entry of  $K^{42}$  into the above muscles in the presence of 0, 3, and  $10\text{mM}$   $Cs^+$  ion, pH 7.4. For these experiments, the rates of ion entry are given in number of moles (or micromoles) per gram of fresh tissue per minute. These rates can be readily converted to number of moles per square centimeter of cell surface per minute by using the average area/weight ratio of frog muscle cells given in Table 8.7 (that is,  $550\text{ cm}^2/\text{g}$ ).

Studies of the entry of  $Cs^{134}$  into each of these fixed-charge systems in the presence of varying amounts of  $K^+$  ion were made and the results are shown for sulfonate exchange resin in Figure 11.11, for dried collodion in Figure 11.12, and for sheep wool in Figure 11.13. All unequivocally show the type of competitive ionic entry that occurs in living cells. Since we cannot imagine the exchange resin, the dried collodion, or the sheep wool to possess ionic carriers or pumps, the kinetics observed must arise from the nature of the fixed-charge system itself.\*

\* The fact that competitive ionic entry has been established in all fixed-charge systems which have been studied has additional significance. It means that, within the surface layer of a two-dimensional fixed-charge system, the majority of ions occupy sites; that is, most ions are associated. Only a minority occupy the interstices. This is a clear demonstration of the thesis that nearly complete counterion association exists in these systems (Chapter 2).



**Figure 11.11. THE EFFECT OF VARYING THE EXTERNAL  $\text{K}^+$ -ION CONCENTRATION ON THE RATE OF ENTRY OF  $\text{Cs}^{134}$  INTO SHEETS OF SULFONATE EXCHANGE RESIN, NALFILM-1 ( $25^\circ\text{C}$ ). The values for the  $\text{K}^+$ - and  $\text{Cs}^+$ -ion concentrations are given in units of 4 mM/l. The rates of entry are in terms of  $10^4$  counts per minute per arbitrary unit surface measured at pH 7.0 to 7.1. (Ling, 1960.)**

#### D. THE RATE OF ENTRY AND THE FREE ENERGY OF ADSORPTION

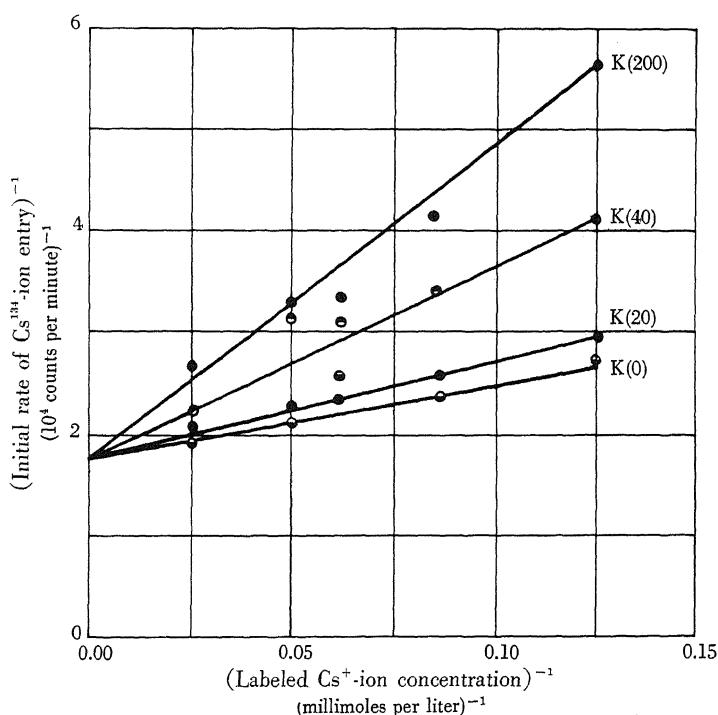
Equation (11-24) provides a means of determining whether ions enter by an adsorption-desorption process or by noncompetitive migration. It also provides a method for finding the dissociation constants  $\kappa_i$ , and through these, the free energy of adsorption since  $\Delta F_i = RT \ln \kappa_i$  and  $\Delta F_j = RT \ln \kappa_j$ , where  $\kappa_i$  is given by equation (11-17). In Table 11.1, the results of several series of experiments are presented. The free energy of adsorption  $\Delta F_i$  which is low in comparison with the calculated adsorption energy  $\Delta E_i$  indicates the relatively large partition function ratio,  $(\text{p.f.})_i^{\text{fr}} / (\text{p.f.})_i^{\text{ads}}$ , for each of these ions. This is due to the fact that the process occurs at the surface;  $(\text{p.f.})_i^{\text{fr}}$  refers to the partition function of the  $i$ th ion in a dilute free solution (see Section 2.4).

The data given in Table 11.2 represents the rates of entry of radioactive  $\text{K}^+$ ,  $\text{Rb}^+$ , and  $\text{Cs}^+$  ions at various temperatures in Ringer's solution where these ions were present in quantities negligible compared with the quantity of  $\text{Na}^+$  ion. At all temperatures tested, the rate of entry for the three alkali-metal ions followed

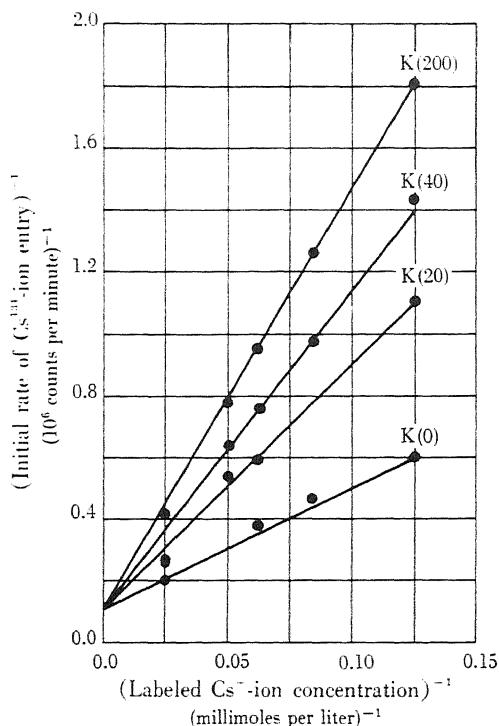
the order  $K^+ > Rb^+ > Cs^+$ . However, the  $\Delta F$  values are such that the adsorption order is  $Rb^+ > Cs^+ > K^+$ . We shall demonstrate that a substantial proportion of the  $i$ th ions enter by the triplet route. Such entry involves two steps: First, a successful occupation of a site by a  $p_i$  ion (again, the proportion of sites occupied varies *directly* with the magnitude of the free energy of adsorption  $\Delta F_i$ ), and second, dislodgment of the  $i$ th ion by a second countercation, predominantly the  $Na^+$  ion. The success of the second step varies *inversely* with the magnitude of the free energy of adsorption  $\Delta F_i$ . Clearly, an optimal  $\Delta F_i$  for a maximal rate of entry must exist; our data indicate that it is close to the  $\Delta F$  of the  $K^+$  ion.

#### E. LACK OF COMPETITION IN INTERSTITIAL PERMEATION

If the adsorption energy of a  $K^+$  ion on the surface fixed ionic sites is very much greater than that of a  $Na^+$  ion (see Chapter 10), the relative probability that a  $Na^+$  ion will successfully compete for the surface sites is very low. But, if the  $Na^+$



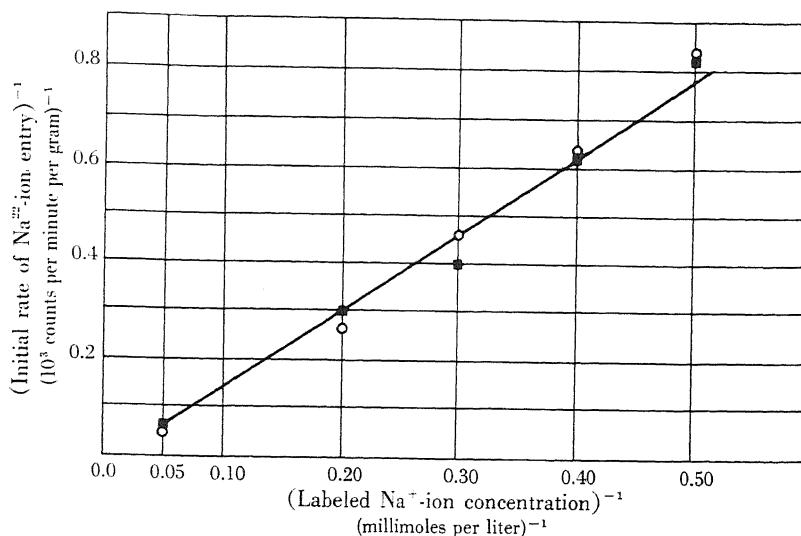
**Figure 11.12. THE EFFECT OF VARYING THE EXTERNAL  $K^+$ -ION CONCENTRATION ON THE INITIAL RATE OF  $Cs^{134}$  ENTRY INTO OXIDIZED COLLODION MEMBRANES.** The  $K^+$ - and  $Cs^+$ -ion concentrations are in units of 4 mM/l. The rates of entry are in terms of  $10^4$  counts per minute per unit surface as defined by the lucite rods of identical dimensions on which the collodion membranes were cast ( $25^\circ C$ , pH 7.0 to 7.1). (Ling, 1960.)



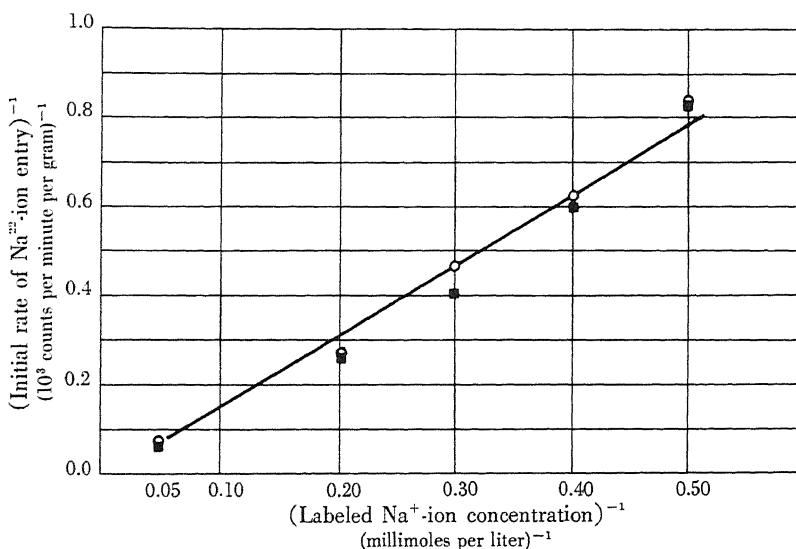
**Figure 11.13.** THE EFFECT OF VARYING THE EXTERNAL K<sup>+</sup>-ION CONCENTRATION ON THE RATE OF Cs<sup>134</sup> ENTRY INTO WOOL. The experiment was performed on 0.03g of virgin, scoured, defatted sheep's wool. The values for the K<sup>+</sup>- and Cs<sup>+</sup>-ion concentrations are in millimoles per liter (25°C, pH 7.0 to 7.1). (Ling, 1960.)

Date	$-\Delta F_i$ , kcal/mole	$-\Delta F_j$ , kcal/mole	$-\Delta F_j$ , kcal/mole	$-\Delta F_j$ , kcal/mole
3-10-54	K : 2.35	Rb : (4mM) 3.88	Rb : (15mM) 3.48	Rb : (25mM) 3.24
3-10-54	K : 2.38	Rb : (4mM) 3.82	Rb : (15mM) 3.68	—
3-11-54	K : 2.63	K : (4mM) 2.70	K : (20mM) 2.37	K : (30mM) 2.47
3-11-54	K : 2.60	K : (4mM) 2.63	K : (20mM) 2.39	K : (30mM) 2.35
8-31-53	Rb: 3.80	K : (2.5mM) 2.73	—	—
4-26-55	K : 2.34	Cs : (3mM) 3.68	Cs : (10mM) 3.21	—

**Table 11.1.** THE FREE ENERGY OF ASSOCIATION OF K<sup>+</sup>, Rb<sup>+</sup>, AND Cs<sup>+</sup> IONS ON THE SURFACE OF MUSCLE CELLS. These data were calculated from studies of ionic-entry rates similar to, and including, the data given in Figure 11.10. For each experimental series more than one free-energy value is obtained, one for the ion whose entry rate is being studied ( $\Delta F_i$ ) and the others for the inhibiting ion ( $\Delta F_j$ ); all values are given in the table.



**Figure 11.14. THE EFFECT OF VARYING THE  $\text{Na}^+$ -ION CONCENTRATION ON THE RATE OF ENTRY OF  $\text{Na}^{22}$  ( $15^\circ\text{C}$ ).** Frog *sartorius* muscles were equilibrated for 15 minutes in solutions containing various combinations of radioactive and nonradioactive  $\text{Na}^+$  ion; they were then washed for five minutes ( $0^\circ\text{C}$ ) in a nonradioactive normal Ringer's solution. Other procedures were similar to those given under Table 11.2. Open circles indicate values obtained for 46mM NaCl; squares indicate values obtained for 92mM NaCl.



**Figure 11.15. THE EFFECT OF VARYING EXTERNAL  $\text{Rb}^+$ -ION CONCENTRATION ON THE INITIAL RATE OF ENTRY OF  $\text{Na}^{22}$  INTO FROG MUSCLE.** Data were obtained by procedures similar to those described under Figure 11.14. Open circles indicate values obtained for 0mM RbCl; squares indicate values obtained for 46mM RbCl.

Date	Ion	Soaking time, min	Temperature						
			0°	5°	10°	15°	20°	25°	30°
7-13-54	K <sup>42</sup>	15	16.1	24.0	23.0	29.1	38.8	40.0	60.2
			16.3	16.8	26.4	34.9	42.4	55.8	49.8
			15.6	18.2	25.8	24.6	36.9	61.2	45.0
			11.3	15.9	24.7	16.2	35.7	48.6	45.7
Average			(14.8)	(18.7)	(25.0)	(29.5)	(38.5)	(51.1)	(47.7)
6-16-54	Rb <sup>86</sup>	15	5.00	8.40	10.28	16.80	18.30	24.60	28.30
7-19-54			4.53	5.55	7.68	14.80	13.40	17.00	20.60
			4.79	9.43	11.90	19.35	18.38	19.98	16.05
7-22-54			5.07	13.00	13.75	15.36	19.97	22.60	31.50
			6.50	9.13	11.91	23.50	15.68	23.60	29.86
7-28-54			3.63	5.10	9.15	11.69	16.67	19.30	21.10
			3.96	6.08	8.88	11.70	14.80	22.65	24.62
8-24-54			3.80	6.08	8.44	16.56	16.32	23.52	—
			4.92	6.20	10.04	13.84	15.95	15.32	20.20
			4.60	6.04	9.32	15.04	20.20	20.44	18.96
			4.00	5.88	8.76	14.52	16.44	17.72	21.00
Average			(4.62)	(7.35)	(10.01)	(15.74)	(16.92)	(20.61)	(23.22)
7-21-54	Cs <sup>134</sup>	15	2.24	2.29	3.43	4.19	6.33	6.03	9.34
			—	2.60	3.62	5.24	5.78	7.55	9.71
8-3-54			2.38	3.51	3.86	4.46	4.24	5.94	7.14
			2.48	3.22	4.73	4.15	4.07	4.69	5.90
8-4-54			2.17	3.50	4.48	5.24	4.54	6.32	8.22
			2.84	3.82	3.36	5.36	4.98	5.42	7.49
Average			(2.42)	(3.16)	(3.91)	(4.77)	(4.99)	(5.99)	(7.97)
9-21-61 <sup>a</sup>	Cs <sup>137</sup>	15	1.33	—	—	—	2.14	—	3.46
10-6-61 <sup>a</sup>			1.75	—	2.39	—	3.11	—	4.06
1-15-62 <sup>a</sup>		60	0.492	—	1.10	—	1.97	—	2.99
			(0.487) <sup>b</sup>	—	(1.09) <sup>b</sup>	—	(1.96) <sup>b</sup>	—	(2.96) <sup>b</sup>
9-21-61 <sup>a</sup>	Na <sup>22</sup>	15	1.36	—	—	—	2.05	—	2.48
10-26-61 <sup>a</sup>			1.98	—	2.33	—	2.60	—	3.71
10-27-61 <sup>a</sup>			1.89	—	2.07	—	2.64	—	3.26

**Table 11.2. THE INITIAL RATES OF ENTRY OF VARIOUS ALKALI-METAL IONS INTO FROG MUSCLES AT VARIOUS TEMPERATURES.** Frog *semilendinosus*, *tibialis anticus longus*, and *iliofibularis* muscles were soaked for 15 minutes in a Ringer's solution containing 2.5 mM/1 of the ion to be studied and the corresponding isotope (K<sup>42</sup>, Rb<sup>86</sup>, and Cs<sup>134</sup>). Na<sup>22</sup> studies were made using frog *sartorius* muscles in a Ringer's solution containing 102 mM/1 of the Na<sup>+</sup> ion. The muscles were then vigorously washed for 15 minutes (for Na<sup>22</sup> experiments, 5 minutes) in nonradioactive Ringer's solution at 0°C, blotted dry, and transferred to a lusteroid tube containing 1cc 0.1N HCl. After being heated in a hot-water bath for one minute, the tube containing the muscle and extract was placed in a scintillation well counter and its total radioactivity assayed. Data for Cs<sup>137</sup> were obtained by a similar experimental procedure except that frog *sartorius* muscles were used. The lower values

ion exists in very high concentration outside the cells, a relatively larger fraction of  $\text{Na}^+$  ion will have sufficient kinetic energy to enter by interstitial permeation. In this process, no site occupation is involved; thus, one would not then expect competition in entry of such ions. Given this condition, the rate of entry of  $\text{Na}^{22}$  should be independent of the presence of other ions, whether they are nonradioactive  $\text{Na}^+$  ions or  $\text{Rb}^+$  ions. Figures 11.14 and 11.15 show experimental data\* which verify both of these expectations.

That  $\text{Na}^+$  ions enter primarily by interstitial permeation follows from the highly unfavorable adsorption energy for the  $\text{Na}^+$  ion in comparison with that of the  $\text{K}^+$  ion on the surface fixed sites (see Chapter 10). In other cells, such as mouse ascites cells, the  $\text{Na}^{22}$  ion competes for entry with both nonradioactive  $\text{Na}^+$  ions and  $\text{K}^+$  ions. This indicates a much smaller selectivity for  $\text{K}^+$  ions over  $\text{Na}^+$  ions on the surface anionic sites of these cells (Figures 11.16 and 11.17).

#### F. FACILITATION OF ION PERMEATION BY WEAKLY ADSORBED IONS

Table 11.2 presents the results of experiments in which the permeation of various radioactive ions was determined at various temperatures. The inward  $\text{Na}^+$ -ion permeability appears to be rather small, a fact which may be interpreted as being due to its ineffectiveness in occupying the surface sites [see equation (11-9)]. However, equation (11-13) predicts that a weakly adsorbed ion like the  $\text{Na}^+$  ion, although ineffective in competing for sites, may be quite effective in serving as the second countercation in triplet formation. This is especially true if the  $\text{Na}^+$  ion is

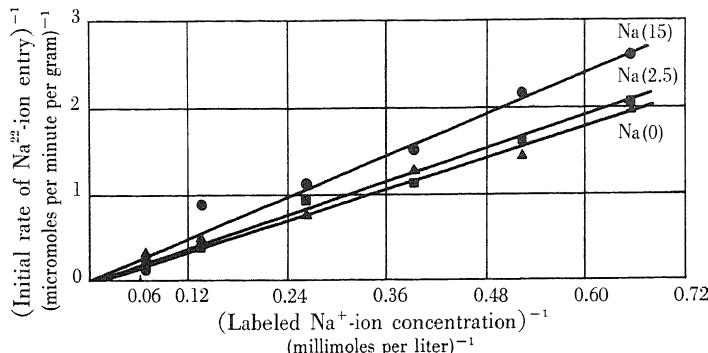
\* To measure the rate of ion exchange across cell surfaces, a "washout" curve like those shown in Figures 11.2 and 11.25 is usually used. If the plot of  $\ln C_i$  versus time is rectilinear, the slope of the curve gives the rate of exchange for ions like  $\text{K}^+$ . However, the fact that  $\text{Na}^+$ -ion exchange is bulk-phase limited makes the common practice of artificially straightening the beginning of the curve and finding the permeability rate from this straight piece totally unacceptable and erroneous. In Chapter 8, we presented, in detail, data that lead to the establishment of a method for estimating the correct rate of exchange; this method was used in these and other studies. Although the rate of entry thus determined is considerably higher than that often given, it is somewhat lower than the true rate of exchange.

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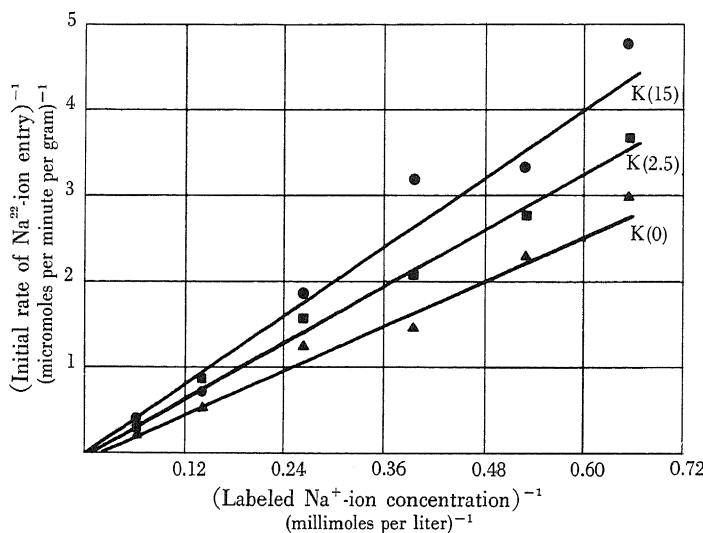
obtained for this series are due to the smaller percentage of connective tissue present in the *sartorius* muscle as compared with the other three (that is, *semitendinosus*, *tibialis anticus longus*, and *iliofibularis*). Connective tissue initially adsorbs the  $\text{Cs}^+$  ion at a more rapid rate than do muscle fibers; by long-term soaking, the effect of the difference in the rate of entry is minimized. Unless otherwise stated, these data have not been corrected for adsorption onto connective tissue. In the cases of  $\text{Rb}^+$  and  $\text{K}^+$  ions, other experiments have shown that the error thus introduced is relatively insignificant. Rates of entry are given in  $10^{-9}$  moles per gram of fresh tissue per minute per millimole of ion in the external solution. For conversion of data to number of moles per square centimeter of cell surface per minute per millimole of external ion, divide by the average area/weight ratio of  $550 \text{ cm}^2/\text{g}$  (see Table 8.7).

<sup>a</sup> Each value in the series is the average of eight *sartorius* muscles.

<sup>b</sup> Data corrected for cation adsorption onto connective tissues.

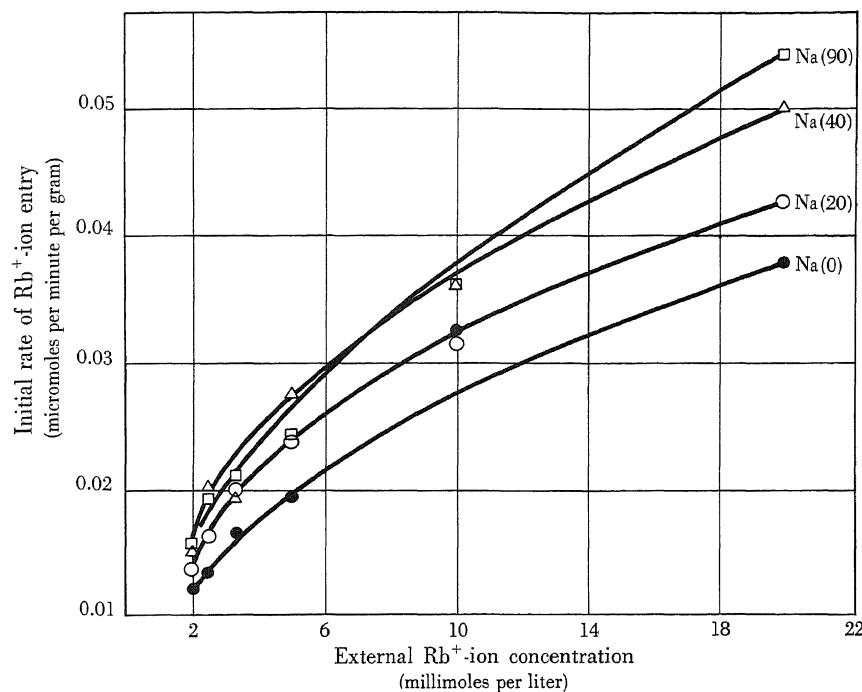


**Figure 11.16.** THE EFFECT OF VARYING EXTERNAL  $\text{Na}^+$ -ION CONCENTRATION ON THE INITIAL RATE OF ENTRY OF  $\text{Na}^{22}$  INTO ASCITES CELLS. Washed ascites cells were incubated for ten minutes at  $23^\circ\text{C}$  in a Ringer's solution containing  $\text{Na}^{22}$  and then separated by centrifugation; the radioactivity was counted.



**Figure 11.17.** THE EFFECT OF  $\text{K}^+$ -ION CONCENTRATION ON THE INITIAL RATE OF ENTRY OF  $\text{Na}^{22}$  INTO ASCITES CELLS. The procedure was similar to that used to obtain the data for Figure 11.16.

present at a high concentration when it may increase the rate of entry of another cation. Figure 11.18 shows that the substitution of isotonic  $\text{NaCl}$  for isotonic glucose significantly increases the rate of entry of the  $\text{Rb}^+$  ion. This increase is proportional to the  $\text{Na}^+$ -ion concentration and strongly indicates the importance of the triplet route for entry of relatively strongly adsorbed ions.



**Figure 11.18.** THE FACILITATION OF Rb<sup>+</sup>-ION ENTRY INTO FROG MUSCLES BY THE Na<sup>+</sup> ION. Experimental procedures were similar to those given under Table 11.2, but at 0°C.

### 11.3. Ionic Diffusion

According to the association-induction hypothesis, cells are bulk-phase fixed-charge systems. We should thus expect that the lowering or removal of rate-limiting surface barriers to diffusion will force bulk-phase diffusion to become rate limiting. A study of the diffusion of ions into or out of the cell under these conditions should reveal the characteristics of bulk-phase diffusion, which should differ significantly from the characteristics of diffusion phenomena in dilute aqueous solution. Since the surface and the bulk phase are both fixed-charge systems, diffusion within the bulk phase should have much in common with surface permeation as discussed in the preceding section. We shall present experimental evidence to support both of these expectations.

#### A. DIFFUSION IN MODELS OF FIXED-CHARGE SYSTEMS

If cell surfaces are fixed-charge systems, ionic permeation through these should possess behavioral patterns which are shared by all other established fixed-charge

systems. Comparative studies of ionic entry into frog muscle, exchange resins, dried collodion, and wool have established this. Similarly, if the bulk phase of the cytoplasm is also a fixed-charge system, diffusion of ions through it should resemble diffusion within other bulk-phase fixed-charge systems. This subject will serve as an introduction to the problems of ionic diffusion within living cells.

Recently a new mathematical technique for the study of radioactive ion exchange was developed by Pitts (1954). His method permits a comparison between theoretical patterns of diffusion and the experimental facts. Spherical systems such as exchange-resin beads containing no radioactive ions are suspended in a solution containing radioactive ions at concentration  $C(0)$ ; the final equilibrium concentration of radioactive ion in the solution is  $C(\infty)$ . We define the function  $Q(t)$ :

$$Q(t) = \frac{C(0) - C(t)}{C(0) - C(\infty)}. \quad (11-25)$$

Values of this function can be found from successive measurements of  $C(t)$ , the radioactivity in the solution, at various times  $t$ . We define another function  $\tau$ :

$$\tau = \frac{Dt}{r_0^2} \quad (11-26)$$

where  $D$  is the diffusion coefficient and  $r_0$ , the radius of the particle. Assigning various arbitrary values,  $10^{-2}$ ,  $10^{-1}$ , 1, 10,  $10^2$ , and so on, to  $\tau$ , we can calculate  $C(\tau)$ , the expected concentration of radioisotope which corresponds to each value of  $\tau$  from the known values of the total volume of the spheres and of the solution and the total ion concentrations in both phases. We define a third function  $Q(\tau)$ ,

$$Q(\tau) = \frac{C(0) - C(\tau)}{C(0) - C(\infty)}. \quad (11-27)$$

Let us plot  $Q(\tau)$  against  $\tau$ . From this curve and the plot of  $Q(t)$  versus  $t$ , the correspondence between  $t$  and  $\tau$  for equal values of  $Q(t)$  and  $Q(\tau)$  can easily be found. Having found this, we can plot  $\log \tau$  against  $\log t$ , since equation (11-26) provides that

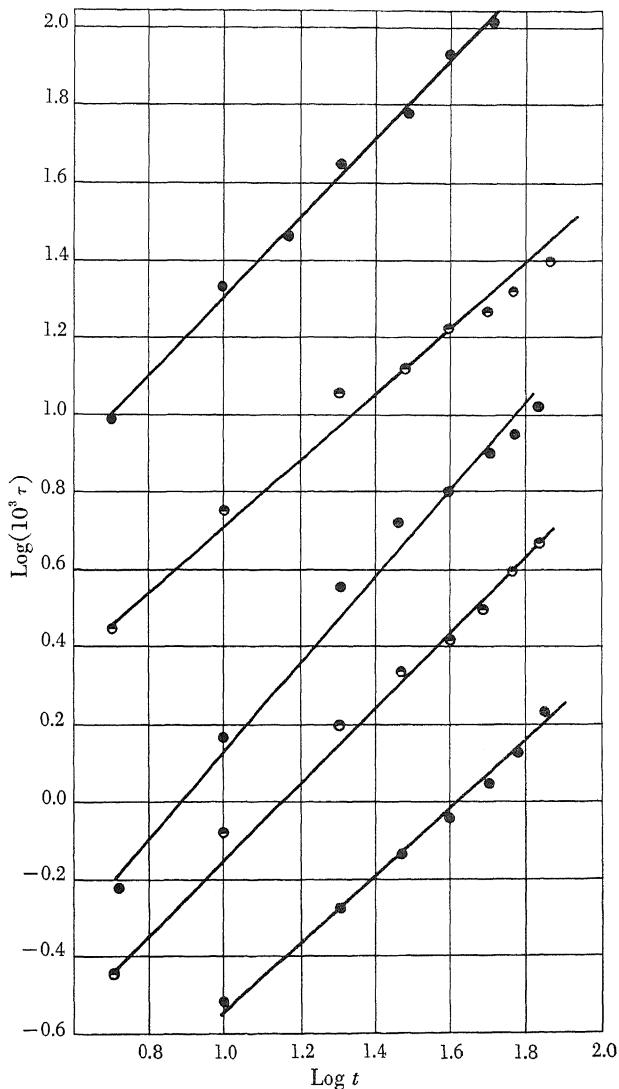
$$\log \tau = \log t + \log (D/a^2). \quad (11-28)$$

This plot should be a straight line with slope near unity in the case of bulk-phase-limited entry. Pitts pointed out that this slope of unity is independent of scattering in the sizes of the spheres.

If the rate-limiting step is at the surface and there is no significant scattering of particle sizes, a plot of  $\log P(t)$  against  $t$  should be a straight line,\* where

$$P(t) = 1 - Q(t). \quad (11-29)$$

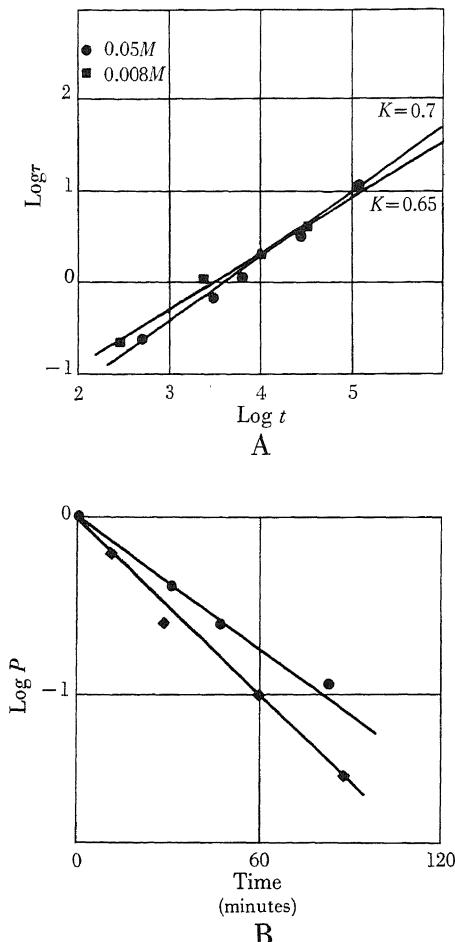
\* This relation is, in essence, that represented by equation (11-3).



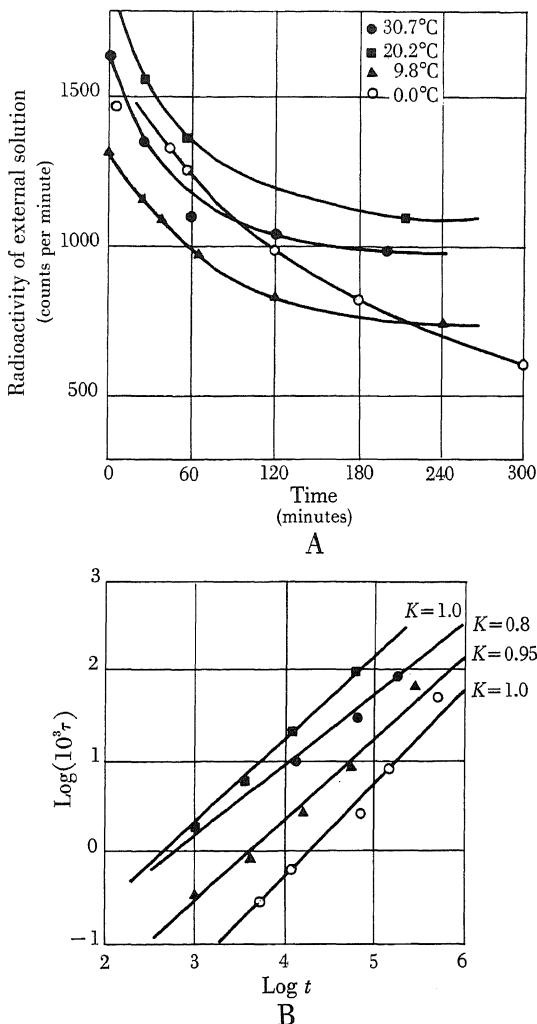
**Figure 11.19.** THE TIME COURSE OF RADIOACTIVE  $\text{Ag}^+$ -ION EXCHANGE IN  $\text{AgCl}$  CRYSTALS, PLOTTED ACCORDING TO EQUATION (11-28). Data from Langer (1942, 1943). The slopes of the curves, from top to bottom, are 1.63, 0.82, 1.06, 0.96, and 0.91. (Figure after Pitts, 1954.)

(1)  $\text{Ag}^+$ -ion exchange in  $\text{AgBr}$  crystals

Pitts (1954) derived these theoretical equations primarily to determine whether the diffusion of  $\text{Ag}^+$  and  $\text{Br}^-$  ions within  $\text{AgBr}$  crystals is surface or bulk-phase limited. The results which are given in Figure 11.19 show that, for the  $\text{Ag}^+$  ion, the rate of exchange is bulk-phase limited.



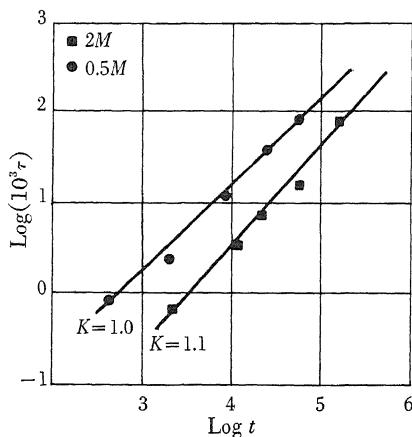
**Figure 11.20.** SURFACE-LIMITED  $\text{Ca}^{++}$ -ION EXCHANGE AT LOW EXTERNAL  $\text{Ca}^{++}$ -ION CONCENTRATIONS. The time course of exchange of  $\text{Ca}^{++}$ -charged cationic-exchange-resin beads (Dowex 50) in a constant volume of external solution containing radioactive calcium at low concentrations ( $0.05M$  and  $0.008M$ ). A, a plot of the data according to equation (11-28); B, a plot according to equation (11-29). In this and the following three figures,  $K$  represents the slope for equation (11-28). (Figures after Sugai and Furuichi, 1955.)



**Figure 11.21. BULK-PHASE-LIMITED  $\text{Ca}^{++}$ -ION EXCHANGE AT HIGH EXTERNAL  $\text{Ca}^{++}$ -ION CONCENTRATION.** The experiments were similar to those described in Figure 11.20, except that the external radioactive  $\text{Ca}^{++}$ -ion concentration is 1.0M. A, the time course of loss of radioactivity from the external solution. Note that these curves are identical to the curves which would be obtained if the resin had been charged with radioactive  $\text{Ca}^{++}$  ion and the time course of loss of radioactivity followed (as for Figure 11.1). B, plots of the data given in A according to equation (11-28); note the slopes are close to unity. (Figures after Sugai and Furuichi, 1955.)

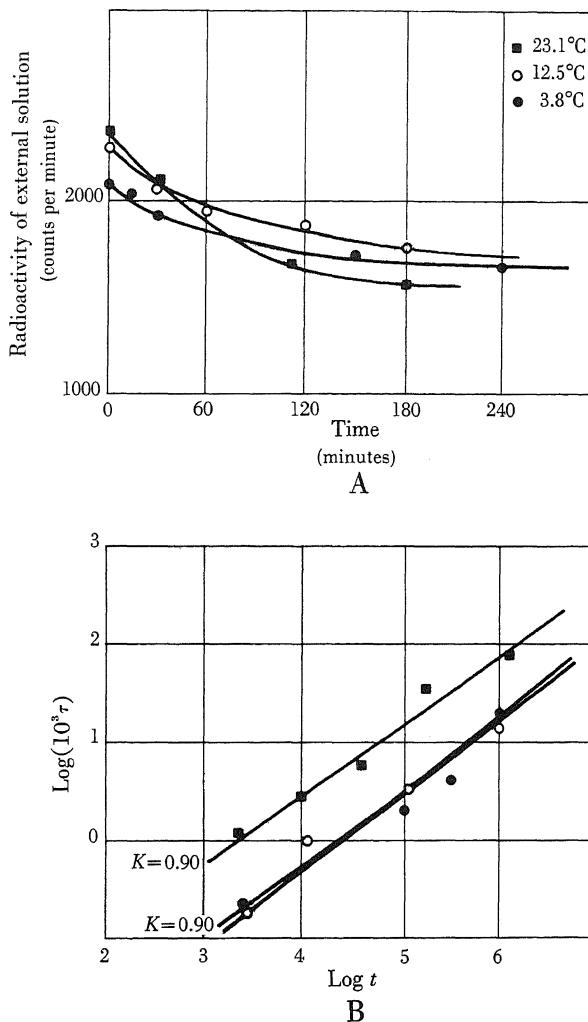
## (2) $\text{Ca}^{++}$ -ion and $\text{Cl}^-$ -ion exchange in exchange resins

Sugai and Furuichi (1955) applied the equation of Pitts in studying the rate of exchange of  $\text{Ca}^{45}$  in  $\text{Ca}^{++}$ -ion-loaded Dowex-50 resin beads and  $\text{Cl}^{36}$  in  $\text{Cl}^-$ -ion-loaded Amberlite IRA-410 beads. Their data, reproduced in Figures 11.20 to 11.23, show that when the external ion concentration is low (that is, less than  $10^{-1}M$ ) the rate-limiting step is at the surface since a plot of  $\log P(t)$  against  $t$  is a



**Figure 11.22. BULK-PHASE-LIMITED  $\text{Ca}^{++}$ -ION EXCHANGE AT HIGH EXTERNAL  $\text{Ca}^{++}$ -ION CONCENTRATION.** Experiments were similar to those described under Figure 11.20, except that the external  $\text{Ca}^{++}$  concentrations were 0.5 and  $2M$ . The data was plotted according to equation (11-28). The data indicate that the critical external concentration which changes the surface-limited exchange shown in Figure 11.20 to bulk-phase-limited exchange is at or below  $0.5M$ . (Figure after Sugai and Furuichi, 1955.)

straight line and the plot of  $\log \tau$  versus  $\log t$  is a line whose slope is not equal to one (Figure 11.20). When the external  $\text{Ca}^{++}$ -ion concentration is raised to  $0.5M$  or more, a plot of  $\log \tau$  against  $\log t$  gives a straight line with a slope close to unity (Figures 11.21 and 11.22) and the  $\log P(t)$ -versus- $t$  plot is not a straight line. These results show that in a fixed-charge system (the exchange resin), bulk-phase-limited diffusion can be revealed by the removal or the widening of the "bottleneck" at the surface through an increase in the concentration of the ion under study. The bulk-phase self-diffusion coefficients for  $\text{Ca}^{++}$  in Dowex-50 and for  $\text{Cl}^-$  in Amberlite IRA-410, reproduced in Table 11.3, are several orders of magnitude lower than the diffusion coefficients of  $\text{CaCl}_2$  and  $\text{KCl}$  in free solution at concentrations similar to the concentrations of those ions within the exchange resin ( $\text{Ca}^{++}$  4 to  $5M$ ).



**Figure 11.23. BULK-PHASE-LIMITED EXCHANGE OF  $\text{Cl}^-$  IN HIGH EXTERNAL  $\text{Cl}^-$ -ION CONCENTRATION.** A, time course of loss of radioactive  $\text{Cl}^-$  from the external solution containing 1.0M labeled NaCl to Amberlite IRA-410 charged with chloride. B, data plotted according to equation (11-28). Note that slopes are close to unity. (Figures after Sugai and Furuichi, 1955.)

Diffusion medium	Diffusing ion	$T, ^\circ\text{C}$	$D, \text{cm}^2/\text{sec}$	Source
AgCl crystals	Ag <sup>+</sup>	—	$\approx 10^{-13}$	Pitts, 1954
Calcium-charged Dowex 50	Ca <sup>45</sup>	1.0	$1.2 \times 10^{-8}$	Sugai and Furuichi, 1955
		9.8	$1.7 \times 10^{-8}$	
		20.2	$5.3 \times 10^{-8}$	
		30.7	$5.8 \times 10^{-8}$	
Chloride-charged Amberlite IRA-410	Cl <sup>36</sup>	3.8	$1.0 \times 10^{-8}$	Sugai and Furuichi, 1955
		12.5	$1.3 \times 10^{-8}$	
		23.1	$2.5 \times 10^{-8}$	
Free solution	CaCl <sub>2</sub> (1.0M)	25.0	$1.220 \times 10^{-5}$	Harned and Owen, 1958
Free solution	K <sup>+</sup> in KCl (1.0M) <sup>a</sup>	25.0	$2.113 \times 10^{-5}$	Friedman and Kennedy, 1955
	(2.0M) <sup>a</sup>	25.0	$2.013 \times 10^{-5}$	
Free solution	Na <sup>+</sup> in NaCl (1.0M) <sup>a</sup>	25.0	$1.234 \times 10^{-5}$	Mills, 1955

Table 11.3. DIFFUSION CONSTANTS IN FREE SOLUTION, EXCHANGE RESINS, AND AgCl CRYSTALS.

<sup>a</sup> Self-diffusion coefficients of the ion.

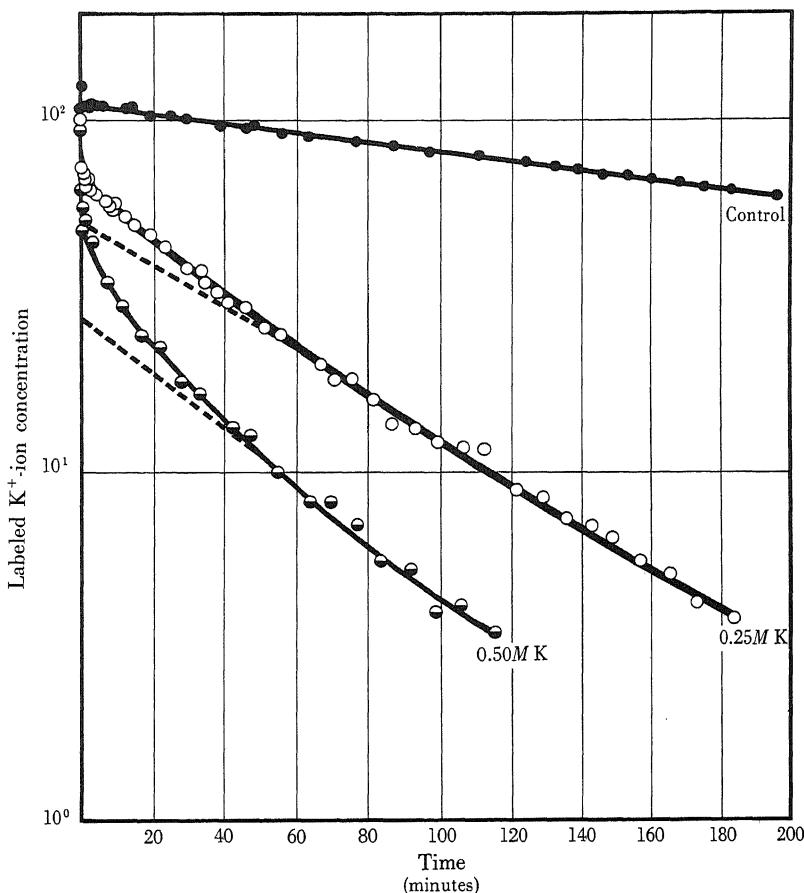
## B. IONIC DIFFUSION IN INTACT CELLS

### (1) Conversion of surface-limited K<sup>+</sup>-ion exchange to bulk-phase-limited exchange

The experimental findings of Sugai and Furuichi suggest another test of the present hypothesis. If muscle cells are bulk-phase fixed-charge systems not unlike exchange-resin beads, a surface-limited ion-exchange process like that found for the K<sup>+</sup> ion should be converted into bulk-phase-limited exchange by an increase in the external K<sup>+</sup>-ion concentration. Figure 11.24 shows that this is the case. A plot of the rate of K<sup>+</sup> exchange, which is unequivocally surface limited in normal Ringer's solution (see Figure 11.2), assumes the curvature characteristics found in semi-logarithmic plots of all bulk-phase-limited exchange processes when the muscle is washed with a highly concentrated (250mM) K<sup>+</sup>-ion solution. These results are analogous to the results of Sugai and Furuichi for exchange-resin beads. The K<sup>+</sup>-ion-exchange curve for muscle washed in the 250mM K<sup>+</sup>-ion solution greatly resembles the curve for Na<sup>+</sup>-ion exchange in a normal Ringer's solution.

### (2) Bulk-phase-limited Na<sup>+</sup>-ion exchange

In Section 11.1E, we demonstrated that Na<sup>+</sup>-ion exchange by frog muscle in normal Ringer's solution is bulk-phase limited as revealed by the washing-out pro-



**Figure 11.24. THE CONVERSION OF SURFACE-LIMITED TO BULK-PHASE-LIMITED  $K^{42}$  EXCHANGE IN FROG MUSCLES.** The surface-limited  $K^{42}$  exchange of frog muscle washed in a stream of normal Ringer's solution containing  $2.5\text{mM}$  KCl (see control muscle and also Figure 11.2) is converted to bulk-phase-limited exchange when the washing solution contains a high  $K^+$ -ion concentration ( $0.25M$ ,  $0.5M$ ). This is analogous to ion exchange in another bulk-phase fixed-charge system, the exchange resin. (See Figures 11.20 to 11.23.) Ordinate:  $10^4$  counts per minute per gram for control muscle;  $10^3$  counts per minute per gram for experimental muscles.

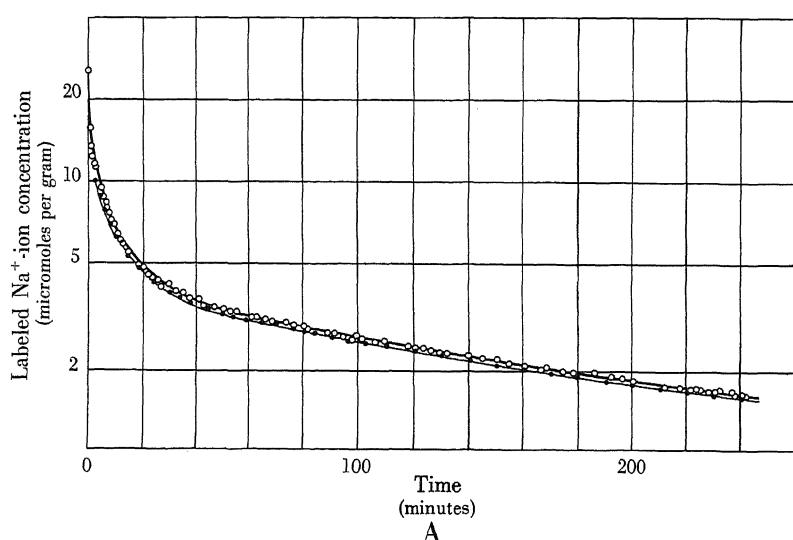
cedure described in Chapter 8. The experimental material used was a single muscle fiber, isolated only after a tedious procedure. It can be shown that either small muscle-fiber bundles or whole *sartorius* muscles are perfectly adequate if suitable corrections are made. First, a correction must be made for the true intercellular space; this consists of channels whose lengths are approximately equal to the thickness of the muscle and in which the ionic self-diffusion constants are the same

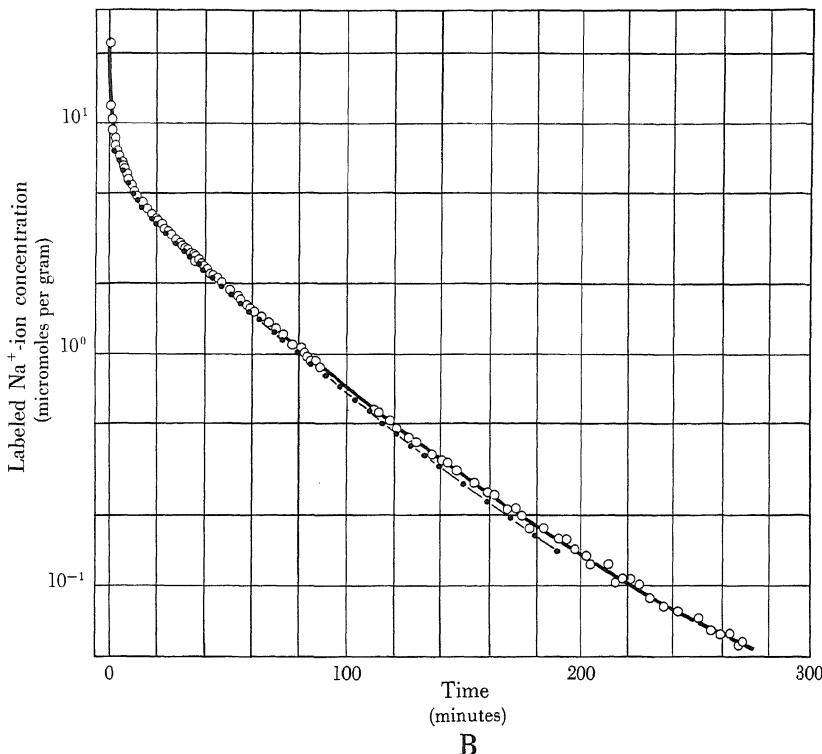
as those in 0.1*M* ionic solutions. The time course for exchange of the true intercellular space can be calculated from the equation given under Table 8.7 and subtracted from the total washout curve. Second, a correction must be made for the exchange of  $\text{Na}^+$  ion from the accompanying connective tissue. This correction has been described in the legends to Tables 8.7 and 8.8 and Figure 8.6.

Typical curves for  $\text{Na}^{22}$ -ion exchange in frog *sartorius* muscles at 0°C and at 25°C are presented in Figure 11.25. At low temperatures, the curve appears to be straight because of the very low rate of exchange. At the higher temperature, the continuous curvature which accompanies a much larger percentage exchange reveals the complex nature of the diffusion process. Although somewhat difficult to analyze, such complexity is to be expected since the nature of the fixed-charge system must vary considerably from one locus to another within the cell. The complexity produced by various components which exchange their  $\text{Na}^+$  ion at different rates suggests one or more modes of diffusion. Diffusion through the cytoplasm into the external solution proceeds rapidly and constitutes a "main stream" of exit. Other  $\text{Na}^+$  ions, occupying favorable adsorption sites, will exchange with the  $\text{Na}^+$  ion in this main stream, at a rate specific to the particular type of site occupied. There are several pieces of experimental evidence to support this concept.

Abelson and Duryee (1949) have demonstrated that 30-minute immersion of frog eggs in a Ringer's solution containing radioactive sodium leads to a substantial concentration of  $\text{Na}^+$  ion in the nucleus (Figure 11.26).\* Radioautographs

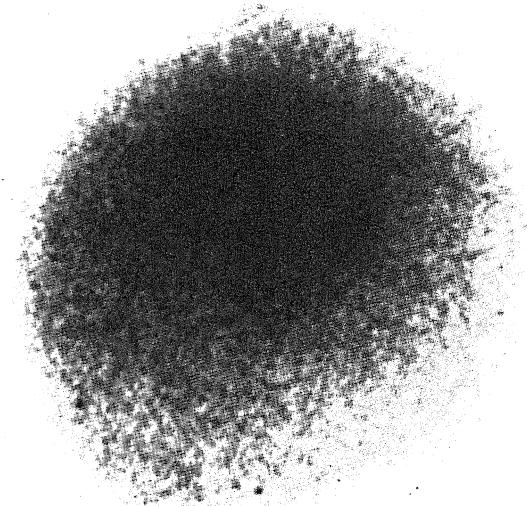
\* The author has repeated these experiments, using similar techniques, and essentially has confirmed their results.



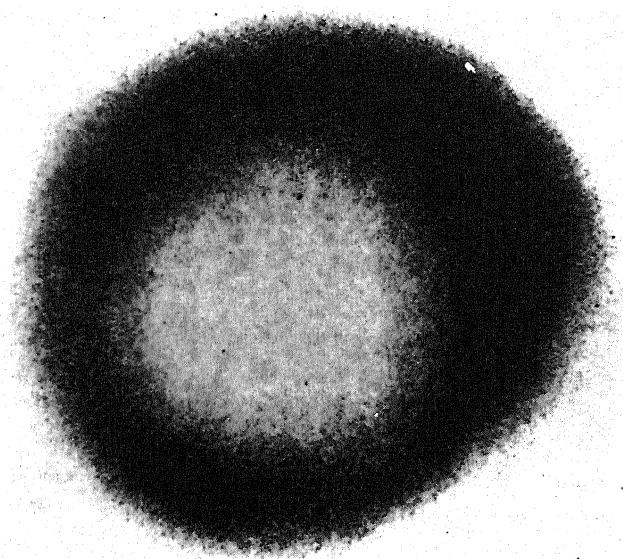


**Figure 11.25. THE TIME COURSE OF LOSS OF RADIOACTIVE  $\text{Na}^{22}$  FROM FROG SARTORIUS MUSCLES.** The muscle was incubated in Ringer's solution containing  $\text{Na}^{22}$  for one day at  $0^\circ\text{C}$ , then washed with nonradioactive Ringer's solution at  $0^\circ\text{C}$  for A and at  $25^\circ\text{C}$  for B. A procedure similar to that described under Figure 11.2 was used. Open circles indicate values obtained from the experiment; black dots indicate values corrected for diffusion from connective tissue and extracellular space.

show the nucleus as a dense circular patch contrasting sharply with the rest of the cell throughout which the silver grain is uniformly distributed but less dense. Other experiments have shown an initial rapid exchange involving about 12 per cent of the total  $\text{Na}^{+}$ -ion content; the exchange rate then decreases and there is relatively little exchange after three hours of immersion. Abelson and Duryee, in another experiment, found ring-shaped silver-grain areas in radioautographs of both dead and living cells; these led them to conclude that, in frog eggs,  $\text{Na}^{22}$ -ion exchange cannot be surface limited. If it were, the cytoplasm would be uniformly darkened with no sharp centripetal gradient like that in Figure 11.27. They calculated that the diffusion coefficient for the fast-exchanging fraction of  $\text{Na}^{+}$  ion in cytoplasm is  $2.6 \times 10^{-7} \text{ cm}^2/\text{sec}$  (the constant is  $1.30 \times 10^{-5} \text{ cm}^2/\text{sec}$  for the diffusion of  $\text{Na}^{+}$  ion in  $0.1M$   $\text{NaCl}$  or  $\text{KCl}$  solution, see Wang, 1952; Mills, 1955).

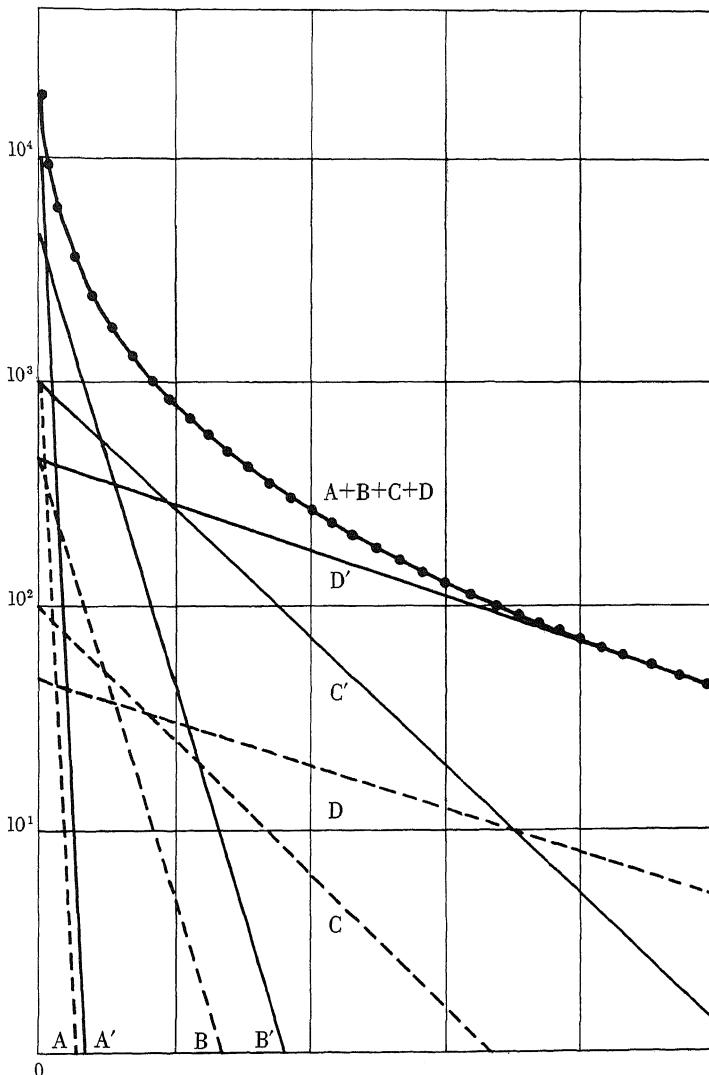


**Figure 11.26. RADIOAUTOGRAPH OF A FROG EGG EXPOSED TO  $\text{Na}^{24}$  FOR 30 MINUTES.** After soaking in Ringer's solution containing  $\text{Na}^{24}$ , the egg was frozen in liquid air and sectioned meridionally. The dark circle in the upper third corresponds to the size and position of the nucleus which appears vividly white in the frozen section, in contrast to the darker cytoplasm surrounding it. (Radioautograph from Abelson and Duryee, 1949.)



**Figure 11.27. RADIOAUTOGRAPH OF A FROG EGG EXPOSED TO  $\text{Na}^{24}$  FOR FIVE MINUTES.** Although this figure was obtained from a dead egg, such a ring-shaped autograph can also be obtained from living eggs. The technique used was the same as that for Figure 11.26. (Radioautograph from Abelson and Duryee, 1949.)

The total washout curve for  $\text{Na}^+$  ion in frog muscle—either a single fiber or an entire *sartorius* muscle—is bulk-phase limited. However, this complex phenomenon cannot be described as a simple bulk-phase diffusion nor can it be characterized by a simple diffusion coefficient because the plot of the time course of loss of radioactive  $\text{Na}^{22}$  ion bends continuously. It does not straighten out as it would if

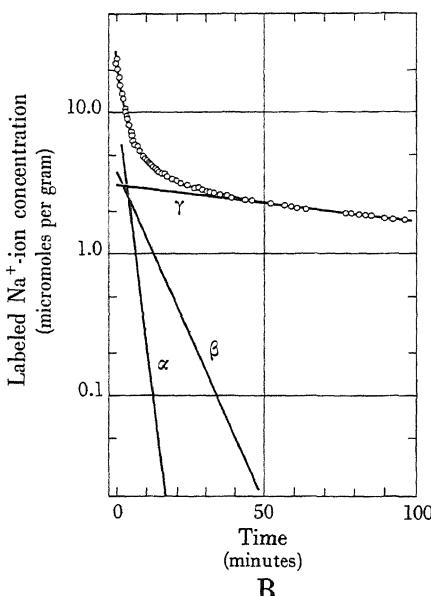
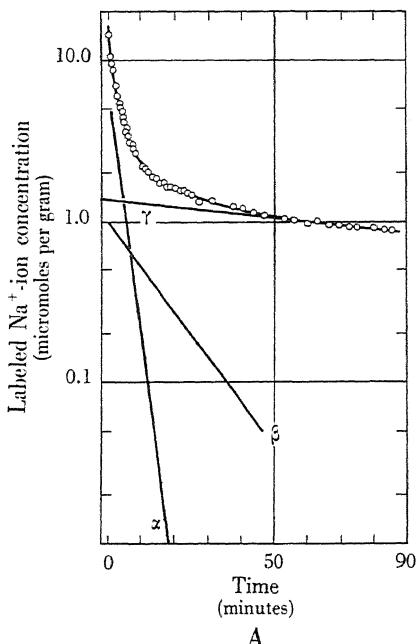


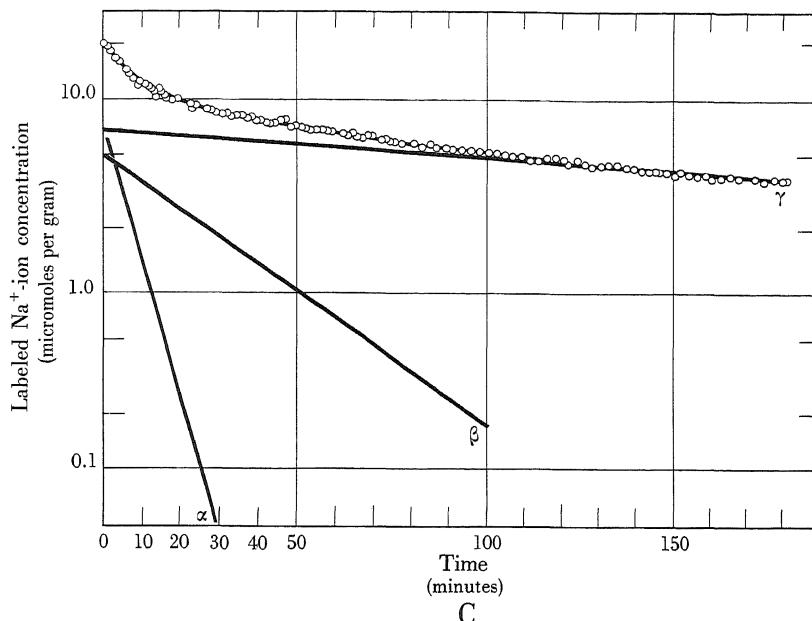
**Figure 11.28. THE RESOLUTION OF AN ARTIFICIALLY CONSTRUCTED COMPOSITE CURVE.** The curves  $A'$ ,  $B'$ ,  $C'$ , and  $D'$ , as well as composite curve  $A + B + C + D$ , are raised by a factor of 10 to avoid confusion.

this diffusion were a simple process like that shown in Figure 11.1. The task of resolving such complex curves at first appears hopeless but we shall show that a fairly accurate resolution is possible.

Let us designate several hypothetical exchange processes occurring in a bulk-phase system as A, B, C, D, and so on. These processes can be arrayed either in series,  $A \leftarrow B \leftarrow C \leftarrow D$ , or in parallel,  $A \parallel B \parallel C \parallel D$ . In any possible composite of these processes, the rate-limiting step or steps can be either a bulk-phase-limited diffusion (as in Figure 11.1) or a single rate-limiting step (as in surface-limited exchange, Figures 11.2 and 11.20). Each of these steps can, to all intents and purposes, be represented by a single straight line in a semilogarithmic plot. We should like to resolve a curve representing several of these single processes into the curves for its separate rate-limiting components. In Figure 11.28, composite curve  $A + B + C + D$  has been artificially constructed from the simple exponential lines, A, B, C, D. The components,  $A'$ ,  $B'$ ,  $C'$ ,  $D'$ , are resolved from the composite curve by first fitting the terminal portion with a straight line ( $D'$ ) and then fitting another straight line with  $(A + B + C + D - D')$ ; this gives  $C'$ . This process is continued until the four straight lines,  $A'$ ,  $B'$ ,  $C'$ ,  $D'$  have been derived. (In the illustration,  $A + B + C + D$ ,  $A'$ ,  $B'$ ,  $C'$ , and  $D'$  have each been raised by a factor of ten to avoid confusion.) Each of the resolved lines,  $A'$ ,  $B'$ ,  $C'$ ,  $D'$ , has approximately the correct slope as well as the correct intercept on the ordinate for its corresponding original line, A, B, C, or D.

Adopting the above method of resolution, we find that the  $\text{Na}^{22}$ -ion-washout



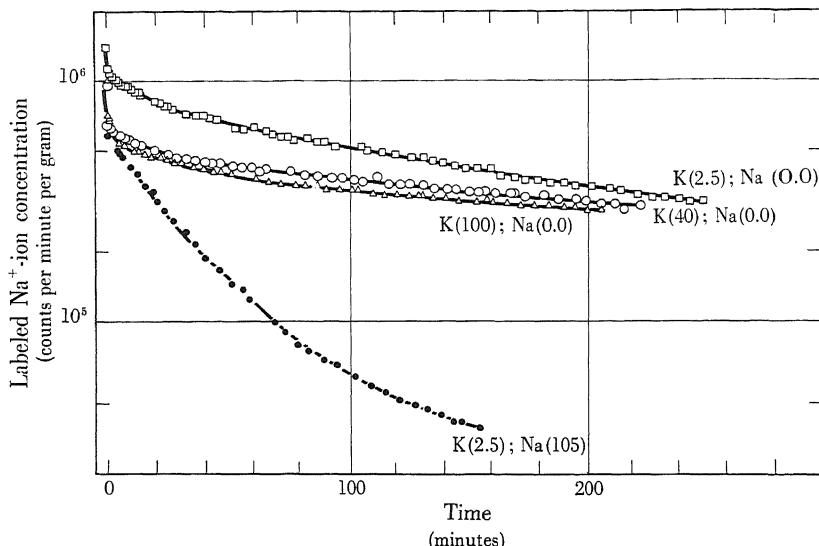


**Figure 11.29. THE EFFECT OF THE LENGTH OF EQUILIBRATION TIME ON THE TIME COURSE OF  $\text{Na}^{22}$  LOSS FROM FROG SARTORIUS MUSCLES ( $0^\circ\text{C}$ ).** Muscles were equilibrated in radioactive Ringer's solution ( $0^\circ\text{C}$ ): A, for two minutes; B, for 10 minutes; C, for one day. The technique used was similar to that used to obtain the data for Figure 11.2. Compare with Figure 11.25. Note the steady increase of labeled  $\text{Na}^+$  ion in the  $\gamma$ -fraction with length of soaking time. Differences in exchange rates of the  $\alpha$ -fractions in A, B, C, arise primarily from the different durations of these experiments, hence the positioning of the  $\gamma$ -fraction approximation which, in turn, influences the estimation of  $\gamma$ -fractions.

curve may be resolved into at least three major fractions which we shall designate as  $\alpha$ ,  $\beta$ , and  $\gamma$ . The nature of these fractions is a problem which is now under active investigation. There are reasons to believe that the  $\gamma$ -fraction corresponds to Abelson and Duryee's slow-exchanging fraction in frog eggs and either  $\alpha$  alone, or  $\alpha$  and  $\beta$  represent their fast-exchanging fraction.

The  $\gamma$ -fraction is probably  $\text{Na}^+$  ion which has been adsorbed onto fixed sites. As seen in Figure 11.29, this fraction progressively increases as the duration of initial soaking is increased. In contrast, the  $\alpha$ -fraction in a muscle soaked for a few minutes differs little from that in one soaked for eight days. If we visualize the  $\alpha$ -fraction as the interstitial  $\text{Na}^+$  ion, its time course represents a diffusion process through channels of interstitial space. This fraction has a diffusion coefficient of  $0.5$  to  $1.5 \times 10^{-8} \text{ cm}^2/\text{sec}$  at  $0^\circ\text{C}$ . Assuming a temperature coefficient of 1.3, this should give a diffusion coefficient of the same order of magnitude as that calculated by Ableson and Duryee for frog eggs at room temperature.

Next we must ask two important questions relating to these fractions. First, are the mechanisms of ion exchange in the bulk phase similar to those for ion exchange on the cell surface? We expect to find a basic resemblance between adsorption-desorption migration at the surface and the exchange of ions adsorbed inside the cell since we maintain that the fixed ions on the surface, as well as those



**Figure 11.30. THE EFFECT OF THE EXTERNAL  $\text{Na}^+$ -ION CONCENTRATION ON THE TIME COURSE OF LOSS OF  $\text{Na}^{22}$  FROM FROG SARTORIUS MUSCLE (25°C).** The muscles were equilibrated in a Ringer's solution in which all but a tracer amount of  $\text{Na}^+$  ion ( $\text{Na}^{22}$ ) was replaced with glucose. Following this equilibration, the muscles were washed (see Figure 8.5) with normal Ringer's solution or a Ringer's solution in which the  $\text{NaCl}$  was replaced by varying proportions of D-glucose and  $\text{KCl}$ . The  $\text{KCl}$  concentrations are indicated in the figure in terms of millimoles per liter.

in the bulk phase, determine ion-exchange behavior. A striking phenomenon observed in surface permeation is that strongly adsorbed ions permeate by the adsorption-desorption process; their rates of entry are higher in the presence of  $\text{Na}^+$  ions than in their absence. Figure 11.30 shows that the  $\text{Na}^{22}$ -washout curve for a frog *sartorius* muscle soaked in a glucose solution (containing 2.5mM  $\text{KCl}$ , 2.0mM  $\text{CaCl}_2$ , and carrier-free  $\text{Na}^{22}$ ) and washed in a similar solution (containing no radioactive  $\text{Na}^+$  ion) is totally different from that for a muscle soaked and washed in normal (high  $\text{Na}^+$ ) Ringer's solution. The  $\alpha$ -fraction seems to have disappeared, confirming our suspicion that the  $\alpha$ -fraction represents the main stream of interstitial  $\text{Na}^+$  ions.

The curve obtained in the above experiment has a slope resembling that of the  $\gamma$ -fraction. Results similar to these obtained in a sodium-free medium can also be obtained even when as much as 100 mM/l of glucose is substituted by KCl. However, by changing the washing solution to 100mM NaCl, the rate of exchange is greatly increased. From these experiments we conclude that the  $\alpha$ -fraction must represent the bulk of the interstitial  $\text{Na}^+$  ions present in the cell because of the high  $\text{Na}^+$ -ion concentration outside. On the other hand, when the total external  $\text{Na}^+$ -ion concentration is low,  $\text{Na}^+$  ions are only found adsorbed onto a relatively small number of high  $c$ -value sites which have a higher preference for  $\text{Na}^+$  ion than, say, for  $\text{K}^+$ . As adsorbed ions, these  $\text{Na}^+$  ions migrate, like  $\text{K}^+$  and  $\text{Rb}^+$  ions, on the surface fixed ionic sites, predominantly by the process of triplet formation. In a cell bathed in normal Ringer's solution containing a high concentration of  $\text{Na}^+$  ion, most of the intracellular  $\text{Na}^+$  ions must be interstitial ions. These ions, therefore, serve as the second cations in triplet formation and thus effectively increase the rate of exchange of strongly adsorbed ions.

We now pose the second question. Why should  $\text{Na}^+$ -ion exchange be bulk-phase rather than surface limited? We have provided enough evidence to establish that normal exchange of  $\text{K}^+$  ion is surface limited and that it may be converted to bulk-phase-limited exchange by a high external  $\text{K}^+$ -ion concentration. We have also shown that, under normal conditions,  $\text{Na}^+$ -ion exchange is a complicated type of bulk-phase-limited exchange. If the bulk phase and the cell surface are part of a fixed-charge system and both strongly prefer the  $\text{K}^+$  ion over the  $\text{Na}^+$  ion, as is shown by the total ionic accumulation pattern, by the permeability studies (Figure 11.2), and by resting-potential measurements (see Chapter 10), one should not expect the limitation of diffusion by fixed ionic sites in the bulk phase to differ from limitation of diffusion on the surface. Thus, diffusion of the intracellular  $\text{Na}^+$  ion in the interstitial space must be similar to the entry of the  $\text{Na}^+$  ion by the salutatory migration route. There is, however, one problem. Under normal conditions, the cellular fixed-charge system has a surface potential (the resting potential) which limits the outward passage of cations. Thus, at room temperature, assuming a surface-potential value of 100mv, on the average only one out of a total of 60  $\text{Na}^+$  ions can leave the cell through this barrier. Why then doesn't this factor give  $\text{Na}^+$ -ion exchange a surface-limited character? This question forces us to consider a mechanism for the removal of the potential barrier.

Free ions like  $\text{Na}^+$  and  $\text{Cl}^-$  associate to a small degree in an ordinary 0.1M NaCl solution. The energy favoring this association is not high enough to offset the large entropy gain on dissociation. We have seen (Section 2.4) that the greatest contribution to the entropy of a free ion is made by its rotational degree of freedom, and that rotational hindrance must greatly decrease the rotational partition function of an ion within a cellular fixed-charge system. Thus, the conditions for complete dissociation of free ions do not hold within the cell, and we have reason to believe that  $\text{Na}^+$  ions occupying the interstitial spaces may, in fact, spend a

considerable portion of their time in the form of a complex with the interstitial anion, usually  $\text{Cl}^-$ . These associated  $\text{Na}^+\text{Cl}^-$  pairs are, in effect, neutral molecules and are not barred by the surface potential from leaving the cell by the saltatory route. With the surface barrier thus effectively removed, one may readily understand why  $\text{Na}^+$ -ion exchange in normal cells is bulk-phase limited. This type of exchange has also been shown by Horowitz and Fenichel (to be published) for nonelectrolytes in several types of cells.

If, as we suggested, there is a substantial fraction of the outward-migrating  $\text{Na}^+$  ions which leave the cell associated with interstitial anions, a change of the anion should alter the normal rate of exchange—particularly that of the  $\alpha$ -fraction. However, the anion chosen must be similar to the chloride ion in that it should not be favored for adsorption onto fixed cationic groups within the cell. To this category belongs, among others, the sulfate ion.

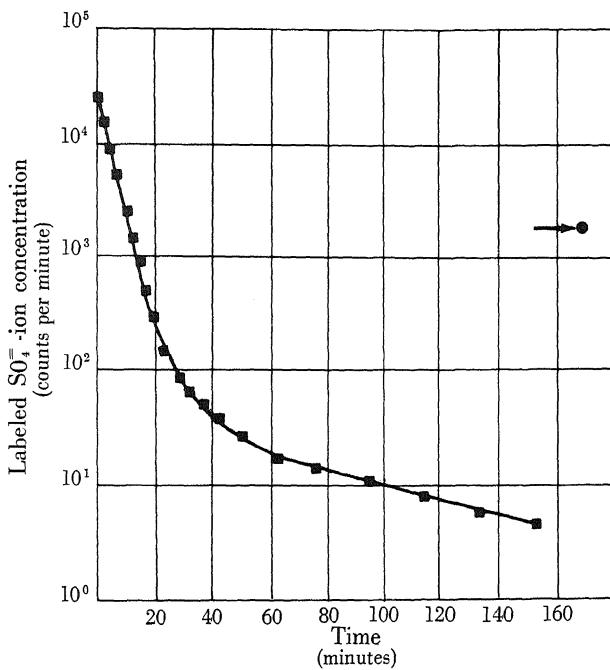
Contrary to earlier belief, this ion readily enters and leaves the cell, as shown in Figure 11.31; here, the washout curve is similar in shape to that of the  $\text{Na}^+$  ion; this indicates that the exchange is bulk-phase limited. Figure 11.32 shows the result of an experiment which was designed so that the normal environment of the cell is preserved in all aspects except that the  $\text{Cl}^-$  ion in the Ringer's solution is substituted by the  $\text{SO}_4^{2-}$  ion. It is evident that the outward-flux rate, particularly that for the  $\alpha$ -fraction, is greatly increased. In Section 2.2, we showed that the probability that an alkali-metal ion will form complexes ( $\text{Na}^+\text{SO}_4^-$  and  $\text{Na}_2\text{SO}_4$ ) with a divalent anion is greater than the probability that the same cation will form complexes with a univalent anion. Thus, the increased rate of exchange of the  $\alpha$ -fraction in an  $\text{SO}_4^{2-}$  environment is due to the existence within the interstitial spaces of a larger fraction of associated  $\text{Na}^+\text{SO}_4^-$  and  $\text{Na}_2\text{SO}_4$  ion pairs.

Association to form a neutral particle will also facilitate  $\text{Na}^+$ -ion migration through the interstitial spaces since the barrier formed by the electrostatic field of the fixed ionic groups and their counterions will be ineffective when the ions are associated in neutral groupings.

## C. IONIC DIFFUSION AND ADSORPTION IN MUSCLE CYTOPLASM

### (1) Theoretical anticipations

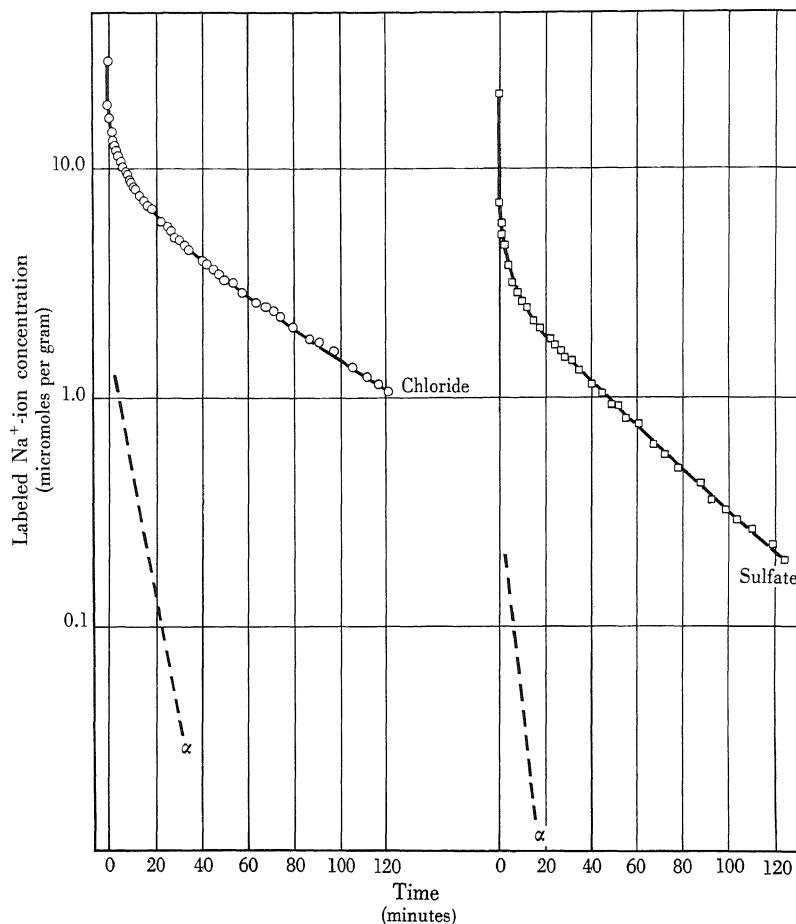
In the history of the development of our understanding of living cells, there has been considerable controversy over the question of whether the intracellular  $\text{K}^+$  ion is bound and nonexchangeable or free as in a dilute salt solution (see Chapter 9). The free-ion concept now seems to be most widely held, for it has been repeatedly shown that the intracellular  $\text{K}^+$  ion is exchangeable and can diffuse rapidly out of a broken cell. Although the association-induction-hypothesis model of ionic accumulation bears certain resemblances to the bound- $\text{K}^+$ -ion concept, it does not support the idea that ions are bound in the sense that they have lost



**Figure 11.31. THE TIME COURSE OF LOSS OF RADIOACTIVE SULFATE ION FROM A FROG *SARTORIUS* MUSCLE (0°C).** The muscle was first equilibrated in a Ringer's solution containing  $\text{S}^{35}\text{O}_4^-$  ion. The loss of radioactivity was detected by measurements of the radioactivity of successive aliquots of washing solution in an apparatus similar to that devised by Levi and Ussing (1948). The aliquots, dried in planchets, were counted with a conventional thin-window Geiger counter. The solid circle shows the amount of radioactivity left in the muscle at the end of the experiment.

their individual identity or are in any sense nonexchangeable (see Ling, 1955a).\* On the contrary, ions which are associated with counterions exchange constantly with similar ions inside and outside cells. As an example we may cite the case of a crystal of AgBr which is very insoluble in water. The insolubility of the crystal indicates that its free energy of association is very much in favor of the crystal-

\* We want to emphasize that although the term "exchangeable" may have had significance in the earlier days, it is a term which is insufficiently delineatory for the present discussion. All ions are exchangeable, given sufficient time.



**Figure 11.32. THE EFFECT OF THE SUBSTITUTION OF  $\text{SO}_4^{2-}$  ION FOR  $\text{Cl}^-$  ION ON THE EFFLUX OF  $\text{Na}^{22}$  FROM FROG SARTORIUS MUSCLES ( $25^\circ\text{C}$ ).** Muscles were equilibrated at  $0^\circ\text{C}$  in Ringer's solution containing  $\text{Na}^{22}$  and washed with nonradioactive Ringer's solutions of otherwise identical composition. Open circles indicate values obtained from a muscle equilibrated and washed in normal Ringer's solution; squares indicate values obtained from a muscle equilibrated and washed in a Ringer's solution in which the  $\text{Cl}^-$  ion had been replaced by  $\text{SO}_4^{2-}$  ion. The  $\alpha$ -fractions, determined by the method discussed in Section 11.3B, are represented by dotted lines.

line associated state. However, we know that the  $\text{Ag}^+$  ion in the bulk of the crystal constantly exchanges with the  $\text{Ag}^+$  ion in the solution (see Section 11.3A).

The physics of self-diffusion and diffusion in ionic crystals has been the subject of many theoretical and experimental studies (see Jost, 1952). Diffusion of ions in a fixed-charge system involves mechanisms similar to those involved in diffusion

through ionic crystals. However, the mechanisms involved depend on whether the ion under consideration is favored for association with the fixed ionic sites within the fixed-charge system. Since, in the majority of cells studied, the  $\text{Na}^+$  ion cannot compete successfully with the  $\text{K}^+$  ion for most of the fixed anionic sites, the diffusion of  $\text{Na}^+$  ion in cells involves a process of jumping from one interstitial position to another. This process is identical to saltatory migration in the entry of ions into cells; the ions which travel in this fashion correspond to the  $\alpha$ -fraction of the  $\text{Na}^+$  ions as pointed out in Section 11.3B.

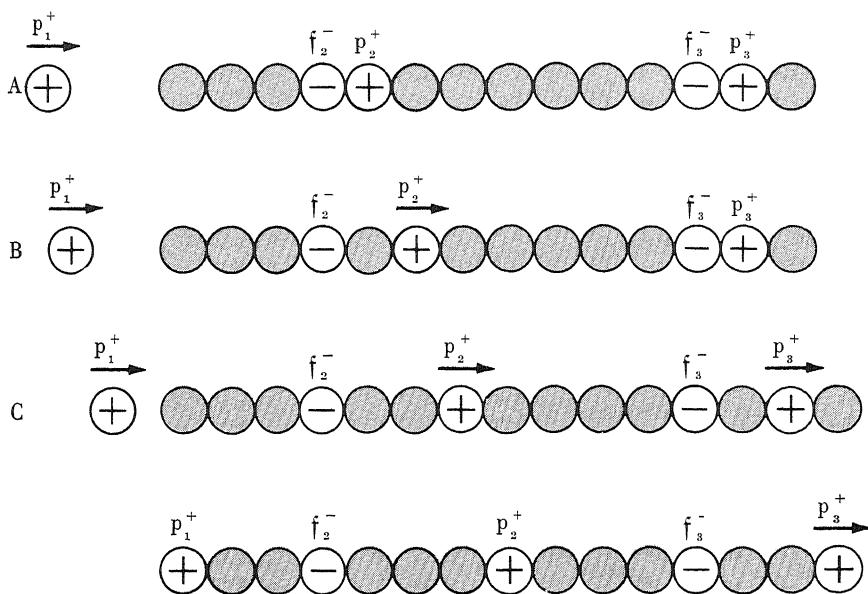
For ions favored by high adsorption energies, the major routes of surface permeation involve doublet and triplet association-dissociation mechanisms. The triplet route, because of its lower activation energy, is the particularly important one.

From Figures 4.6 to 4.8 it is evident that  $\text{Na}^+$  and  $\text{K}^+$  ions tend to assume higher configurations at low  $c$ -values and lower configurations at higher  $c$ -values. The modal  $c$ -value in muscle cytoplasm is such that the  $\text{K}^+$  ion is strongly preferred over the  $\text{Na}^+$  ion (see Table 9.2); this necessitates a fairly high  $c$ -value. Consequently, a large proportion of the intracellular  $\text{K}^+$  ions must assume the 0-configuration. The  $\text{Na}^+$  ion, because of its high extracellular concentration and unfavorable adsorption energy on cytoplasmic sites, must constitute the bulk of the interstitial ions; thus it must be the ion which most often functions as the third ion in the formation of triplets with the adsorbed  $\text{K}^+$ -fixed-anion pair. Each time an interstitial  $\text{Na}^+$  ion approaches one of these pairs along the minimum energy path, the effect of its electric field on the fixed anion causes a decrease of the pair's effective  $c$ -value. When the  $c$ -value decreases, the  $\text{K}^+$  ion takes on a higher configuration. Consequently, the distance between the  $\text{K}^+$  ion and the anion increases and  $\epsilon_{t_{\text{KNa}}}^+$ , the activation energy for the detachment of the  $\text{K}^+$  ion, must decrease. This process necessitates a rapid rate of self-diffusion or exchange of the  $\text{K}^+$  ion (see Figure 11.33; also Jenny and Overstreet, 1939). To a somewhat smaller degree, this mode of ionic migration also applies to ions such as  $\text{Rb}^+$  and  $\text{Cs}^+$ .

(2) Experimental studies on the diffusion and accumulation of alkali-metal ions in cytoplasm

(a) *Diffusion in cut muscles.* In the exchange of  $\text{Na}^+$  ion (Figure 11.29), the  $\alpha$ - and  $\gamma$ -fractions of ions diffusing in cytoplasm are clearly discernible; that the migration of  $\text{K}^+$  ions in cells is a similar process has also been shown by lowering the surface barrier to diffusion through the application of external  $\text{K}^+$  ion in high concentration (Figure 11.24). Removal of the normal surface barrier can also be achieved by sectioning the muscle cells, thus exposing the cytoplasm directly to ions in the bathing solution. Such experiments offer considerable insight into the nature of cytoplasm as a fixed-charge system.

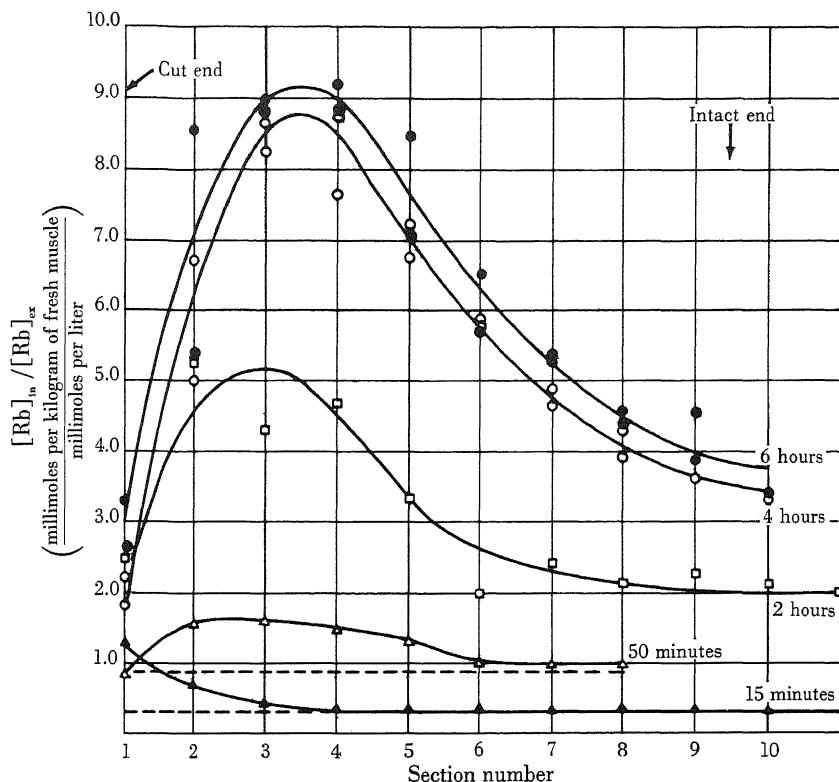
As mentioned in Chapter 9, cut fibers from rat diaphragm muscles accumulate



**Figure 11.33. THE MIGRATION OF CATIONS THROUGH A FIXED-CHARGE SYSTEM BY TRIPLET FORMATION.** This diagram represents the events that occur as an interstitial cation ( $p_1^+$ ) approaches a fixed anion-counterion pair ( $f_2^- p_2^+$ ). A, as the cation  $p_1^+$  approaches the fixed anion  $f_2^-$ , it exerts an attractive force on the electron cloud of  $f_2^-$ ; thus,  $p_1^+$  effectively decreases the  $c$ -value of this fixed anion. B and C, consequently  $p_2^+$  tends to assume a high configuration with more water molecules interspersed between  $f_2^-$  and  $p_2^+$  (water molecules are represented as shaded circles). At this greater distance of separation from  $f_2^-$ ,  $p_2^+$  has to overcome a much lower activation energy to migrate from  $f_2^-$  toward an adjoining fixed anion-counterion pair  $f_3^- p_3^+$ . D, in this process, it acts on  $f_3^- p_3^+$  as  $p_1^+$  acted on  $f_2^- p_2^+$ , thereby initiating a chainlike migration. This type of migration, which is very effective when  $p_2^+$  and  $p_3^+$  are like the  $K^+$  ion which readily shifts its configuration number with a  $c$ -value change, becomes less effective when  $p_2^+$  and  $p_3^+$  are like the  $Cs^+$  ion, which tends to remain at a fixed low configuration in spite of  $c$ -value changes (see Figure 4.6). Chainlike migration can account for the high cytoplasmic conductance found in normal resting cells as well as the mobility of the  $K^+$  ion in the cytoplasm.

$Rb^+$  ion against a concentration gradient (Menozzi *et al.*, 1959). Similar experiments have been performed in our laboratory on frog *sartorius* muscle. This tissue offers two advantages: it is easy to isolate intact and its fibers run parallel from one end of the muscle to the other. If such fibers are cut at one point, a local contracture, extending 1 to 2 mm, develops. Nonetheless, when they are kept in a Ringer's-bicarbonate solution, muscles cut across one end survive for many hours.

In the following experiment muscles were cut at the pelvic end and equilibrated at 25°C with a Ringer's-bicarbonate solution containing 2.5mM  $Rb^+$  ion and no  $K^+$  ion. Paired muscles were left intact and equilibrated in the same solution. At



**Figure 11.34. SPATIAL DISTRIBUTION OF  $Rb^+$  ION IN FROG SARTORIUS MUSCLE INCUBATED WITH ONE END CUT ( $25^\circ C$ ).** Frog sartorius muscles were cut with a sharp razor near their pelvic ends and placed in a Ringer's-bicarbonate solution equilibrated with 95%  $O_2$  + 5%  $CO_2$  and containing 2.5mM  $Rb^+$  ion tagged with  $Rb^{86}$ , but with no  $K^+$  ion. At the end of the specified time a muscle was taken out, blotted dry, and cut into a number of more or less uniform sections with a "guillotine" consisting of a battery of uniformly spaced razor blades. After weighing, each section was soaked in 1*N* HCl and the radioactivity assayed. The ordinate represents the ratio of the concentration of  $Rb^+$  ion in the sections (expressed in millimoles per kilogram of fresh muscle weight) to the extracellular  $Rb^+$ -ion concentration (in millimoles per liter of solution). Section numbers are counted from the cut pelvic end. Dotted lines represent the  $Rb^+$ -ion concentration in a paired control muscle allowed to remain intact and equilibrated in the same way as the corresponding cut muscle.

varying intervals of time, muscles were removed from this solution and cut into small sections 2 to 3 mm in length with a "guillotine" consisting of a battery of evenly spaced razor blades. Each of these sections was analyzed for its  $Rb^+$ -ion content. The results of this experiment (Figure 11.34) show with some accuracy the spatial distribution of the  $Rb^+$  ion taken up by the muscle. After 15 minutes the highest concentration of  $Rb^+$  ion is found at the cut end and progressively

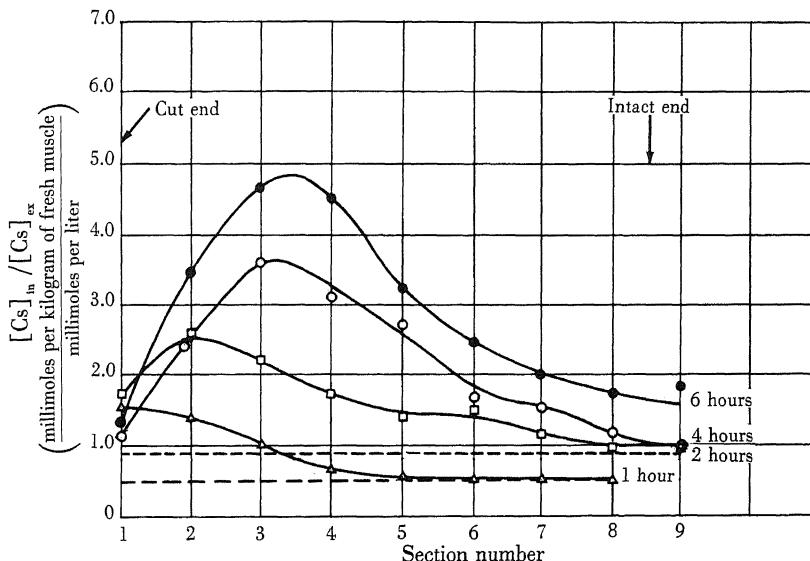
decreases in succeeding sections. If, in a given pair, we compare the cut muscle with the uncut muscle, we find that the rate of entry of  $\text{Rb}^+$  ion into the intact end of the cut muscle does not differ from that into the uncut muscle. As time progresses, the relatively fast-moving front of the ions which entered initially through the cut end moves steadily toward the intact end very much in the way ions diffuse into a capillary. In the meantime, the sections near the cut end continue to accumulate more and more  $\text{Rb}^+$  ion until, after six hours, a high concentration is reached. This is nine times the  $\text{Rb}^+$ -ion concentration in the Ringer's solution. Here again we can see evidence of a fast-moving  $\alpha$ -fraction and a more slowly exchanging  $\gamma$ -fraction. This experiment and others discussed below show that the cytoplasm acts as the seat of ionic selectivity. Thus, these experiments offer powerful support for the association-induction hypothesis.

Hodgkin and Keynes (1953) measured the diffusion coefficient (and mobility) of  $\text{K}^{42}$  in giant squid axon. They showed that the  $\text{K}^{42}$  which enters the cell can move freely with an average diffusion coefficient of  $1.5 \times 10^{-5} \text{ cm}^2/\text{sec}$ . From these data, the authors concluded that "the bulk of potassium inside an axon exists as free ions," and that "the  $\text{K}^{42}$  which enters an axon exists in the axoplasm in much the same state as in a  $0.5M$   $\text{KCl}$  solution." This thesis was directed as a disproof of the crude concept of "bound, inexchangeable" potassium ions; nonetheless this work of Hodgkin and Keynes has frequently been quoted as disproving the present hypothesis in its 1951-1952 form. A few words need to be said to clear up this confusion. Since its inception, the present theory has regarded  $\text{K}^+$  and  $\text{Na}^+$  ions as being free in the sense that they are not covalently linked to other atoms or molecules (Ling, 1951, 1952, 1955a). The question, therefore, is whether the measured diffusion coefficient belongs *uniquely* to the  $\text{K}^+$  ion in a  $0.5M$  free solution. Here we consider several points related to this question.

The variation in the data of Hodgkin and Keynes (diffusion coefficients ranging from 0.73 to  $2.28 \times 10^{-5} \text{ cm}^2/\text{sec}$  for the same procedure on different preparations which took up labeled  $\text{K}^+$  ion to concentrations varying from  $7\text{mM}$  to  $86\text{mM}$ ) does not justify the claim that the  $\text{K}^+$  ion is in a particular state through identification of a *unique* diffusion constant. Thus there is no unique self-diffusion constant for  $0.5M$   $\text{K}^+$  ion although there are unique self-diffusion constants for  $\text{K}^+$  ion in a  $0.5M$   $\text{KCl}$  solution ( $2.135 \pm 0.004 \times 10^{-5} \text{ cm}^2/\text{sec}$ ; note the very small standard error; see Friedman and Kennedy, 1955), and in  $0.5M$   $\text{KI}$  solution ( $2.030 \pm 0.030 \times 10^{-5} \text{ cm}^2/\text{sec}$ ; Mills and Kennedy, 1953). The anions in squid axon are neither chloride nor iodide but are rather proteins, amino acids, and so on. The self-diffusion constant of the  $\text{K}^+$  ion as a salt of these complex anions cannot justifiably be thought to equal either of the above values.

The diffusion coefficient of ions in a fixed-charge system is not necessarily lower than that in free solution. In fact, J. W. McBain and Peaker (1930) and Mysels and McBain (1948) long ago demonstrated that the self-diffusion coefficient of the  $\text{K}^+$  ion on the surface of glass is somewhat higher than that in dilute solution. The glass surface, as an anionic fixed-charge system, adsorbs the  $\text{K}^+$  ion and allows it to have a slightly higher self-diffusion coefficient than it has in free solution (see Nielsen *et al.*, 1952). The mechanism we have proposed for this is illustrated in Figure 11.33. The data of Hodgkin and Keynes can be accounted for by an enhanced self-diffusion coefficient for a fraction of the intracellular  $\text{K}^+$  ion in combination with a slower fraction which exchanges continually. The exact value obtained in any particular experiment will thus depend on how much labeled  $\text{K}^+$  ion has entered the cell and how much of it has exchanged with originally adsorbed  $\text{K}^+$  ion.

(b) *Selectivity in cut muscles.* Table 11.4 gives the results of analyses of the Rb<sup>+</sup>-, K<sup>+</sup>-, and Na<sup>+</sup>-ion concentrations in sections of *sartorius* muscles which have been equilibrated with the pelvic end cut for four hours in Ringer's solution containing 2.5mM Rb<sup>+</sup> ion, 2.5mM K<sup>+</sup> ion, and 102.0mM Na<sup>+</sup> ion. While Rb<sup>+</sup> ion accumulated in sections close to the cut end, all sections maintained a high degree



**Figure 11.35. SPATIAL DISTRIBUTION OF Cs<sup>+</sup> ION IN FROG SARTORIUS MUSCLE INCUBATED WITH ONE END CUT (25°C).** Technique the same as that used in obtaining Figure 11.34, except that 2.5mM Cs<sup>+</sup> labeled with Cs<sup>134</sup> was used instead of Rb<sup>+</sup> ion.

of selective K<sup>+</sup>-ion accumulation and Na<sup>+</sup>-ion exclusion. The selectivity for Cs<sup>+</sup> ion in such muscles is shown by Figure 11.35 which gives results of an experiment similar to that depicted in Figure 11.34 except that the Cs<sup>+</sup> ion was used instead of the Rb<sup>+</sup> ion. The results are similar to those shown in Figure 11.34 except that the selectivity ratios attained are considerably lower. From Figures 11.34 and 11.35 and Table 11.4, we can deduce that the selectivity of the naked cytoplasm follows the order K<sup>+</sup> > Rb<sup>+</sup> > Cs<sup>+</sup>. This result has also been found for intact cells when all external ions are at fairly high concentrations (Ling, unpublished; see also Table 9.5 for data on rat muscles).

(c) *The high activation energy for K<sup>+</sup>-Rb<sup>+</sup> exchange.* The rising Rb<sup>+</sup>-ion concentration near the cut end of the muscle in Figure 11.34 and Table 11.4 represents an exchange of the Rb<sup>+</sup> ion for the K<sup>+</sup> ion (and/or for fixed and other cations) on

fixed anionic sites. As discussed in Section 11.2A, this adsorption phenomenon requires an activation energy of considerable magnitude. On the basis of the present theory, which predicts similar mechanisms for normal surface permeation and diffusion in cytoplasm, and from our study of the effect of temperature on

Frog No.	Muscle group index	Temp	Time	Rb, $\mu\text{M/g}$				K, $\mu\text{M/g}$	
				Sec- tion 1 (cut)	Sec- tion 2	Sec- tion 3	Sec- tion 4	Sec- tion 1 (cut)	Sec- tion 2
I, II, III	A	25°C	4 hr	8.45	8.65	3.29	2.72	38.09	76.20
	B	0°C	24 hr	2.10	2.89	2.53	2.11	47.30	56.43
IV, V, VI	A	25°C	4 hr	12.87	12.24	7.80	5.75	35.10	62.08
	B	0°C	24 hr	2.35	3.13	4.70	4.74	14.71	31.26
VII, VIII, IX, X	A	25°C	4 hr	14.33	10.91	7.70	6.80	37.49	68.73
	B	0°C	24 hr	3.99	5.69	8.42	7.22	18.60	40.62

Frog No.	Muscle group index	Temp	Time	K, $\mu\text{M/g}$				Na, $\mu\text{M/g}$	
				Sec- tion 3	Sec- tion 4	Sec- tion 1 (cut)	Sec- tion 2	Sec- tion 3	Sec- tion 4
I, II, III	A	25°C	4 hr	80.00	53.86	42.26	20.94	24.12	17.14
	B	0°C	24 hr	72.22	78.01	40.65	22.72	20.27	15.12
IV, V, VI	A	25°C	4 hr	69.81	68.63	40.48	19.77	23.40	26.28
	B	0°C	24 hr	61.63	77.51	56.50	37.51	24.73	18.42
VII, VIII, IX, X	A	25°C	4 hr	72.76	75.31	36.04	22.34	19.00	20.53
	B	0°C	24 hr	60.60	69.79	44.35	37.91	23.85	15.42

**Table 11.4. TEMPERATURE EFFECT ON Rb<sup>+</sup>- AND K<sup>+</sup>-ION ACCUMULATION AND Na<sup>+</sup>-ION EXCLUSION IN THE CYTOPLASM OF CUT MUSCLE CELLS.** *Sartorius* muscles were cut at the pelvic end and incubated in 80ml of Ringer's bicarbonate (95% O<sub>2</sub> + 5% CO<sub>2</sub>), containing Rb<sup>+</sup> (2.5mM), K<sup>+</sup> (2.5mM), Na<sup>+</sup> (102mM). One of each pair was kept at 25°C for four hours, the other at 0°C for 24 hours. At the end of the specified time, the muscles were cut into four equal sections—section 1 including the cut end. Corresponding sections from three or four different muscles were combined for analysis of Rb, K, and Na by flame photometry.

permeation (see Table 11.2), we can anticipate that lowering the temperature to 0°C will substantially inhibit the accumulation of Rb<sup>+</sup> ion near the cut end. This is demonstrated in Table 11.4. After four hours at 25°C, the level of Rb<sup>+</sup> ion in the section containing the cut end was five times the external ion concentration; on the other hand, the paired muscle equilibrated at 0°C failed to selectively accumu-

late Rb<sup>+</sup> ion at the cut end even after 24 hours. Nevertheless, selective K<sup>+</sup>-ion accumulation and Na<sup>+</sup>-ion exclusion were maintained almost independently of temperature (see muscles 1A and 1B in particular).

In summary, we see that the processes of entry of an ion into the cell through its normal surface as well as diffusion and accumulation within the cytoplasm are basically the same.\*

\* Although we have already presented data in Chapters 8 and 10 which are sufficient to disprove the membrane-pump concept of ion accumulation, in deference to its long history and wide acceptance the complete incompatibility of this thesis with the present findings will be reviewed in a forthcoming paper.

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# 12

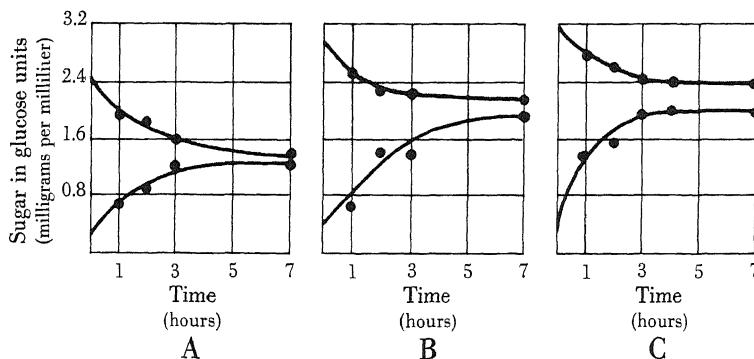
## SELECTIVE DISTRIBUTION AND PERMEABILITY OF NONELECTROLYTES

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Living cells, whether individual unicellular organisms or parts of a complex biological system, are always found in dilute aqueous solutions. Therefore any substance which has a profound effect on cells must have an appreciable solubility in water. We have said a great deal about the interactions of ions, one type of water-soluble substance, with biological systems. We have also encountered urea, another water-soluble compound. Urea does not carry a net charge but it is highly soluble because it, like water, is polar and thus forms H-bonds with the medium. Similar nonionic H-bond-forming compounds, often called nonelectrolytes, have received considerable attention from biologists; these include alcohols, aldehydes, ketones, ethers, esters, and amides. The primary task of this chapter is to show how the observed interactions of these compounds with cells—often difficult to reconcile with the membrane theory—follow as natural consequences of the association-induction hypothesis.

### 12.1. Selective Distribution of Nonelectrolytes

Widely varying ratios for the partition of sugars between plasma and red blood cells in various animals have been reported (for reference, see Andreen-Svedberg, 1933; Goodwin, 1956). In Figure 12.1, we have reproduced the data of Kolotilowa and Engelhardt (1937) which show that, at equilibrium, the nonfermentable sugars, galactose, arabinose, and xylose, do not reach the same concentration in the intracellular water of red blood cells that they reach in the medium. The work



**Figure 12.1. THE UPTAKE OF NONFERMENTABLE SUGARS BY RABBIT ERYTHROCYTES.** The upper curves were obtained from experiments which assayed sugar in the bathing medium, the lower curves from experiments which assayed sugar in the erythrocyte water. The sugars used were A, galactose; B, arabinose; C, xylose. (Figure after Kolotilowa and Engelhardt, 1937; see Troschin, 1958.)

of Helmreich and Cori (1957), Kipnis and Cori (1957), and Norman *et al.* (1959) confirmed the findings of Levine and Goldstein (1955) that a number of nonutilizable or slowly utilizable pentoses can enter ascites and muscle cells. The partition ratios between the intracellular and extracellular phases again vary with the nature of the sugars and that of the cells (Figures 12.2 and 12.3). Xylose, galactose, and sucrose appear to have reached equilibrium in these experiments (Figures 12.2, 12.3); the fact that these sugars occupy only a fraction of the intracellular water cannot be due to the rate of entry or the permeability. We cannot interpret this phenomenon as a steady state representing a balance between permeation and utilization because xylose is not utilizable.

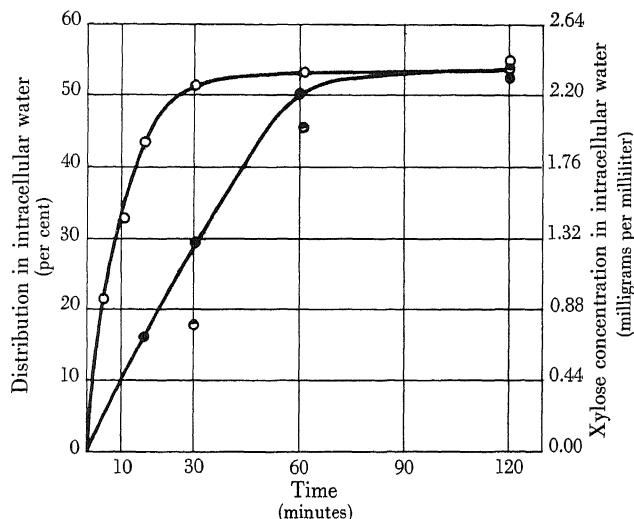
We suggest that the selective exclusion and accumulation of nonelectrolytes, like selective ionic accumulation, is the consequence of a metastable equilibrium state. For this state we write

$$K_D = \frac{C_i^U}{C_i^I}, \quad (12-1)$$

where  $C_i^I$  and  $C_i^{II}$  are the concentrations of the  $i$ th nonelectrolyte in the free-solution and fixed-charge phases, respectively;  $K_D$ , the distribution coefficient, is related to  $F_i^I$  and  $F_i^{II}$ , the free energies of the  $i$ th nonelectrolyte in the free solution and fixed-charge phases, respectively, by the equation,

$$\Delta F_i^{I,II} = F_i^I - F_i^{II} = RT \ln K_D. \quad (12-2)$$

The exact value of  $F_i^I$  is, at the moment, of no concern; it is important only because it is not equal to  $F_i^{II}$ . If  $F_i^I \neq F_i^{II}$ , then  $K_D \neq 1$ , a familiar result in thermodynamics.



**Figure 12.2. THE TIME COURSE OF XYLOSE PENETRATION INTO RAT DIAPHRAGM.** Open circles indicate data from cut diaphragm; solid circles indicate data from intact diaphragm; half-open circles indicate data from the diaphragm of the living animal. (Figure after Kipnis and Cori, 1957.)

Thus if the free energy of the nonelectrolyte in the two phases is different, its distribution must also be different.

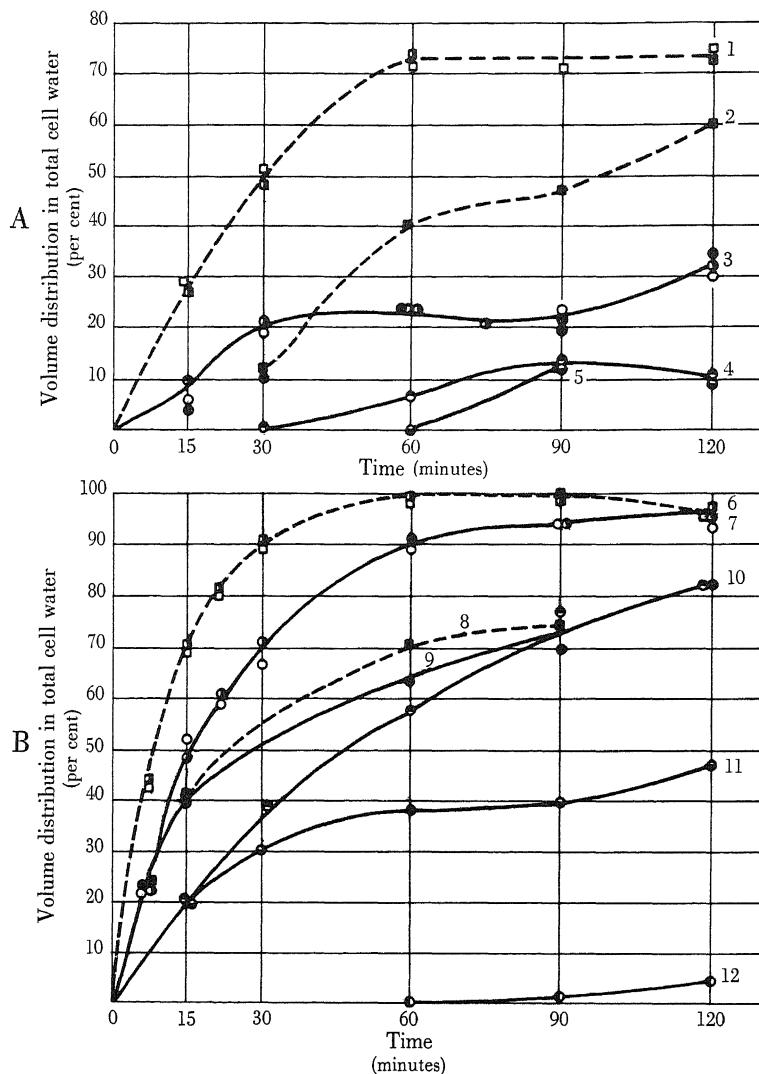
From thermodynamics we know that

$$F = E - TS. \quad (12-3)$$

This equation enables us to evaluate  $F$ , the Helmholtz free energy, by analyzing  $S$ , the entropy, and  $E$ , the energy, separately.

#### A. THE ENTROPY OF NONELECTROLYTES IN A FIXED-CHARGE SYSTEM

Due to the heterogeneous nature of most fixed-charge systems, we can speak of the entropy of a nonelectrolyte within such systems only in terms of an *average*.



**Figure 12.3. THE VOLUME OF DISTRIBUTION OF VARIOUS SUGARS IN THE CELL WATER OF RAT DIAPHRAGM.** A, studies on intact fibers: 1, the time course of uptake of galactose □ and D-xylose ■ in the presence of added insulin; 2, L-xylose ■, in the presence of added insulin; 3, galactose ○, D-xylose ●, and L-xylose ● in the absence of insulin; 4, mannitol ●; and 5, sucrose ●. B, studies with cut fibers: 6, the time course of uptake of galactose and D-xylose in the absence of added insulin; 7, galactose and D-xylose in the absence of added insulin; 8, L-xylose in the presence of insulin; 9, L-xylose in the absence of insulin; 10, mannitol; 11, sucrose; and 12, insulin ●. (Figure after Norman *et al.*, 1959.)

In Chapter 2, we extensively discussed how restriction of the rotational and configurational entropies for both charged and H-bonding molecules follow from the nature of the living-cell fixed-charge system. Since these restrictions hold for the molecules now under consideration, the entropy of a nonelectrolyte within a fixed-charge system must, in general, differ from the entropy of the same nonelectrolyte in free aqueous solution.

### B. THE ENERGY OF THE NONELECTROLYTE IN A FIXED-CHARGE SYSTEM

The high solubility of the organic nonelectrolytes is fundamentally an expression of the H-bond-forming groups they carry. However, just as the  $c$ - and  $c'$ -values of charged groups can vary from one group to another, the  $c$ - and  $c'$ -value analogues of H-bonding groups also vary from molecule to molecule, in both the proton-donating and the proton-accepting capacities. Thus the protein backbone offers one class of H-bonding sites, the water molecules in the lattice around, say, a fixed anion and counterion pair (see Figure 4.3) offer another, and the water molecules in a free solution, a third. These differences in H-bonding sites lead to a significantly different average H-bonding energy for the same nonelectrolyte in a free dilute aqueous solution and within the fixed-charge system.

Thus we see that, from the energy as well as the entropy point of view, we can expect that  $F_i^e \neq F_i^{ex}$  and hence that  $K_D \neq 1$ . This, for example, is the case in ion exchange resins; see Table 12.1. Wheaton and Bauman (1953) have shown that in such resins, a type of fixed-charge system, there is a specific distribution coefficient for each nonelectrolyte in each type of resin. Furthermore, in agreement with our theoretical considerations, these data show that selective exclusion and accumulation depend on both the nature of the fixed-charge system and the nature of the counterions.\* There is neither a cell membrane nor a pump in such exchange resins; they are, however, fixed-charge systems. The fact that such systems show the same type of ability selectively to exclude nonelectrolytes as is observed in living cells may be viewed as a confirmation of the basic theme of the association-induction hypothesis.

Useful as is the model of the exchange resin, it is not a living cell. The living cell is a unique proteinaceous fixed-charge system which has the ability to assume more than one metastable cooperative state. When such a cooperative change occurs, it is likely that the  $c$ - and  $c'$ -values, and their analogues, of the polar groups are profoundly altered and, with this, the H-bonding energy as well as the entropy of the nonelectrolytes within the fixed-charge system must follow to engender a different  $F_i^{ex}$ . From this, we expect, first, that the equilibrium distribution of different organic nonelectrolytes in a particular cell may vary with the

\* This is to be expected from the fact that the H-bonding capacity of the water within the fixed-charge system varies with respect to the nature of both the fixed polar groups and their counterions as well as with the configuration these groups adopt.

state of the cells as characterized by the  $c$ - and  $c'$ -value ensembles of the cell proteins, and, second, that variation of such an ensemble of  $c$ - and  $c'$ -values by hormonal action or excitation, for example, would lead to a change of distribution coefficient for different nonelectrolytes. There are many experimental observations of the effect of insulin and stimulation on sugar exclusion. Levine and Goldstein

Nonelectrolyte	$K_D$		
	Dowex 50-X8, H <sup>+</sup>	Dowex 50-X8, Na <sup>+</sup>	Dowex 1-X7.5, Cl <sup>-</sup>
methanol	0.61	—	0.61
ethylene glycol	0.67	0.63	—
glycerine	0.49	0.56	1.12
D-glucose	0.22	—	—
sucrose	0.24	—	—
phenol	3.08	—	17.7
acetone	1.20	—	1.08
formaldehyde	0.59	—	1.06
ethylene diamine	—	0.57	—

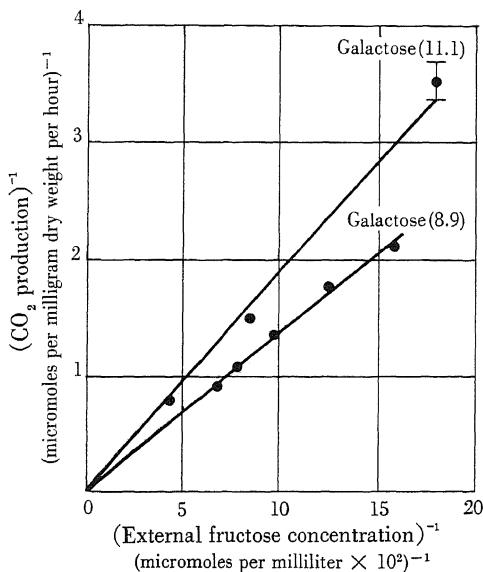
**Table 12.1. THE DISTRIBUTION COEFFICIENT FOR NONELECTROLYTES IN ION EXCHANGE RESINS CHARGED WITH DIFFERENT COUNTERIONS.** The distribution coefficient  $K_D$  is defined as the ratio of the solute concentration inside the resin to that outside when the initial concentration of the solute in the external aqueous phase is 0.05 per cent. (Wheaton and Bauman, 1953.)

(1955), Helmreich and Cori (1957), Kipnis and Cori (1957), and Norman *et al.* (1959) all confirm the theoretical expectations just presented.

## 12.2. Selective Permeability of Nonelectrolytes

The effect on the mode of penetration of cations by the presence of fixed anionic groups on the cell surface has already been discussed. In this section we shall broaden the concept of permeation through association to include the structures near the cell surface that possess H-bonding sites. These may be carboxylic and amino groups, as well as sites carried by the proton-donating group NH and the proton-accepting group C=O of the polypeptide chains. Thus the water molecules in the I, II, and III configurations around fixed anionic and cationic sites and also water molecules attached to the backbone all occupy H-bonding sites. These sites differ from the "sites" in liquid water in that bonds formed at the liquid-water sites are of lower energy, and in that steric and other factors will further limit the

type of molecules that can combine. The entry of water or other H-bonding substances thus entails the dissociation of already existing bonds, followed by the association of the entrant substance with the fixed-charge system through newly formed H-bonds and eventually the dissociation and entry of the substance.



**Figure 12.4. THE COMPETITIVE INHIBITION OF FRUCTOLYSIS BY GALACTOSE IN EHRЛИCH ASCITES TUMOR CELLS.** The substrate concentration is expressed as micromoles per milliliter and the velocity is expressed as micromoles of CO<sub>2</sub> produced per milligram dry weight of tumor cells per hour. The values, 11.1 and 8.9, refer to galactose concentration in micromoles per milliliter. (Figure after Nirenberg and Hogg, 1958.)

The mechanism of entry of nonelectrolytes is similar to the mechanism proposed for the entry of cations and should follow similar kinetics. Structurally similar molecules may be expected to prefer certain sets of H-bond-forming sites. In this case, competitive inhibition will result, as in the case of the rates of entry of different sugars, for example, the competition between glucose, sorbose, and fructose for entry into human red blood cells (Widdas, 1954). Similarly, galactose shows a competitive inhibitory effect on fructose utilization by intact Ehrlich ascites cells while it shows no such effect on homogenates of these cells. Nirenberg and Hogg (1958) interpreted these, their results, as being due to a "competition for a binding site which appears to be a functional part of the transport mecha-

Diaphragm preparation	Pentose concentration, mg/ml	Insulin, unit/ml	Intracellular pentose content after incubation for		
			5 min	10 min	15 min
Cut	1.00	0	—	—	0.41 (2)
Cut	2.50	0	—	—	1.06 (2)
Cut	4.50	0	—	1.59 (3)	1.92 (6)
Cut	4.50	0 <sup>a</sup>	—	—	—
Cut	5.00	0	1.06 (3)	1.71 (3)	2.18 (3)
Cut <sup>b</sup>	5.00	0	—	1.76 (2)	2.12 (2)
Cut	5.00	0.4	2.33 (3)	3.20 (3)	3.38 (3)
Intact	4.50	0	—	—	0.70 (2)
Intact	4.10	0.4	—	—	1.95 (4)

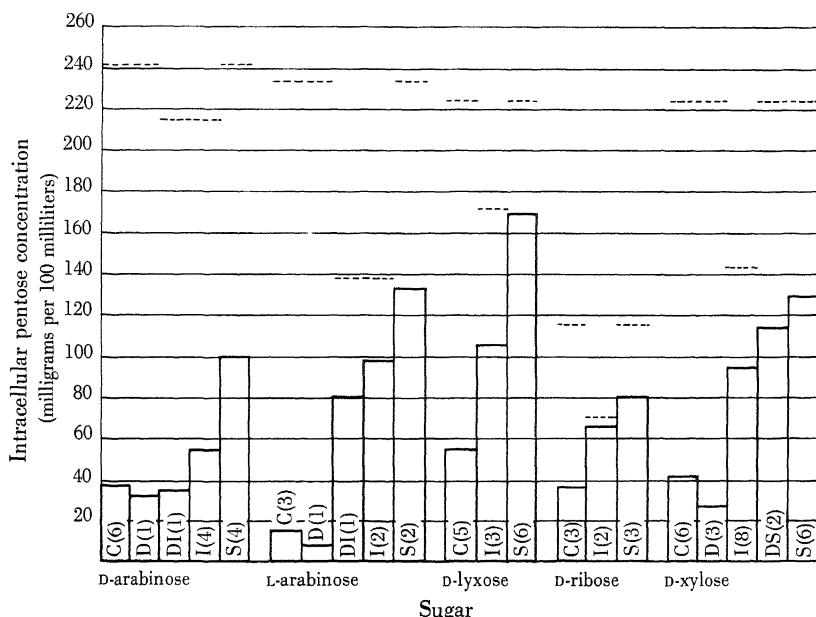
Diaphragm preparation	Pentose concentration, mg/ml	Insulin, unit/ml	Intracellular pentose content after incubation for		First-order velocity constant, $k (\text{min})^{-1}$	Final volume distribution, %
			30 min	60 min		
Cut	1.00	0	0.50 (2)	0.52 (2)	0.114	52
Cut	2.50	0	1.28 (6)	1.27 (2)	0.116	51
Cut	4.50	0	2.40 (18)	2.46 (12)	0.105	55
Cut	4.50	0 <sup>a</sup>	3.66 (11)	—	—	81
Cut	5.00	0	2.63 (20)	2.66 (8)	0.108	53
Cut <sup>b</sup>	5.00	0	2.58 (6)	2.70 (6)	0.103	54
Cut	5.00	0.4	3.53 (11)	3.58 (6)	0.221	72
Intact	4.50	0	1.27 (4)	2.48 (4)	0.022	54
Intact	4.10	0.4	3.20 (4)	3.24 (4)	0.063	80

**Table 12.2. THE RATE OF PENETRATION AND THE DISTRIBUTION EQUILIBRIUM OF D-XYLOSE IN ISOLATED RAT DIAPHRAGM IN THE PRESENCE AND IN THE ABSENCE OF INSULIN.** Values are expressed as milligrams per milliliter of intracellular water. The numbers of experiments are given in parentheses. (Table from Kipnis and Cori, 1957.)

<sup>a</sup> Insulin (0.5 to 1.0 unit per 100 grams body weight) was injected subcutaneously 15 to 30 minutes prior to the removal of the diaphragm; no insulin was added *in vitro*.

<sup>b</sup> Diaphragms obtained from alloxan-diabetic rats.

nism" (Figure 12.4). We suggest that these sites are the fixed H-bonding sites; as such, they would be indistinguishable from "carriers" by kinetic analysis (Le-Fevre, 1948). Yet they would require no continual expenditure of metabolic energy, a concept that is susceptible to criticism, as mentioned already (see Chapter 8).



**Figure 12.5. THE INTRACELLULAR CONCENTRATION OF PENTOSES IN RAT GASTROCNEMIUS MUSCLE.** Animals were sacrificed two hours after the injection of 100 milligrams of pentose per 100 grams of rat. C, control; D, diabetic; I, insulin; S, stimulated. The dotted lines represent the corresponding plasma values. The numbers of experiments are given in parentheses. (Figure after Helmreich and Cori, 1957.)

Remembering the importance of  $c$ - and  $c'$ -values in determining the H-bond-forming capacities of both the polar groups and the backbone it is to be expected that any agent or action which alters the  $c$ - and  $c'$ -values will alter not only the accumulation or exclusion of nonelectrolytes, as already mentioned, but also their permeability.

The experiments of Kipnis and Cori (1957) and of Helmreich and Cori (1957) previously mentioned show that insulin, as well as electrical stimulation, increases the rate of entry of pentoses into muscle (Table 12.2, Figure 12.5). Both phenomena may be interpreted as being due to variation of the  $c$ - and  $c'$ -values by the respective agents (see Chapters 15 and 16). The increased rate of penetra-

tion of glucose through the blood-brain barrier and across crystalline lens, brought about by insulin and by growth hormone may be explained similarly (Ross, 1952).

## B I B L I O G R A P H Y

### BOOKS AND MONOGRAPHS

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Hechter, O., and Lester, G., Cell permeability and hormone action. *Recent Progr. in Hormone Research* 16, 139-186 (1960). An up-to-date review of nonelectrolyte behavior as part of contribution to the symposium. The article is followed by a discussion by various prominent workers in this area. Hechter and Lester in their interpretation of biological phenomena invoked Gregor's concept of selective ionic accumulation in exchange resins due to swelling-pressure effect. For criticism of Gregor's concept, see, for example, Cruickshank, E. H., and Meares, P., The thermodynamics of cation-exchange. *Trans. Faraday Soc.* 53, 1299-1308 (1957). Note also that Gregor's theory fails to explain the inverted order of  $\text{Na}^+$ -versus- $\text{K}^+$ -ion selectivity in carboxylic and sulfonic exchange resins (see Section 9.1A).

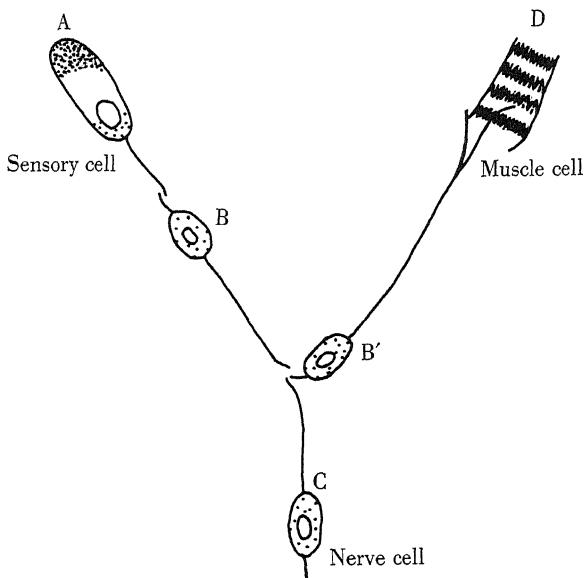
# 13

## EXCITATION AND INHIBITION

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Two important attributes shared by most living organisms are sensitivity to changes in the external environment, and activity which is coordinated among the



**Figure 13.1. A TYPICAL PERCEPTION-MODULATION-RESPONSE CHAIN.** Cell A, a typical sensory cell, contains a specialized portion particularly susceptible to excitation by a specific type of external stimulus. Excitation leads to the release of a chemical transmitter whose action excites the nerve cell B. This cell then releases a chemical mediator which acts on the nerve cell B'. The response of B' to this stimulus is modified by the neuron C. If the net effect of B and C on B' is excitation, an all-or-none impulse will be fired to the effector cell, D. The response of the effector cell is determined by the liberation of a transmitter substance at the B'-D junction. Essentially the same pattern is followed in hormonal action; in this case the transmitter substance travels over a longer distance through the bloodstream rather than the short distance from a nerve ending to, for example, a muscle motor end plate.

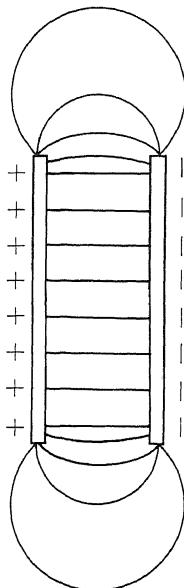
various parts of the organism. The basis of both attributes is formed by the properties of living protoplasm which allow it to be excited by various types of stimuli and to transmit to distant loci a representation of the information thus received. The reception of information from the outside world is usually handled by special sense organs such as the eyes and ears. Internally, messages are transmitted through the nervous system in the form of electrical impulses and through blood or tissue fluids by chemical mediators. Once the message reaches its destination, it must be converted to a form which can be recognized by the responsive organ. In its turn, the response may be suppressed or enhanced by information received from other sources.

This sequence is represented by the diagram in Figure 13.1. First, let us discuss the simplest case: The sensory cell is excited by a stimulus; when the excitation reaches the level known as the threshold, it is transmitted to a conducting nerve cell which carries the message, coded in terms of frequency-modulated nerve impulses, along the nerve axon. At the distal end of the axon, the impulses release chemical mediators which excite other cells and elicit a response such as the contraction of a muscle fiber. In this chapter, we shall discuss these steps in a somewhat inverted order: the conduction of nerve impulses, the release of the chemical mediator by a nerve impulse, and finally, the perception of various forms of stimuli and their conversion to nerve impulses. We shall also discuss complex cases in which other participating neurons can modify the responses of the simplest reception-response chain, notably through inhibition.

### 13.1. The Mechanism of Electrical Excitation and the Conduction of Impulses

Physiologically, the resting potential of nerve or muscle provides a basis for a transient variation, the action potential. This is the physiological code unit for messages sent over long distances. In Chapter 10, we suggested that the outermost functional layer of voluntary muscle and nerve cells is composed of a protein or protein complex bearing fixed, predominantly anionic sites with a *c*-value of about  $-2.7\text{\AA}$ . At this *c*-value, the adsorption of the  $\text{K}^+$  ion is greatly favored over the adsorption of the  $\text{Na}^+$  ion. Equation (10-8) therefore predicts a highly  $\text{K}^+$ -ion-sensitive resting potential in which the cellular phase is negative with reference to the external solution. With respect to dissociation, those  $\text{K}^+$  counterions associated with sites on or near the surface of a cell have an advantage not enjoyed by counterions embedded in the interior. Surface counterions undergo a much greater configurational and rotational entropy gain when they dissociate into the contiguous free solution than do the internal counterions when they dissociate into the bulk phase (see Section 2.4). In other words, when compared with that of a similar  $\text{K}^+$  ion within the fixed-charge system, the free energy of dissociation for

the surface K<sup>+</sup> counterion should be smaller in magnitude. Because the *c*-value ensemble is influenced by each dissociation, the *c*-values of the surface sites are poised in a delicate state of balance although similar fixed-site–counterion pairs in the bulk phase of the fixed-charge system are not. Thus, the surfaces of nerve and muscle fibers as well as those of many other cells are particularly sensitive to



**Figure 13.2. ELECTRIC LINES OF FORCE.** Lines of force originate at charges of one sign and terminate at charges of opposite sign.

environmental changes, often responding with an all-or-none shift in the metastable *c*-value ensemble.

If a pair of electrodes is introduced into a NaCl solution and an electromotive force is applied, the positively charged Na<sup>+</sup> ions will move toward the negatively charged cathode and the negatively charged Cl<sup>-</sup> ions will move toward the positively charged anode. According to electrostatics, the field between the electrodes may be represented by lines of force whose density is related to the charge on the electrodes. Diagrammatically, the lines of force start at charges of one sign and terminate at charges of opposite sign (Figure 13.2). If a densely charged phase, a muscle or a nerve fiber, for example, is introduced between the two electrodes, the lines of force will converge on the surface charges and terminate there, leaving the interior of the cell relatively unaffected. This is called the Faraday cage

effect.\* The surface charges at which the lines of force terminate are subjected to the same physical forces which produce the electrolytic separation of anions and cations in a salt solution. As in electrolysis, these surface charges tend to move toward the sources of the lines of force and thereby to reach a state of lower energy. In muscle and nerve, the surface charges consist mostly of  $K^+$  ions and fixed anions.† Thus, most of the lines of force originating at the cathode terminate at the  $K^+$  counterions and those from the anode terminate at the free and fixed anions. The anode produces a relatively weak effect because there are few counteranions close to the cell surface, the major anionic components being the protein carboxyl groups which cannot migrate. A much stronger effect is produced by the lines of force originating from the cathode because the  $K^+$  ions may easily be removed from surface sites. In consequence, a migration of  $K^+$  ions from the surface follows the application of an electric field of sufficient strength and duration. Since each desorption initiates a  $c$ -value change at neighboring sites, a threshold is eventually reached and an indirect  $F$ -process follows,‡ leading to a general  $c$ -value shift such that  $Na^+$ -ion adsorption is favored over  $K^+$ -ion adsorption. We then say that part of the fiber surface has been excited by the cathodic field.

Once an action potential is created, an electromotive force develops between the active part of the muscle or nerve fiber and adjacent regions; the active part is then cathodic with respect to the contiguous quiescent region. Like the cathode, the relatively cathodic active region may bring about desorption of  $K^+$  ions and the activation of the next section of nerve or muscle fiber. The formal picture is similar to that conventionally drawn to illustrate the membrane theory of excitation. However, a relatively simple mechanism is provided for the phenomenon usually described ambiguously as a permeability change. The importance of such local circuits in the propagation of impulses is well established. Their purely electrical aspects are best illustrated by saltatory transmission along myelinated nerve fibers (see Hodgkin, 1951).

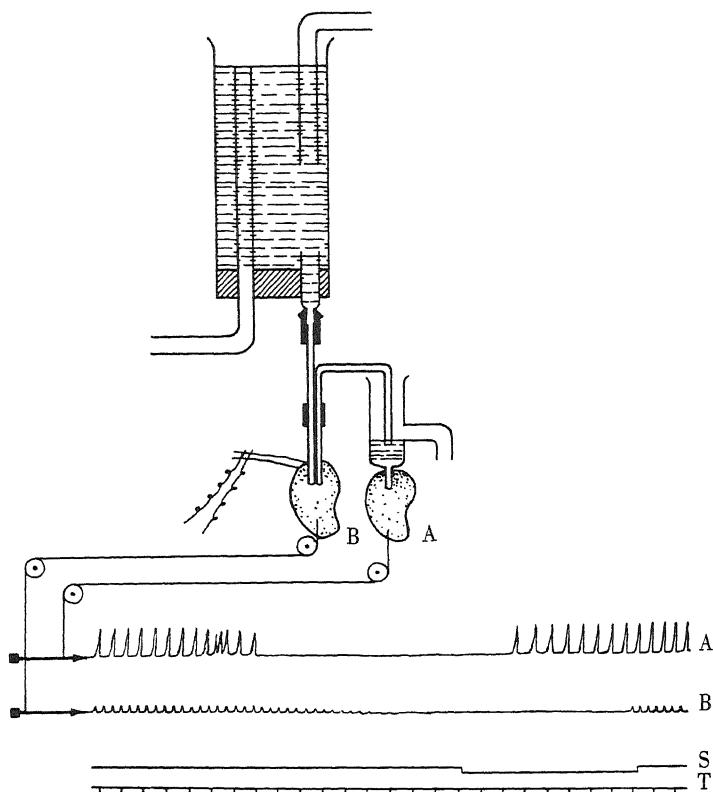
### 13.2. The Release of Chemical Mediators by the Nerve Impulse and the Generation of the End-Plate Potential

Loewi, in a series of papers (1921, 1936), established that the liberation of a chemical substance which follows stimulation of the vagus nerve brings about the arrest

\* At a phase boundary like that separating the muscle or nerve cell from the plasma, both the vacated anionic sites in the cellular phase and their counterions outside the cell congregate close to the interface (see Figure 10.6), rather than distributing themselves uniformly in the intracellular and extracellular phases. In this way, a minimum electrostatic potential energy, compatible with the ambient temperature, is maintained. A familiar example is the distribution of gas molecules or dust particles around the earth; this follows a fundamentally similar statistical distribution with its maximum density close to the surface of the earth.

† That the surface fixed sites are essentially anionic was shown in Section 10.4.

‡ The desorption of  $K^+$  ion is equivalent, but opposite in effect, to the addition of the  $H^+$  ion during the acid titration of hemoglobin which served as the experimental basis for postulating the indirect  $F$ -process (see Section 7.4C).

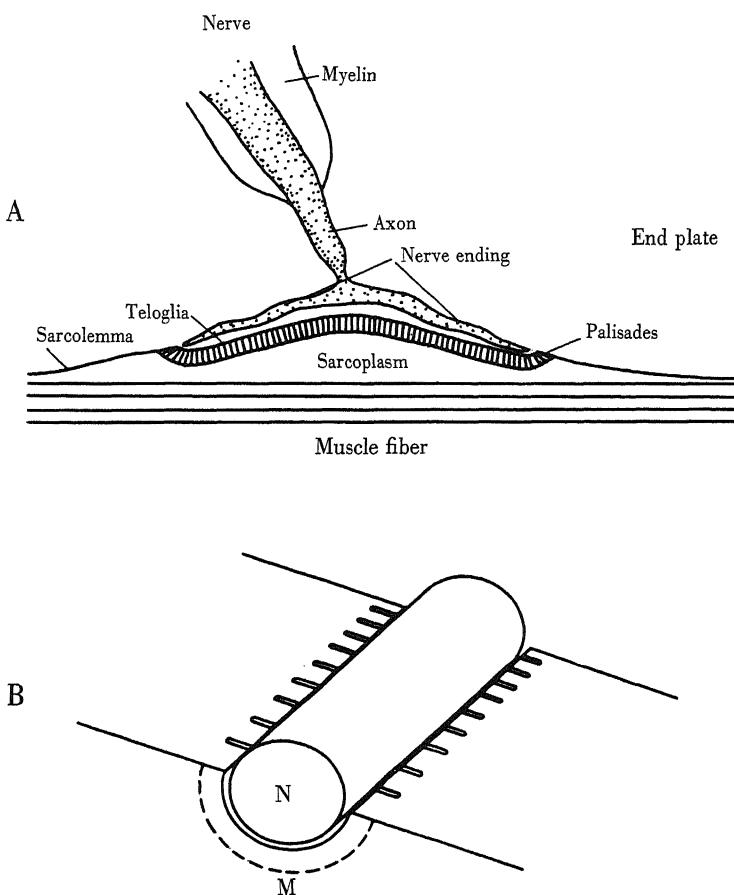


**Figure 13.3. LOEWI'S EXPERIMENT.** An isolated heart B with intact nerves is perfused at constant pressure with a Ringer's solution. The perfusion fluid passes to a second isolated heart A. Contractions from both hearts are recorded by writing levers; time T is given in 5-second intervals. The vagus fibers from the first heart are stimulated (S) for 40 seconds; this heart is quickly arrested. Slowing of the second heart A is apparent within 15 seconds after arrest of the first; it stops shortly thereafter. The experiment shows that a chemical substance, later identified as acetylcholine, is released by the vagus nerve during stimulation. (Diagram after Bain, 1932.)

of heart-muscle contractions (Figure 13.3). The substance was later identified as acetylcholine. This was the first experimental evidence to establish unequivocally the existence of chemical mediation; subsequent work in this field has been extensive. One of the most exciting series of experiments showed that acetylcholine acts as a chemical mediator at the neuromuscular junction, translating the electrical nerve impulse into a signal which initiates muscular contraction (Dale *et al.*, 1936; G. L. Brown *et al.*, 1936; Brown, 1937). An overwhelming collection of experimental facts has now firmly established this key role of acetylcholine in neuro-

muscular transmission (Eccles *et al.*, 1941; Kuffler, 1949; Hunt and Kuffler, 1950; Fatt, 1954).

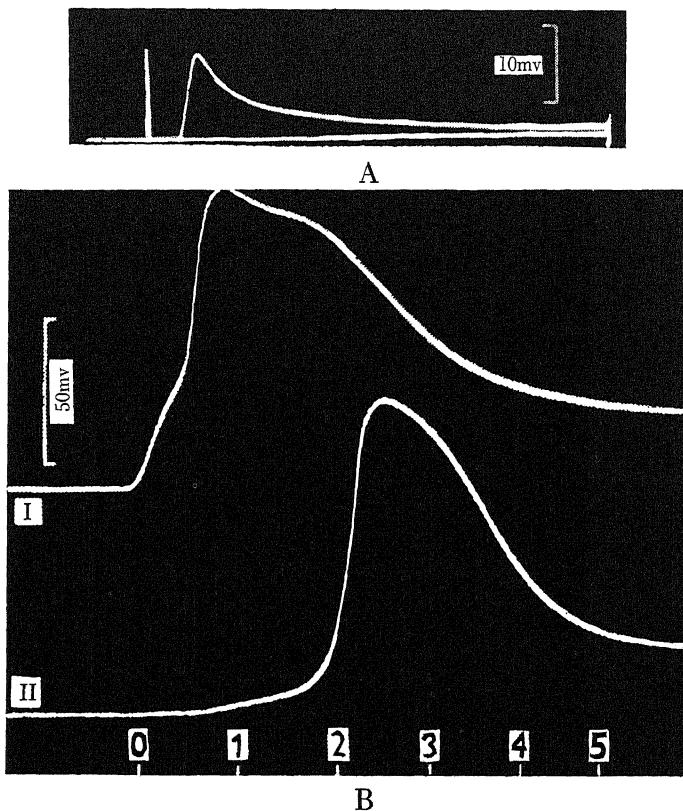
Histological investigation has shown that muscle cells are in close contact, but not continuous, with the nerve which innervates them (Figure 13.4). Thus, when a nerve impulse arrives at the neuromuscular junction, acetylcholine is released from the nerve terminal, diffuses through the small intervening space, and reacts with a specialized patch of muscle-fiber surface called the end plate. The acetylcholine sets up a local partial depolarization of the cell surface at this locus, which, at rest, has a surface potential of the same magnitude as the rest of the muscle fiber. Such local potential variations are commonly called end-plate potentials.



**Figure 13.4. THE REGION OF THE MUSCLE END PLATE.** A, a cross sectional diagram. B, the nerve ending N is actually buried in a groove on the muscle surface M. (A after Acheson, 1948, after Couteaux; B after Birks *et al.*, 1960.)

Figure 13.5 shows that these end-plate potentials may be recorded with a Gerard-Graham-Ling microelectrode inserted in the muscle fiber at the end-plate region (see Alexander and Nastuk, 1953, Figure 4); these potentials cannot be recorded at a distance from the end plate.

The role of acetylcholine in the creation of end-plate potentials is strongly indicated by the demonstrations, on one hand, that this compound is secreted by the stimulated nerve-muscle preparations (Dale *et al.*, 1936) and, on the other hand, that a potential variation similar to the end-plate potential can be generated



**Figure 13.5. THE END-PLATE POTENTIAL OF A FROG MUSCLE FIBER.** Potentials were recorded with the bathing Ringer's solution grounded and a microelectrode inserted into a single muscle fiber at the end-plate region. A, the propagated muscle impulse is prevented by the drug, curare. This also reduces the size of the end-plate potential. B, recording BI is from a normal end plate. However, due to the superposition of the action potential, only the rising phase of the end-plate potential is evident. For recording BII, the microelectrode was placed away from the end-plate region; only an action potential is seen. (Figures from Fatt and Katz, 1951.)

by the local application of acetylcholine at the end plate, *but not elsewhere on the muscle fiber* (Buchthal and Lindhard, 1937; Kuffler, 1943; Nastuk, 1953). The magnitude of the potential change varies with the concentration of acetylcholine applied (see Figure 13.15).

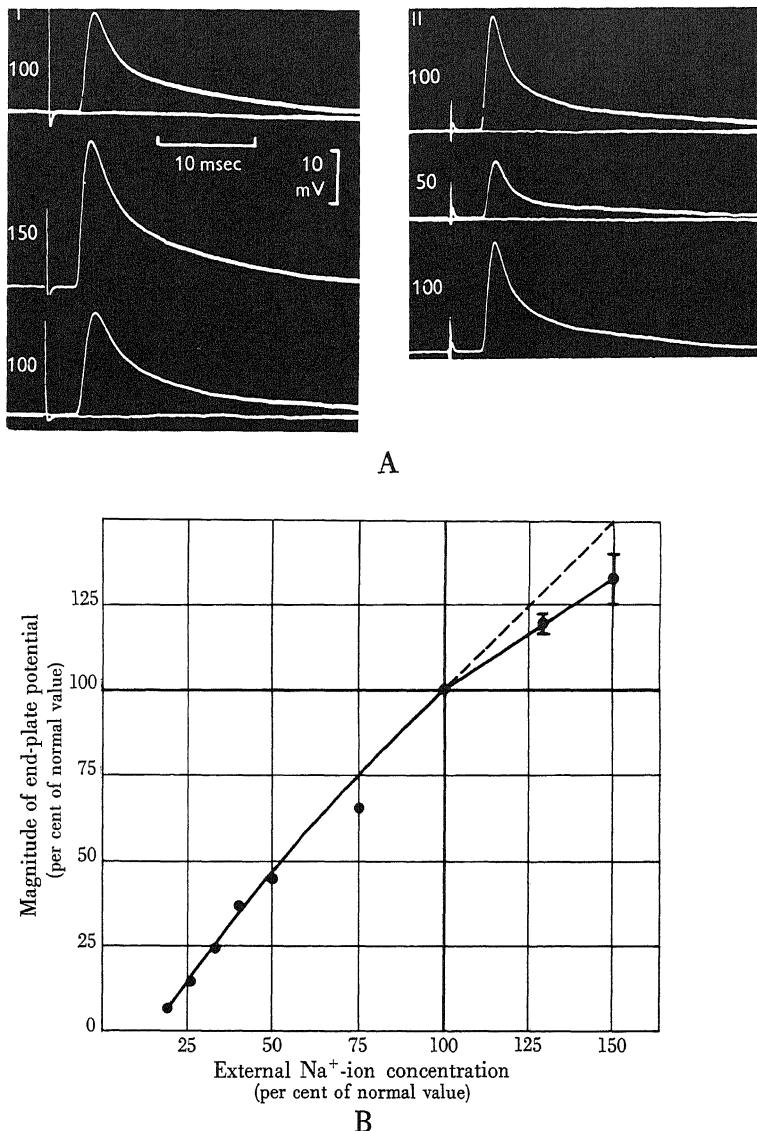
#### A. THE MECHANISM OF THE END-PLATE POTENTIAL

The end-plate potential is manifested in basically the same way as the action potential, that is, by a variation in the resting potential. We have already suggested that the mechanism of the action potential is a change from a cooperative state where the *c*-value is such that the  $K^+$  ion is preferred to an alternative cooperative state where the  $Na^+$  ion is preferred. This scheme seems applicable to the end-plate potential also, for it is now established that, as in the case of the action potential, the magnitude of the end-plate potential depends largely on a high external  $Na^+$ -ion concentration\* as shown by Figure 13.6; compare this with Figure 10.4 (see also Fatt, 1950; Nastuk, 1953). But there is a large difference between the magnitudes of the propagated action potential (approximately 100mv) and the end-plate potential (approximately 50mv); and there is no reversal of polarity (the overshoot) in the end-plate potential. These variations, however, may readily be interpreted as results of a difference in the level of the active-state *c*-values. In the case of the action potential, the *c*-value of the surface anions is such that  $-\Delta E_{Na} \gg -\Delta E_K$  and equation (10-8) predicts an inverted potential such as that shown in Figure 10.3. In the case of the end plate, the modal *c*-value of the anionic sites is such that the difference between  $\Delta E_{Na}$  and  $\Delta E_K$  may be considerably smaller. Equation (10-8) then predicts a smaller potential variation with no overshoot.

#### B. THE ROLE OF ACETYLCHOLINE IN THE GENERATION OF THE END-PLATE POTENTIAL AND ITS LIBERATION FROM THE NERVE ENDING

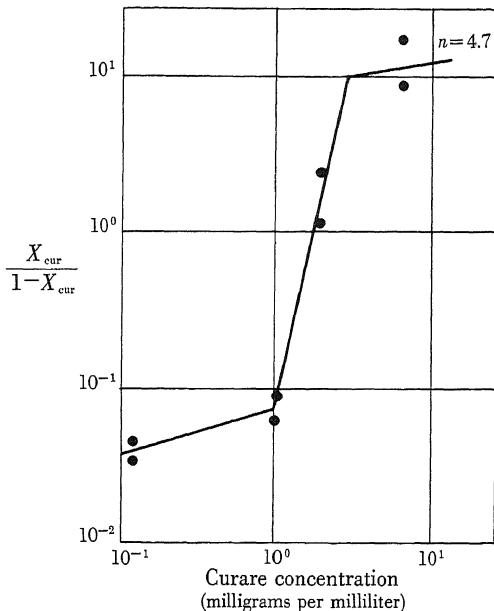
The acetylcholine released during activity is continuously resynthesized by the enzyme, choline acetylase (Quastel *et al.*, 1936; Nachmansohn and Machado, 1943). Since the function of acetylcholine depends on its explosive release in a relatively large quantity (estimated at about one million molecules per nerve impulse, see Acheson, 1948; Rosenblueth, 1950), there must be a means of storing it. We suggest that the mechanisms of storage and liberation, as well as the generation of the end-plate potential, follow a pattern presented earlier in this monograph as a basic function of the protein fixed-charge system. Specifically, we suggest the following mechanism.

\* The  $Na^+$  ion may be replaced by other ions such as quaternary ammonium and onium ions, as in the case of the action potential (see Nastuk, 1959; Koketsu and Nishi, 1959; also del Castillo and Katz, 1955); this phenomenon is explained in Section 10.2.



**Figure 13.6. THE RELATION BETWEEN EXTERNAL  $\text{Na}^+$ -ION CONCENTRATION AND THE MAGNITUDE OF THE END-PLATE POTENTIAL.** A, individual end-plate potentials are recorded with an intracellular microelectrode in muscle fibers treated with curare. The numbers, 150, 100, 50, indicate per cent of normal  $\text{Na}^+$ -ion concentration. I and II represent two series of experiments where the effects of increasing and decreasing  $[\text{Na}^+]_{\text{ex}}$ , respectively, are shown. These effects are perfectly reversible when the muscles are returned to a normal  $[\text{Na}^+]_{\text{ex}}$ . B, the collected data are tabulated. The height at the peak of the end-plate potential as a per cent of the normal height is plotted against the external  $\text{Na}^+$ -ion concentration, also expressed as per cent of the  $\text{Na}^+$ -ion concentration in normal frog Ringer's solution. (Figures from Fatt and Katz, 1952b.)

First, the synthesized acetylcholine is "stored" as the associated counterion of fixed sites on proteins at the nerve ending. At rest, these fixed sites have a *c*-value which favors adsorption of acetylcholine over adsorption of the  $\text{Na}^+$  ion.\* This adsorption is autocooperative and thus release of the adsorbent must be an all-or-none process. Evidence to support this concept may be derived from the data of



**Figure 13.7. ADSORPTION OF CURARE BY PROTEINS ISOLATED FROM ELECTRIC TISSUE OF THE ELECTRIC EEL.** A log-log plot of the data of Ehrenpreis (1960) showing that the adsorption of curare onto the "acetylcholine-receptor" protein is, in the middle concentration range, markedly autocooperative with an *n*-value of 4.7. There is strong evidence that acetylcholine and curare compete for the same sites (Section 13.3B); this suggests a similar autocooperative adsorption of acetylcholine. The data was replotted with the assumption that the total binding capacity is 0.371 mg of curare per mg of receptor protein.

Ehrenpreis (1960). These data, when plotted according to equation (5-12), show that the adsorption of curare is highly autocooperative (Figure 13.7) with an *n*-value of 4.7 (see Section 7.4B). Since curare and acetylcholine appear to compete for the same sites (see below), these data suggest a similar autocooperative adsorption of acetylcholine.

\* We shall make no commitment at this time as to whether these proteins, bearing gangs of acetylcholine-adsorbing sites, are located on or in anatomically demonstrable vesicles which have been suggested to be sacs enclosing acetylcholine (B. Katz, 1958; Birks *et al.*, 1960).

Second, the front of an action potential advancing along the nerve fiber engenders an all-or-none indirect *F*-process in the gangs adsorbing acetylcholine. This change forces the *c*-value ensemble to shift so that the relative affinities for the  $\text{Na}^+$  ion and for acetylcholine are reversed and adsorption of the  $\text{Na}^+$  ion is favored over adsorption of the acetylcholine ion (for evidence of  $\text{Na}^+$ -ion participation, see Fatt and Katz, 1952b).

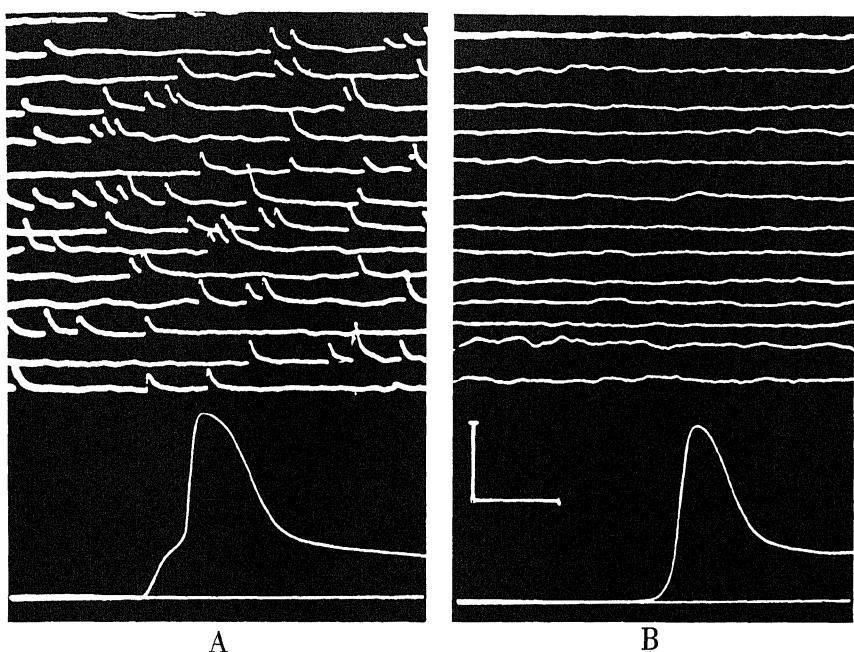
Third, the rejected acetylcholine ions are greatly preferred by certain fixed surface sites on the muscle end plate. These sites are cardinal to gangs of sites which, prior to the adsorption of acetylcholine, have a *c*-value such that the  $\text{K}^+$  ion is greatly preferred over the  $\text{Na}^+$  ion. The adsorption of acetylcholine leads to an indirect *F*-process and to a new *c*-value (say, about  $-2.3\text{\AA}$ ) at which the relative preference for the  $\text{Na}^+$  ion is increased (see Figure 13.6), creating the end-plate potential on the cell surface (for evidence, see Fatt and Katz, 1952b). We postulate that the cardinal sites which adsorb the acetylcholine ions also constitute either the whole or part of the enzymatically active sites of choline esterase (see Nachmansohn and Wilson, 1956); thus the adsorption of acetylcholine is followed by its rapid hydrolysis into acetate and choline. These components, no longer preferred by the sites, diffuse away; the *c*-value of the gang cooperatively reverts to that of the resting muscle surface and the normal end-plate resting potential is once more established, thus completing the cycle.\* Although the above sequence of events follows naturally from the present general theory, support for it is, to a large extent, derived from the work of Katz, Fatt, and del Castillo† on the miniature end-plate potentials.

A Gerard-Graham-Ling microelectrode inserted into a frog *sartorius* muscle fiber usually shows a persistent, unvarying resting potential (Figure 13.8B); however, Fatt and Katz (1952a) discovered that if the amplification in the recording system is high, certain regions of the fiber, corresponding to the location of the end plate, show spontaneous electrical activity (Figure 13.8A). In their time courses and in their responses to specific drugs and to denervation, these miniature potential variations resemble the much larger end-plate potentials generated in response to activity of the nerve supplying the end plate. It has been shown that both the temporal and the spatial distributions of these miniature potentials are random and that their frequency increases sharply with a rise of temperature (see Katz, 1958). Indeed, the investigators have suggested that these potentials represent the impact of unit "packets" of acetylcholine liberated from the nerve ending. According to the present interpretation, each packet represents a number of

\* This scheme also offers a mechanism for conversion of metabolic energy stored in acetylcholine during its synthesis to biological work (electrical). Another brief discussion of this will be given in Section 15.5 in connection with reversible contractile processes.

† These authors have interpreted their experimental evidence in the light of the membrane theory and may not agree with the interpretation presented here.

acetylcholine ions which have been adsorbed onto a cooperative gang of fixed ionic sites; their liberation follows the thermal detachment of a cardinal adsorbent.\*

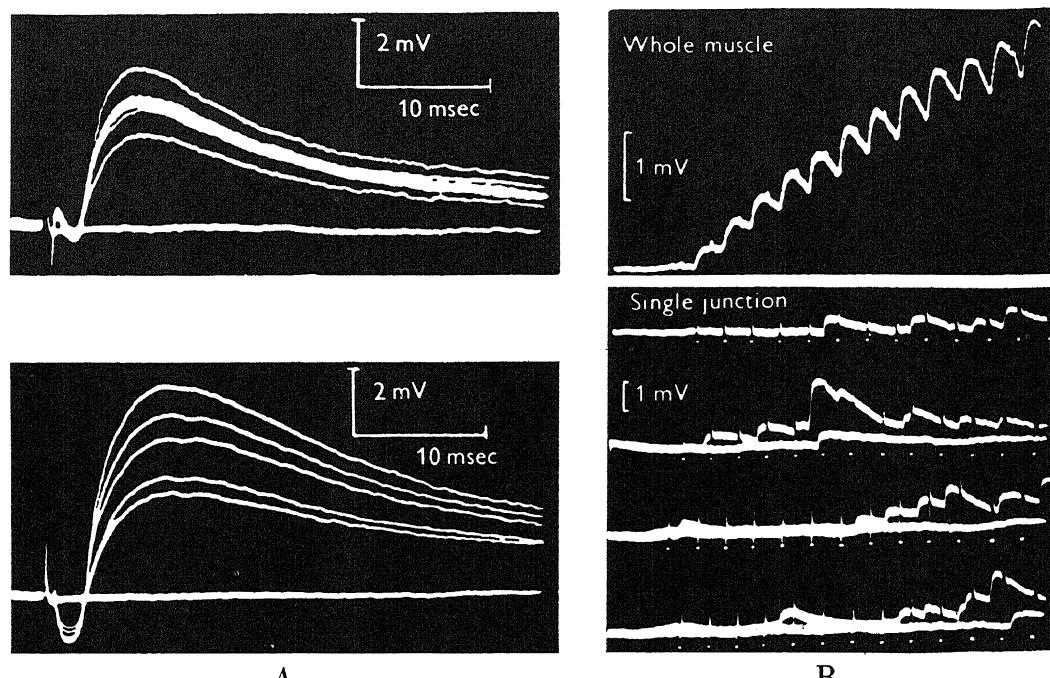


**Figure 13.8. MINIATURE END-PLATE POTENTIALS.** Localization of spontaneous activity in the muscle *extensor digitorum longus IV* by intracellular recording. A, recorded at the end plate; B, recorded two millimeters away in the same muscle fiber. The lower records, taken at high speed and low amplification, show the response to a nerve stimulus (shock applied at beginning of sweep); the upper records taken at low speed and high amplification show the spontaneous activity at the end plate, A, and the lack of spontaneous activity in the areas away from the end plate, B. Voltage and time scales: 50mv and 2msec for the lower records, and 3.6mv and 47msec for the upper records. (Figures from Fatt and Katz, 1952a.)

Further experimentation with end-plate potentials revealed other interesting facts. For example, del Castillo and Katz (1954a) have shown that the normal end-plate potential represents a quantal summation of individual miniature end-plate potentials and that the  $\text{Ca}^{++}$  ion increases and the  $\text{Mg}^{++}$  ion decreases the number

\* Although the average kinetic energy  $\frac{3}{2} kT$  at room temperature is only 0.9 kcal/mole, a very small value compared with the adsorption energy of the cardinal adsorbent (perhaps 10 kcal/mole), a small per cent of the particles could possess much greater kinetic energy and effectively dislodge a particle which would resist the average thermal bombardment.

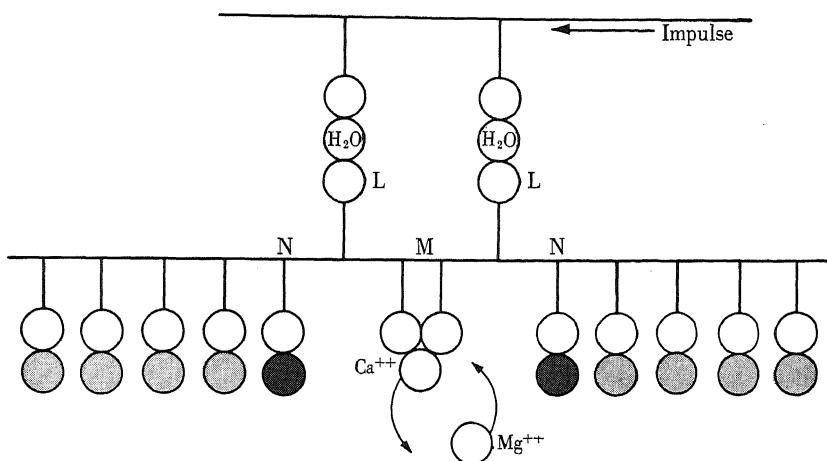
of quanta released in response to a nerve volley, but neither affects the size of an individual quantum (Figure 13.9). These ions have no effect on the rate of generation of the spontaneous miniature potentials. Cathodic polarization applied to



**Figure 13.9. THE QUANTAL NATURE OF THE END-PLATE POTENTIAL.** A, records from single neuromuscular junctions showing the end-plate responses to successive nerve stimuli in the presence of high ( $0.018M$ )  $Mg^{++}$ -ion concentration, which diminishes "quanta" of acetylcholine liberated at each impulse. A stepwise fluctuation in the amplitude of successive potentials is observed (del Castillo and Engbaek, 1954). B, Recruitment of end-plate potentials during a tetanizing stimulation of the nerve (100/sec). Muscles were treated with high ( $0.014M$ )  $Mg^{++}$ -ion and low ( $0.0009M$ )  $Ca^{++}$ -ion concentrations. The upper record, from a whole *sartorius* muscle, represents the average response of hundreds of end plates and shows the quantal nature of the facilitation effect of one stimulus on a successive one. The lower record is an intracellular recording from a single end plate of the muscle *extensor longus digitorum IV*; stimuli are indicated by dots. Note that at the single end plate, even though the same frequency of stimuli is applied, the responses are quantal and do not show the gradual increase. (Figure from del Castillo and Katz, 1954a.)

the nerve ending does produce a graded increase in the rate of firing of the miniature potentials; this effect can be reduced greatly by the  $Mg^{++}$  ion. We suggest the following scheme, which accounts for all of these diverse facts, as a mechanism for the release of acetylcholine. Referring to Figure 13.10, we designate site N as the cardinal site whose adsorbent controls, in an all-or-none manner, the release of a quantum of acetylcholine. An adjacent site L, perhaps bound by a salt linkage of

low configuration with another protein at the nerve ending, is cardinal to site N. When a nerve impulse arrives, either site L undergoes a change of adsorbent or the adsorbent shifts to another configuration causing, by induction, a *c*-value shift at site N. This shift releases the adsorbed acetylcholine from the gang. The *c*-value of site L, in turn, is controlled by site M which prefers to adsorb the alkaline-earth ions (and possibly represents a pair of rather closely located chelating sites). The cardinal adsorbents,  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  ions, then modulate, in an all-or-none



**Figure 13.10.** GANGS OF SITES ADSORBING ACETYLCHOLINE AND CARDINAL SITES, L AND N, WHICH CONTROL THE AUTOCOOPERATIVE RELEASE OF THE ACETYLCHOLINE. Cardinal site M, which selectively adsorbs divalent cations, consists of a closely placed pair of anionic sites and is capable of modulating the adsorbent at L. Darkest circles represent acetylcholine; lighter circles can represent either acetylcholine or a different counterion.

fashion, the adsorbent on site L (or the configuration of the adsorbent at L). This determines the reactivity of site L to the nerve impulse or to cathodic polarization but has no effect on site N *per se* so that the rate of thermal desorption at N remains unchanged with variations of  $\text{Ca}^{++}$ - and  $\text{Mg}^{++}$ -ion concentrations. On the other hand, any effects which depend on the all-or-none shift of adsorbent at site L are modulated by the concentration of alkaline-earth ions.

### 13.3. The Generator Potentials—Their Function in Excitation and Inhibition

A number of investigators have demonstrated that rhythmic responses of nerve and muscle to electrical and chemical stimuli are usually initiated by a

transient local depolarization which varies in magnitude with the intensity of the stimulus (Adrian and Gelfan, 1933; Fessard, 1936). It has been suggested that the initiation of the nerve impulse by sensory stimuli follows a similar pattern (Pantin, 1937). The term "generator potential," introduced by Granit (1947) has now gained wide acceptance; the occurrence of such potentials in all types of sensory organs is becoming increasingly clear. In Section 13.2, we discussed the neuromuscular junction and the end-plate potential at length, both because of their intrinsic interest, and because the end-plate potential is a thoroughly studied example of the generator potential. We should now like to show that this generator potential serves as the universal first step in the activation of an excitable cell. Such a cell may be a sensory cell for perception of a particular type of environmental change, an interneuron which integrates and coordinates various types of information derived from within and without the organism, or an effector cell such as a muscle or gland cell which carries out the response. We shall now discuss the ways in which the generator potential serves as the fundamental instrument directing the complex behavior of a living organism in terms of excitation and inhibition.

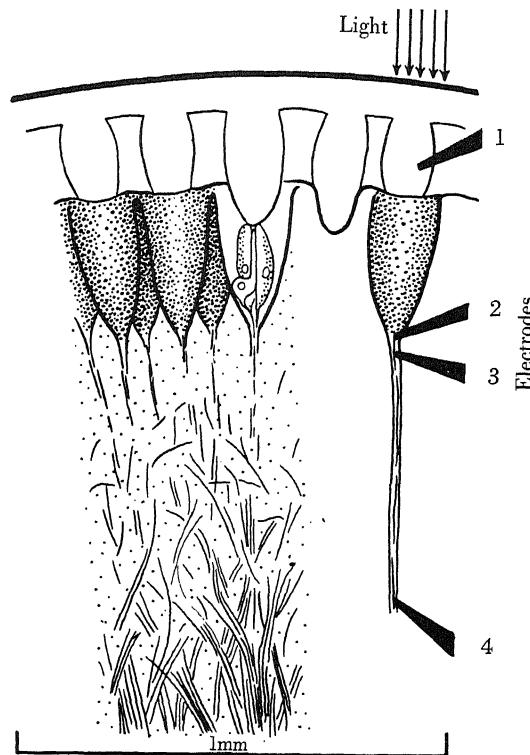
#### A. THE LOCATION OF THE GENERATOR POTENTIAL AND ITS GRADED RESPONSE

##### (1) Photoreceptors

Granit, in his monographs (1947; 1955), introduced the theory of the generator potential and extensively discussed the subject, particularly with reference to visual perception. For the cursory presentation possible in the present monograph, however, the work of Hartline and co-workers (1952) seems most illuminating (Figures 13.11 and 13.12). Using fine cotton-wick electrodes, these workers recorded the potential variation from a single visual cell of the lateral eye of the horseshoe crab (lead 1-2) and from the nerve strands emerging from the ommatidium cell body (lead 3-4). Figure 13.12A shows the direct relationship between the intensity of the impinging light and the magnitude of the generator potential (specifically, the "retinal" action potential, lead 1-2) and between the generator potential and the frequency of discharge of nerve impulses.

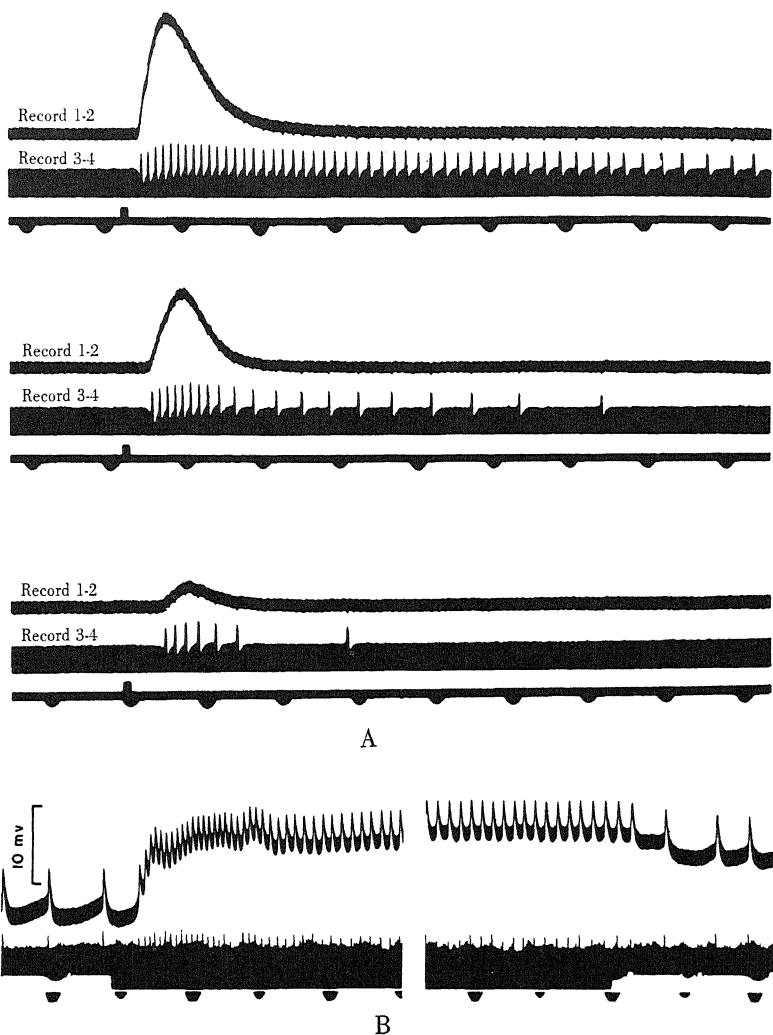
##### (2) Mechanoreceptors

A variety of sensations can be elicited by mechanical stimuli; auditory, pressure, and proprioceptive receptors all respond to mechanical (pressure) changes. A specific example of a mechanoreceptor is the dendritic processes of the sensory neurons of the lobster and of the crayfish; these processes are attached to the strands of a receptor muscle. Passive stretching following contraction of the mus-

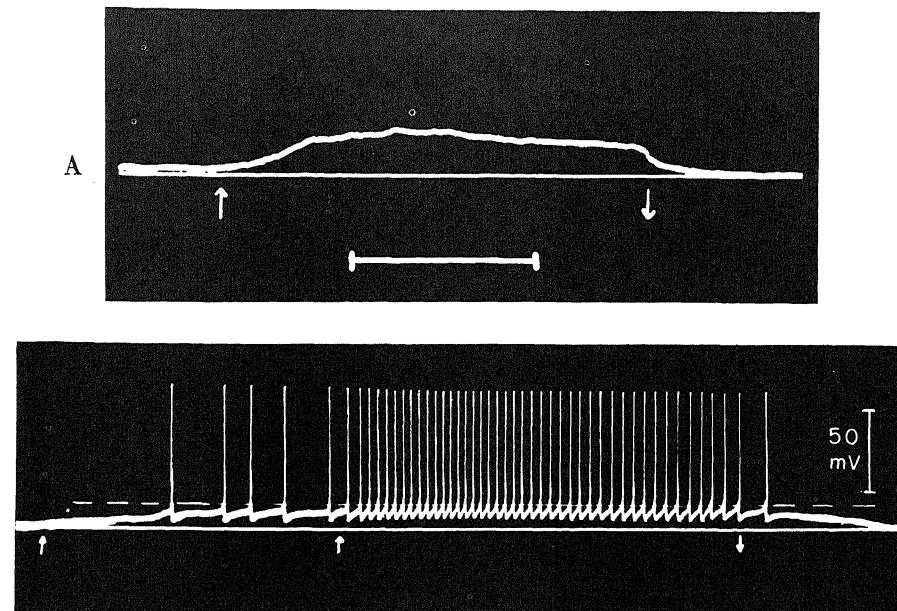


**Figure 13.11.** A SECTION OF LATERAL EYE OF *LIMULUS* IN A PLANE PERPENDICULAR TO THE SURFACE OF THE CORNEA. The transparent cornea at top contains the crystalline cones of the ommatidia; the heavily melanin-pigmented conical bodies of these form a layer on the inner surface of the cornea. On the left, a group of ommatidia is represented with indications of bundles of nerve fibers traversing the plexus behind the ommatidia, collecting in larger bundles that join to become the optic nerve. One of these ommatidia has been represented as if the section had passed through it, revealing the sensory component, also sectioned. On the right, an ommatidium with its nerve-fiber bundle is represented as it appears after having been isolated by dissection and suspended in air on electrodes (moist cotton wicks from chlorided silver tubes filled with seawater) represented by the solid black triangles. (Figure after Hartline *et al.*, 1952.)

cle excites these neurons. Figure 13.13 illustrates the generator potential created in response to weak extension; when it reaches a certain threshold, repetitively firing impulses are set up (Eyzaguirre and Kuffler, 1955). The direct relationship between the height of the generator potential and the amount of stretch is shown in Figure 13.14 which was obtained from studies of another type of mechanoreceptor, the sensory nerve terminals in the muscle spindles of the frog (Katz, 1950).



**Figure 13.12. ACTION POTENTIALS (GENERATOR POTENTIALS) OF AN ISOLATED OMMATIDIUM AND ITS NERVE STRAND.** A, potentials from an ommatidium and its nerve were recorded simultaneously in response to short flashes of light (0.02sec) at three intensities (relative values, top to bottom, 1.0, 0.1, 0.01). The signal for the light flash appears as a black square near the beginning of each record just above time marks. The preparation used was the same as that for Figure 13.11. Record 1-2 tracings were recorded between wick electrodes 1 and 2, record 3-4 tracings recorded between electrodes 3 and 4. B, simultaneous recordings of the potentials arising within an ommatidium (upper trace) and from the nerve bundle attached to the ommatidium (lower trace) in response to prolonged illumination. The black band fused to the underside of the lower trace indicates the duration of illumination. The potentials from the ommatidium were recorded between a Gerard-Graham-Ling microelectrode (tip diameter less than  $1\mu$ ) inserted into it, and an indifferent electrode in the solution covering the eye. Wick electrodes were used for recording potentials from the nerve as for A. The interval between time marks at the bottom was 0.2sec. (Figures from Hartline *et al.*, 1952.)



**Figure 13.13. POTENTIAL CHANGES IN A SLOWLY ADAPTING RECEPTOR CELL FROM AN ABDOMINAL SEGMENT OF A CRAYFISH.** An intracellular microelectrode inserted into the cell body registers the changes in potential during stretch. A, weak extension, starting at the first arrow, reduces the resting potential and sets up the generator potential, reflecting the intensity and time course of stretch. B, when the generator potential reaches a critical potential level (broken line), propagated impulses are set up which continue as long as adequate stretch is maintained. (Figures from Eyzaguirre and Kuffler, 1955.)

### (3) Chemoreceptors

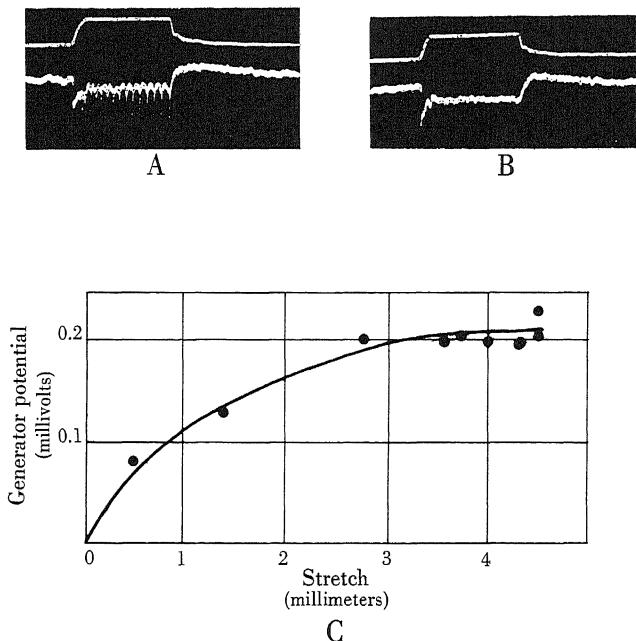
The chemoreceptor most extensively studied is, of course, the muscle end plate which has already been discussed in detail. The direct relationship between the concentration of the chemical mediator (acetylcholine) and the size of the generator potential (end-plate potential) in this preparation is shown in Figure 13.15, taken from Fatt (1950). Similarly, Figure 13.16 shows that the magnitude of the slow potential of olfactory epithelium in response to odorous substances varies directly with the "strength" of the stimulus, that is, the concentration of the substance (Ottoson, 1956).

## B. THE MECHANISM OF EXCITATION AND INHIBITION IN THE NERVOUS SYSTEM

### (1) Excitation

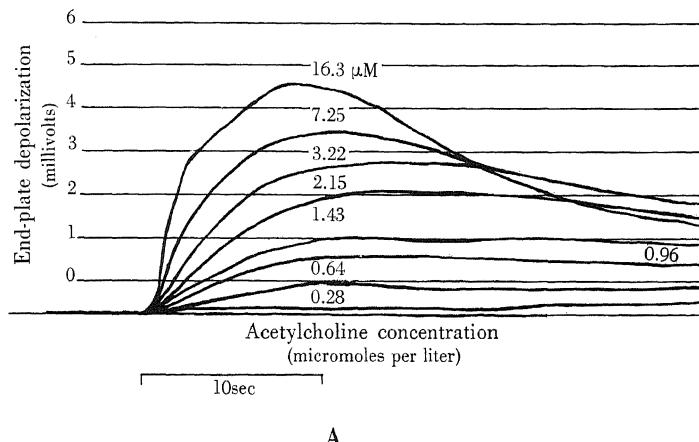
When the generator potential reaches a certain critical level, it triggers a repeated action potential of constant height. The mechanism is, of course, identical with

that proposed in Section 13.1 for electrical excitation. The potential difference between adjacent regions of a nerve or muscle creates an intense local electric field which acts on many selectively adsorbed  $K^+$  ions, reducing the activation energy for their desorption. This desorption, in turn, initiates an indirect *F*-process which

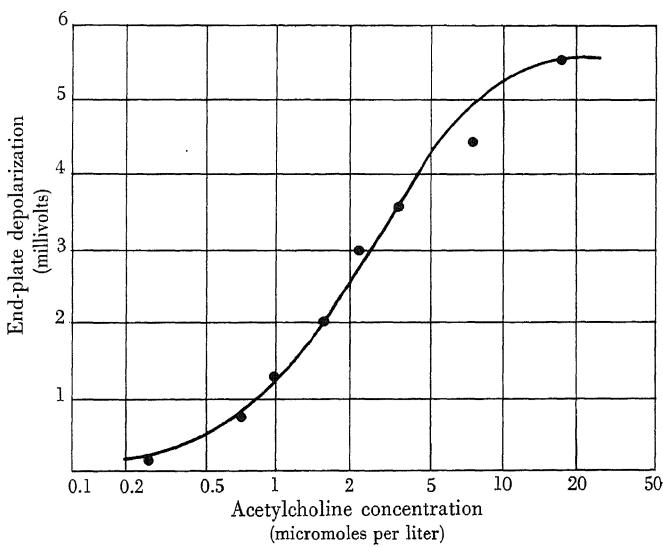


**Figure 13.14. THE RELATIONSHIP BETWEEN EXTENSION OF A MUSCLE SPINDLE AND THE MAGNITUDE OF THE GENERATOR POTENTIAL IN THE SENSORY AXON.** A, the upper tracing indicates the extent of stretch of the muscle-spindle preparation (initial length 16mm); the lower tracing shows potential changes in the sensory axon. Note the similarity to Figure 13.13B. Application of an anesthetic (cocaine) abolishes repetitive firing but not the generator potential as shown in B from such an anesthetized preparation. C, the relationship between the magnitude of the generator potential and the degree of stretching in anesthetized preparations. (Figures after Katz, 1950.)

changes the *c*-value ensemble of the entire gang. That the end-plate potential acts directly through the electric field set up between it and adjacent portions of the nerve or muscle is strongly suggested by the experiments of Fatt and Katz (1951) reproduced in Figure 13.17 and Table 13.1 (see also Figure 13.5). The threshold voltage necessary to initiate the action potential appears to be the same whether it is produced by the direct application of an electric-current pulse or by an in-



A



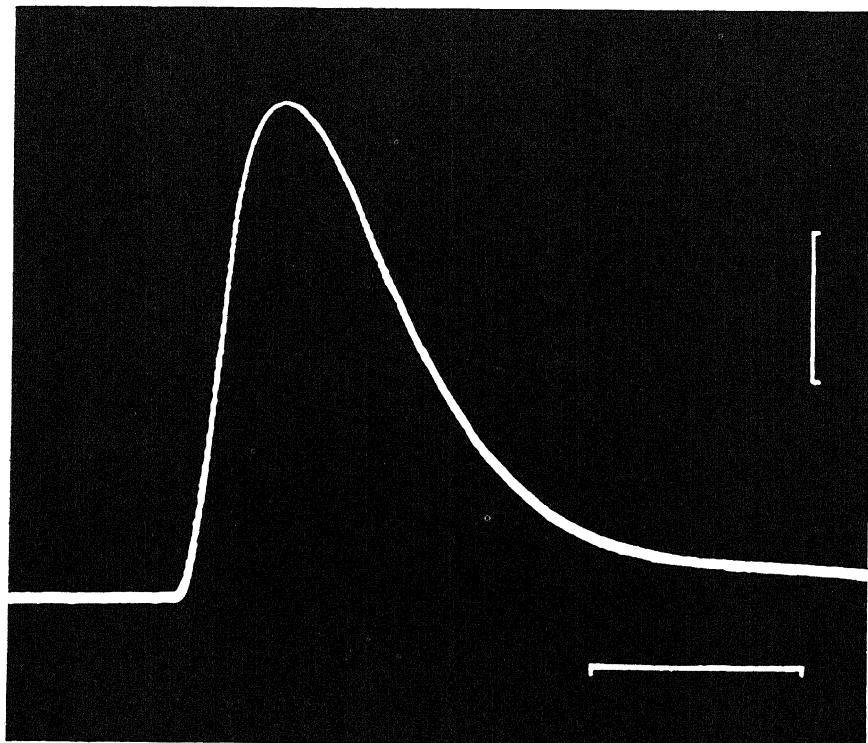
B

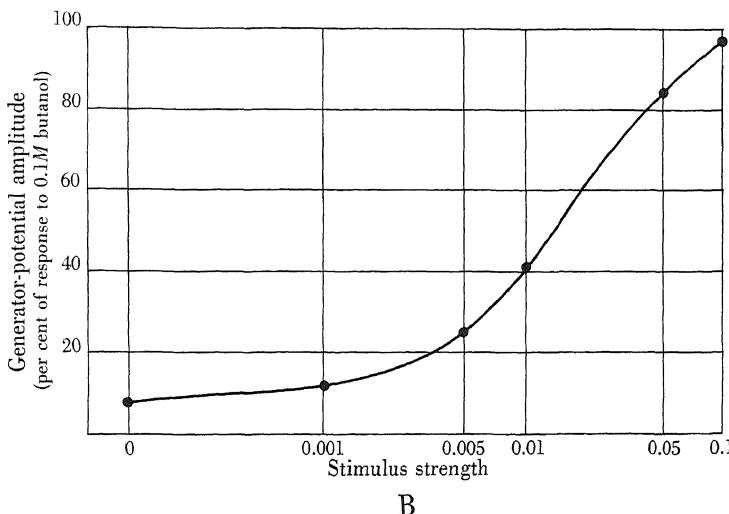
**Figure 13.15. THE RELATIONSHIP BETWEEN THE CONCENTRATION OF ACETYLCHOLINE AND THE MAGNITUDE OF DEPOLARIZATION OF THE END-PLATE REGION OF A MUSCLE FIBER.** A, the tracings show the depolarization at the end-plate focus of the muscle *extensor longus digitorum IV* following the application of various concentrations of acetylcholine. The muscle had previously been bathed in sodium-free solution containing prostigmine bromide  $10^{-6}M$  and was kept in that solution between tests. The acetylcholine concentration is indicated on each trace. B, the relationship of depolarization at the end plate to the concentration of applied acetylcholine producing the depolarization. In this case, the absence of the  $\text{Na}^+$  ion in the external medium prevents the generation of the action potential as curare does. (Figures after Fatt, 1950.)

direct invocation of the end-plate potential. As the height of the generator potential is increased above the threshold voltage, the firing frequency of the action potential increases.

The activation of the cell surface adjacent to the end-plate region depends on the simultaneous shifting of the *c*-value ensembles of a number of gangs. The attainment of this condition is a statistical matter. The probability of attainment is directly proportional to the probability that the individual gangs will fire. This, in turn, is a function of the height of the generator potential because the probability that a single gang will fire is proportional to  $\exp(-\Delta E + \psi_{gen})/RT$ . Therefore, the higher the value of  $\psi_{gen}$ , the larger this probability is, and so is the firing frequency of the action potential.

We have limited our discussion to positive responses to stimuli and the failure of such responses. However, there is another physiological means of controlling excitation. This is inhibition, long recognized by physiologists (see Sherrington, 1920) and found chiefly in the interaction of nerve cells, or neurons, at least in the vertebrates.



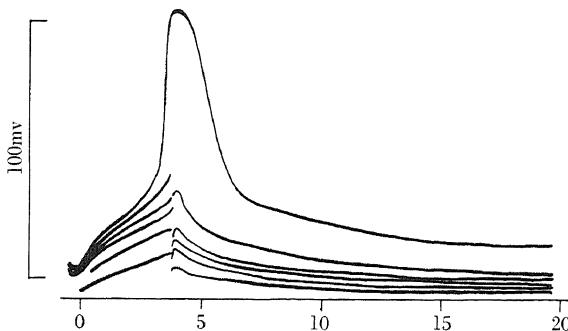


B

**Figure 13.16. THE RELATIONSHIP BETWEEN THE MAGNITUDE OF THE OLFACTORY-EPI-THELIUM GENERATOR POTENTIAL AND STIMULUS STRENGTH.** A, an oscillographic tracing of the time course of potential change in response to butanol (0.5cc) recorded between one active electrode on the surface of the olfactory epithelium and an inactive electrode in contact with the moistened cotton wool on which the isolated preparation was placed. The vertical line represents one millivolt; the horizontal line, two seconds. Upward deflection in this record indicates negativity of the active electrode. B, the relationship of the amplitude of the generator potential to strength of stimulus. The ordinate represents the amplitude of the slow potential at its peak. The abscissa represents the stimulus strength in units corresponding to the concentration of the solutions of butanol used for odorizing the stimulating air. Volume of air, 0.5ml. (A *from* and B *after* Ottoson, 1956.)

## (2) Inhibition

Neurons are specialized cells with long processes extending several feet in some cases. Many of these long processes, or axons, from several nerve cells run together to make up nerve trunks. The cell body of the neuron also possesses many shorter branches called dendrites (Figure 13.18). The work of Edwards and Ottoson (1958) has established that the nerve impulse is generated in the axon a distance from the cell body. It is triggered by a generator potential created at this point. This generator potential represents the integrated response to signals coming from the multiple connections which the neuron makes with other neurons through synapses on the cell body and through dendrites. The potential change produced at a particular locus spreads decrementally over a considerable portion of the cell surface (see Figure 13.19), summing with potential changes initiated at other loci. Thus, if two excitatory stimuli arrive on the cell simultaneously, the generator



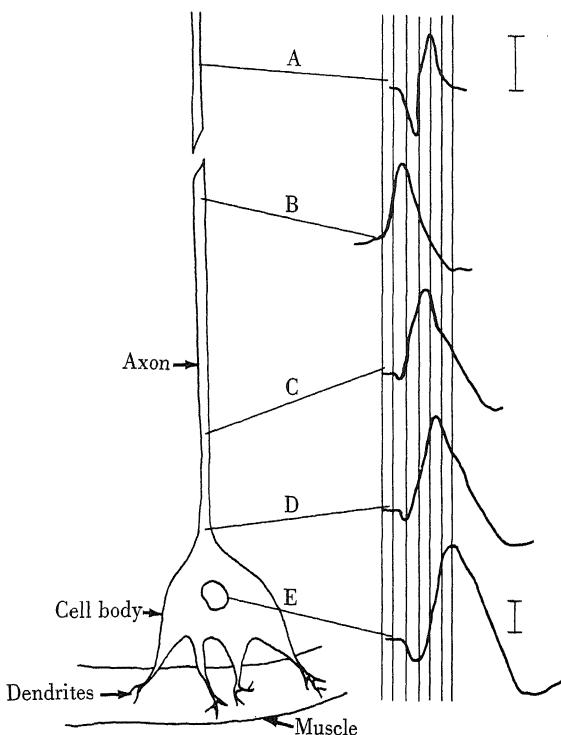
**Figure 13.17. INITIATION OF AN ACTION POTENTIAL BY AN ARTIFICIALLY APPLIED ELECTRIC POTENTIAL.** Two microelectrodes were inserted into the same region of a single muscle fiber. An outward current pulse was applied to one; the other was used to record changes in the cellular potential. The tracings show several subthreshold pulses that failed to generate an action potential and one superthreshold stimulus that succeeded. Time is given in milliseconds. (Figure after Fatt and Katz, 1951.)

potential measured at, for example, a certain locus on the axon, will represent a summation of the two stimuli and the nerve impulse created will be augmented. A more complex phenomenon ensues when some of the stimuli impinging on the cell are inhibitory.

The claw muscles of crustacea are supplied by single nerve fibers which may be isolated and stimulated separately. Studies of this kind have revealed that a single muscle fiber may receive both an excitatory and an inhibitory nerve. Stimulation of the excitatory nerve leads to the contraction of the muscle with the development of a nonpropagated action potential. This action potential is very

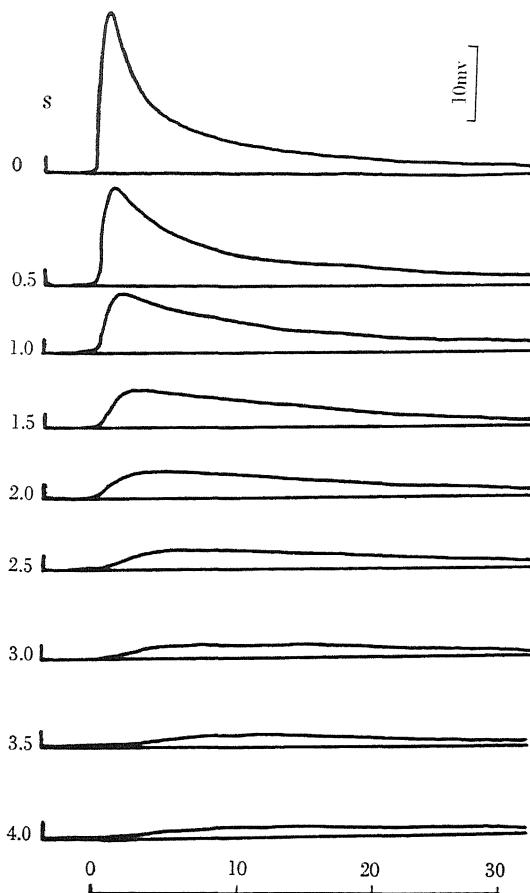
Calcium conc., mM	Direct stimulation			End-plate potential		
	Resting potential, mv	Step height, mv	No. of experiments	Resting potential, mv	Step height, mv	No. of experiments
3.6	86	39(31-50)	20	91	41(25-50)	71
1.8	85	36(30-41)	7	91	33(26-44)	25

**Table 13.1. INITIATION OF AN ACTION POTENTIAL BY ARTIFICIALLY APPLIED ELECTRIC CURRENT PULSES AND BY NATURALLY GENERATED END-PLATE POTENTIALS.** These data show that the extent of local depolarization which effectively elicits an action potential is substantially the same whether produced artificially by applied current or naturally during normal neuromuscular transmission. (Table from Fatt and Katz, 1951.)



**Figure 13.18. THE SITE OF ACTION-POTENTIAL INITIATION IN A NERVE CELL.** Simultaneous recordings were made with two microelectrodes placed outside the cell surface of the lobster stretch receptor. One electrode was kept fixed at the cell body E, while the other was moved along the axon to positions, D, C, B, A. The impulse is generated by stretching of the muscle strand and the time relations of the potentials are indicated by vertical grids. The data indicate that the impulse is generated at a locus close to B for the following reasons: First, the impulse with the shortest latency period is seen at this locus, and second, there is no early positive deflection in B as there would be if the negative action potential were generated elsewhere, thereby creating an earlier positivity. The vertical lines give time in intervals of 0.1msec. (Figure after Edwards and Ottoson, 1958.)

much like the end-plate potential produced at the frog muscle neuromuscular junction and has been called an end-plate potential. Stimulation of the inhibitory nerve causes the potential to shift toward a constant level which is usually not far from the resting level; thus, stimulation of the inhibitory nerve alone (Figure 13.20) causes little potential change (see Kuffler and Eyzaguirre, 1955). However, if an inhibitory impulse is applied after an excitatory impulse, the

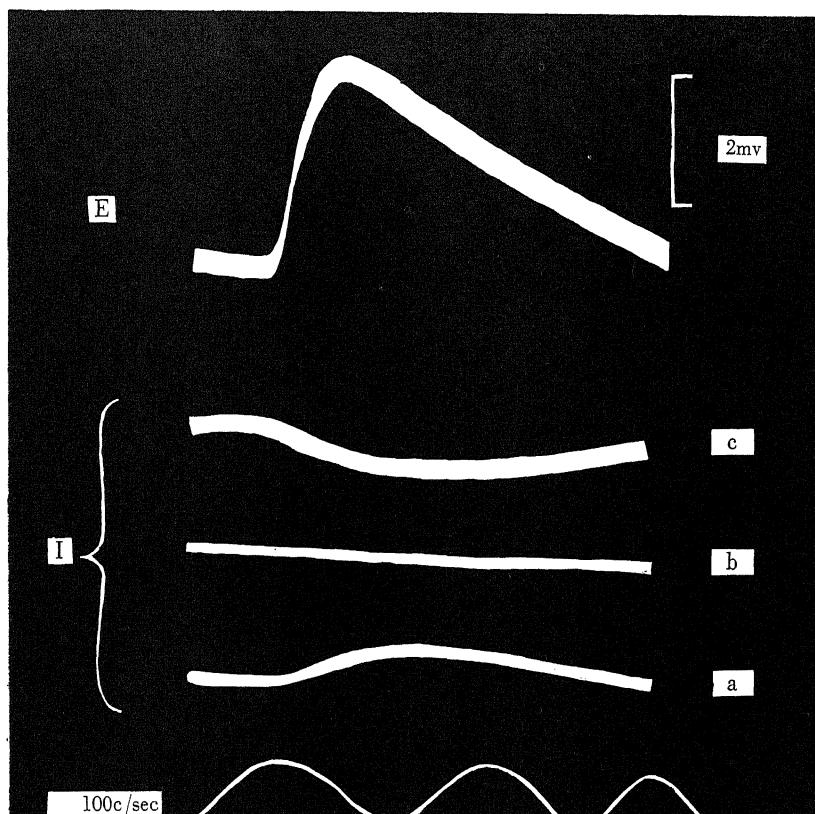


**Figure 13.19.** END-PLATE POTENTIAL IN A SINGLE CURARIZED MUSCLE FIBER, RECORDED WITH THE INTRACELLULAR ELECTRODE AT VARIOUS DISTANCES FROM THE END PLATE. The data show the spatial decrement of the end-plate potential magnitude. The numbers give the distance from the end-plate focus in millimeters  $\times 0.97$ . *s*: stimulus artifact. Time is given in milliseconds. (Figure after Fatt and Katz, 1951.)

amplitude of the end-plate potential and the amount of contraction are decreased (Fatt and Katz, 1953).

Kuffler and Katz (1946) suggested that the inhibitory impulse is transmitted by a chemical mediator which functions like curare,\* a drug which is known to prevent the depolarizing action of acetylcholine at the end plate (Cowan, 1936;

\* The chemical relationship of this interesting drug (first used in a South American arrow poison) to the normal transmitter, acetylcholine, is itself interesting and has been worked out by Bovet (1951).

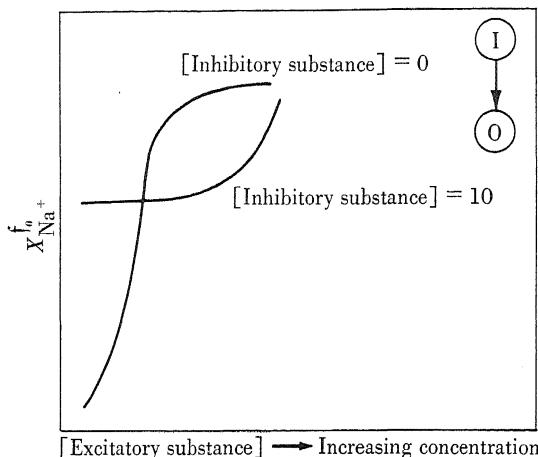


**Figure 13.20. THE LEVELING EFFECT OF AN INHIBITORY IMPULSE ON THE RESTING POTENTIAL.** The muscle whose contraction brings about the opening of a hermit-crab claw is controlled by two nerves. One is a motor axon which can excite the muscle, creating in it a nonpropagated end-plate potential E. The other is an inhibitory nerve, stimulation of which, I, leads to a change of the resting potential toward a certain level. It makes no difference whether the potential was initially a, lower (resting potential lowered to 48mv); c, higher (resting potential raised to 95mv); or b, at the same level (resting potential at 73mv). (Figure from Fatt and Katz, 1953.)

Kuffler, 1943). They also suggested that the excitatory mediator and the inhibitory mediator act competitively (Kuffler and Katz, 1946) for a single receptor site (Fatt and Katz, 1953). It is the opinion of the author that these suggestions are highly plausible and, when incorporated into the general thesis of the present theory, form a nearly complete explanation of the mechanism of neural inhibition.

We have suggested that the end-plate potential arises from an all-or-none change of the  $c$ -value of the surface sites from a high preference for the  $K^+$  ion over the  $Na^+$  ion to a moderate preference for the  $Na^+$  ion over the  $K^+$  ion. A cardinal site on the external surface of the cell controls this cooperative change and

may combine with either the excitatory transmitter, acetylcholine, or the inhibitory transmitter functionally similar to curare. Referring to Figure 13.21, which is similar to Figure 6.1, this external cardinal site can be represented by site I and the gang of sites, by the single site 0 (for the equivalence of reactions of single sites and gangs of sites, see Section 7.4C). Let the  $\text{Na}^+$  ion be the critical adsorbent.



**Figure 13.21. THEORETICAL INTERPRETATION OF THE MECHANISM OF EXCITATION AND INHIBITION (NARCOSIS).** In the absence of the inhibiting substance, increasing concentration of the excitatory substance induces a progressive increase in the fraction of sites preferring the critical adsorbent (the  $\text{Na}^+$  ion), which can effect depolarization (compare with Figures 13.15 and 13.16). After the introduction of the inhibiting substance at a certain concentration, say 10 arbitrary units per liter, an increase in the concentration of the excitatory substance becomes, within a large concentration range, totally ineffective in raising the mole fraction of adsorbed critical adsorbent. The result is the preservation of a particular magnitude of polarization.

In the absence of inhibitory substance, we should expect the preference for the  $\text{Na}^+$  ion to increase progressively with increasing concentration of the excitatory substance. This should lead to progressive depolarization of the resting potential in the region of the end plate [see equation (10-12)]. In fact, the action of one excitatory substance, acetylcholine, on the end-plate potential has already been studied by Fatt and the result, reproduced in Figure 13.15, shows a general correspondence with the theoretical curve.

If we introduce the inhibitory substance into the system at a certain concentration, say 10 arbitrary units per liter, the theoretical curve predicts that the gang of sites will retain a constant relative preference for the  $\text{Na}^+$  ion independent

of variation in the concentration of the excitatory substance over a great range (0 to  $10^{-1}$ ).<sup>\*</sup> Since the composition of Ringer's solution and hence the  $\text{Na}^+$ -ion concentration remains constant, equation (10-12) predicts a constant potential independent of the excitatory substance (acetylcholine). This curve also shows that addition of the inhibitory substance to a system already containing a variable concentration of the excitatory substance tends to drive the resting potential toward a constant level. This hypothesis, synthesized from the theory of Kuffler, Katz, and Fatt, and the main theme of the association-induction hypothesis, is supported by two lines of experimental evidence; both of these will be discussed briefly below.

(a) *Competition by the excitatory and inhibitory transmitters for a single cardinal site.* The crustacean claw muscles resemble the "slow fibers" which are abundant in the *rectus abdominus* muscle of the frog (Kuffler and Vaughan-Williams, 1953). The entire surface of each of these muscles resembles the end-plate region of a "fast" or twitch muscle; both respond to excitatory stimuli with a nonpropagated potential and a contracturelike shortening. The studies of Ariëns *et al.* on contractions of the frog *rectus abdominus* muscle show that acetylcholine and curare compete for the same site (1956, Part 3, p. 4, legend of Figure VIII<sub>1</sub>). By analogy, there is reason to believe that the inhibitory and excitatory mediators may also compete for the cardinal site which controls the surface sites and thus determines the resting potential. However, although this assumption is simplifying and fits the known facts best, it is not the only possible interpretation. For example, the two mediators could compete for adjacent but different sites. In this case, the detailed mechanism would have to be based on Figure 6.3 rather than on Figure 6.1. Only future investigation can determine which explanation is correct.

(b) *Chemical transmission of the inhibitory impulse.* A large body of evidence which supports the chemical (rather than electrical) nature of transmission of excitatory stimuli may be marshalled to demonstrate the chemical nature of inhibitory-impulse transmission. For example, the action of the inhibitory nerve impulse may be simulated by a number of simple chemical compounds: one compound which has been studied extensively in this regard is  $\gamma$ -aminobutyric acid (Hayashi, 1956; Kuffler and Edwards, 1958; Kuffler, 1960). A suitable concentration (about  $10^{-5}M$ ) of  $\gamma$ -aminobutyric acid stops excitatory stimuli and tends to drive the resting potential toward a constant value—an action which simulates those of inhibitory stimuli. It was also noted that, like inhibitory stimuli, the application of  $\gamma$ -aminobutyric acid causes an increase of cellular  $\text{K}^+$ -ion conductance. This fact forms the basis for an alternative interpretation of inhibitory action based on the membrane theory (Fatt and Katz, 1953; Trautwein and Dudel, 1958; Kuffler, 1960). The present theory regards the conductance change

\* The same basic mechanism probably underlies the action of many types of narcotics which may act at loci other than nerve synapses or neuromuscular junctions.

as secondary; this is in accord with the observation that another substance,  $\beta$ -guanidopropionic acid, blocks excitatory stimuli *without* producing a conductance change (Kuffler, 1960).

Because conductance changes have been noted so often in the recent literature, we shall discuss further this observation of increased  $K^+$ -ion conductance in response to  $\gamma$ -aminobutyric acid. If one maintains, as the membrane theorists have done, that the  $K^+$  ion is entirely free within the cytoplasm, the only explanation for increased conductance is a change in permeability. However, if the argument of the present hypothesis that the intracellular  $K^+$  ion is adsorbed is correct, an increase in  $K^+$ -ion conductance across the cell surface could be due to a release of adsorbed intracellular  $K^+$  ion in response to  $\gamma$ -aminobutyric acid. A strong preference of intracellular fixed charges for this compound (see Section 9.3C for selective accumulation of amino acids) is suggested by the fact that  $\gamma$ -aminobutyric acid is constantly and rapidly removed from the surrounding fluid close to the surface of the cell as shown by the observation that stirring the solution can partially restore its action (Edwards and Kuffler, 1959). Other evidence demonstrates that an increase of  $K^+$ -ion efflux from muscles is due to the release of intracellular adsorbed  $K^+$  ion and not to an increase in the permeability constant; this will be given in detail in Section 15.5.

### 13.4. The Activation of Sensory Receptors

We have suggested mechanisms for the liberation of acetylcholine at a nerve ending in response to a nerve impulse as well as for the creation of the generator potential. When such a graded generator potential reaches a certain level, it fires an all-or-none electric impulse; this is transmitted along a nerve and eventually to another nerve or effector cell. We shall conclude our consideration of excitation phenomena by reviewing other modes of stimulation which may activate specific sensory receptors and produce a generator potential.

#### A. MECHANICAL STIMULATION

Mechanical deformation is the mode of activation of a number of sensory organs including the familiar sonic receptor, the ear. Mechanical deformation in a cellular structure must depend basically upon the breaking of reversible bonds, prime among which must be the salt linkages. The sundering of these pre-existing salt linkages leads to a *c*-value-ensemble change and a resultant indirect *F*-process which engenders a graded generator potential.

#### B. THERMAL STIMULATION

The molecular mechanism concerned in the stimulation of temperature (heat and cold) receptors by small temperature changes must resemble the molecular mecha-

nism for heat denaturation of proteins. That is, with an increase in temperature there is a decrease of adsorbed water and a consequent instability of the *c*-value ensemble; this is followed by a cooperative change to the activated state (see Section 7.4C).

### C. CHEMORECEPTION

We have discussed in some detail the mode of action of one type of chemical transmitter, acetylcholine. In general, the mechanism for reception of chemical stimuli is one of the most straightforward receptor mechanisms. For a receptor to react to a chemical stimulus, it must combine with the chemical. The theory that combination with a receptor must be quantitatively related to the magnitude of the taste sensation has been cogently advanced by Beidler (1955).\* Relative to the monovalent cations, we need only assume that chemoreceptor cells on different taste-bud surfaces possess anionic groups with different *c*-values.† One type of receptor would have fixed anionic sites whose *c*-values were greater than  $-0.5\text{\AA}$ . Such sites would respond to acid ( $\text{H}^+$ ) but hardly at all to salt ions; such a pattern has been observed for some chemoreceptors (see Pfaffman, 1951, p. 1148). A second group may have a *c*-value of about  $-0.8\text{\AA}$ . This group would be sensitive to acid and, to some extent, to  $\text{Na}^+$  ion. Such chemoreceptors have also been observed (Beidler, 1952). A third type of receptor site may have a *c*-value of about  $-1.5\text{\AA}$ . These would be sensitive to the alkali-metal ions in the sequence:  $\text{Li}^+ > \text{Na}^+ > \text{NH}_4^+ > \text{K}^+ > \text{Rb}^+ > \text{Cs}^+$ ,‡ which approximates the order found for the taste buds of rodents (Beidler, 1953) as shown in Table 13.2. A fourth group of sites with a *c*-value of  $-3.0\text{\AA}$  would have the preferential binding order:  $\text{NH}_4^+ > \text{K}^+ > \text{Na}^+ > \text{Li}^+$  as has been shown to exist for human taste sensation and for the carnivores§ (Beidler *et al.*, 1955; Pfaffman, 1951) also shown in Table 13.2.

### D. THE MECHANISM OF PHOTOACTIVATION

Scheibe, in 1927, suggested that light absorption could detach an electron from an ion. Since then, Franck and his school, as well as Pauling, and Farkas and Farkas,

\* The low free energy of association between alkali-metal ions and receptor sites found by Beidler agrees with the present theory. Since the active sites are at the surface, a high adsorption energy is counteracted by a large entropy gain on dissociation, and thus the free-energy change is expected to be small.

† This model assumes that the anionic sites in each receptor type are homogeneous in their *c*-values. Other models could be constructed in which two types of anionic site, one of high and one of low *c*-value, would be found in differing proportions in the receptors. Such mixtures could also generate the various orders.

‡ As reported, the order is  $\text{Li}^+ > \text{Na}^+ > \text{NH}_4^+ > \text{Rb}^+ > \text{K}^+ > \text{Cs}^+$ ; the small difference between the  $\text{Rb}^+$  and  $\text{K}^+$  effects might be insignificant (compared with fluctuation of points on the standard curve for the  $\text{Na}^+$  ion).

§ We have repeatedly suggested that the  $\text{NH}_4^+$  ion may serve as the prototype for amino groups. If it does, the preferential sensitivity of carnivores to  $\text{NH}_4^+$  ions gains significance in that meat always contains large amounts of amino acids, peptides, and proteins rich in amino groups. Rodents, whose taste buds are relatively indifferent to the  $\text{NH}_4^+$  ion, relish a vegetarian diet.

have extensively studied electron-transfer spectra.\* The theory of Platzman and Franck arose from such investigations. This theory attributes the ultraviolet absorption spectra of halide ions to an electron-transfer process during which electrons bound to the negative ion are shifted and become bound principally to the water of hydration in immediate contact with the halogen atom. Based on the

Chemical stimulus 0.5M	Rat, 100+	Hamster, 9	Guinea pig, 10	Rabbit, 6	Dog, 7	Cat, 15
LiCl	1.1	1.0	1.1	1.1 <sup>a</sup>	0.06	0.10
NaCl	1.0	1.0	1.0	1.1 <sup>a</sup>	0.18	0.20
NH <sub>4</sub> Cl	0.90	0.66	0.62	1.0	1.0	1.0
KCl	0.50	0.36	0.38	0.92	0.41	0.30

**Table 13.2. RELATIVE SENSITIVITY OF TASTE RECEPTORS TO VARIOUS MONOVALENT CATIONS IN RODENTS AND IN CARNIVORES.** The tongue of an anesthetized animal was placed in a flow chamber to allow rapid stimulation of a well-defined area. The response was measured from the exposed chorda tympani nerve, whose total electrical activity was summed with the aid of an electronic integrator and the magnitude of the response was measured as the total height of the integrated electrical activity. The relative magnitudes of response given in the table are the ratios for a given response to a standard (which is either 0.5M NaCl or 0.5M NH<sub>4</sub>Cl, depending upon which is more effective). The number beneath each species name indicates the total number of animals used in the experiment. (Table from Beidler *et al.*, 1955.)

<sup>a</sup> These values were calculated from the initial rather than the steady-state level of receptor activity.

ideas of Franck, J. Weiss (1939, 1946) suggested a photoreduction and photo-oxidation mechanism between dyes and other molecules involved in electron transfer; this mechanism was consistent with his theory of fluorescent quenching (see also Lumry and Eyring, 1956). Thus, the photoactivated reduction of the binding energy of electrons (ionization potential) can transform a molecule into a strong reducing agent; also, the partially vacated ground state of the excited molecule can increase its tendency to accept electrons, hence, increase its oxidizing power (see Section 7.2C for the relationship of the ionization potential and electron affinity to oxidation-reduction potentials).

Dyes and pigments, both *in vivo* and *in vitro*, associate strongly with protein molecules by ionic and ion-dipole bonds. A transfer of electrons from or to the protein molecule resulting in the photooxidation or photoreduction of the protein is thus possible. The release of electrons from illuminated dyes to an electroscope

\* Franck and Scheibe, 1929; Pauling, 1929; Farkas and Farkas, 1938; Rabinowitch, 1942; Platzman and Franck, 1952.

was demonstrated by Zwaardemaker and Hogewind (1919). These authors were impressed by the fact that this phenomenon cannot be observed unless the photoelectrically active substances are in a colloidal system containing blood, albumin, or gelatin. In other words, proteins are necessary for the transfer of electrons from the photoactivated dyes to the electroscope. The local injection or removal of electrons to or from the protein molecule should induce a variation in the *c*-value by direct and indirect *F*-effects with concomitant variation in the oxidation-reduction potentials of groups like SH and S—S. In other words, these and other functional groups may indirectly serve as special sources or sinks\* for electrons taken up or released through photoactivated molecules.

(1) The interaction of photoactivated dyes and the protein molecules onto which they are adsorbed

Proteins with dye molecules adsorbed often undergo dimensional changes on being exposed to light. Erythrosin and the dichromate ion adsorbed onto gelatin, when exposed to light, cause the hardening of this protein (Lumière *et al.*, 1905; Meisling, 1916); this phenomenon is important for photocopying and photomechanical processes. Similarly, dyes like eosin and methylene blue adsorb onto deoxyribonucleic acid, bringing about a sharp change of viscosity on exposure to light (H. Koffler and Markert, 1951). Finally, we have the observation of Rubinshtein *et al.* (1951) that light may bring about the contraction of actomyosin systems treated with the photoactive dye, erythrosin (Figure 13.22). A theory of the mechanism of such contractile systems will be presented in a later chapter. The proposed mechanism of such protein changes involves a *c*-value shift and the alteration of the preferred partners of the fixed anionic and cationic groups. The photoactivation of all contractile protein systems through this mechanism is in accord with the hypothesis of local electron transfer presented above.

(2) The mechanism of the photodynamic effect

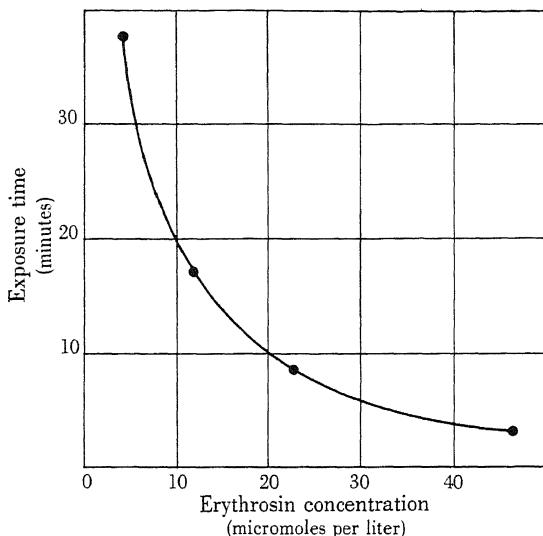
Raab (1900) observed the ability of many fluorescent dyes to sensitize living organisms to light. W. Straub (1904) hypothesized that photodynamic action in biological systems is fundamentally similar to photosensitized oxidation *in vitro*. This view was developed by Gaffron (1933) and in particular by H. F. Blum (1941). Their scheme may be represented by the equations



where D and D' represent the inactive and the activated dye molecule, and X and X', the inactive and the activated substance being oxidized. Reviewing this field,

\* Such sinks would be similar to the "electron traps" of Gurney and Mott (1938) in their theory of photographic sensitization.

Blum concluded, "The dye is taken up by the cell so that it is in intimate association with oxidizable substances upon which the structure of the cell is dependent, probably protein. When the photosensitizer molecule is activated by capture of a quantum of radiant energy, it transfers its activation to this substrate which then



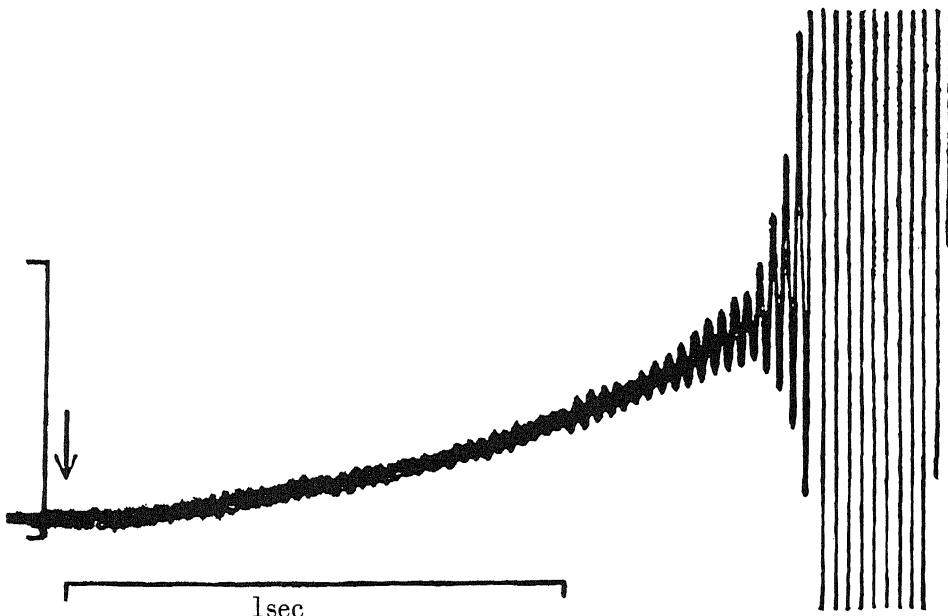
**Figure 13.22. THE CONTRACTION OF ACTOMYOSIN GEL IN RESPONSE TO LIGHT EXPOSURE.** The time of exposure to light necessary to induce the syneresis (dehydration) of actomyosin gel is plotted against the concentration of erythrosin present in the solution. (Figure after Rubinshtein *et al.*, 1951.)

reacts with  $O_2$ , a reaction which would not occur in the normal course of cell structure" (Blum, 1941, p. 82).\*

We have presented evidence that dyes adsorbed onto protein *in vitro* can, by absorbing light, cause changes in the protein molecule; other evidence indicates that this may also be the case in living cells. For example, Doniach (1939) showed that dye which has been exposed to light is totally ineffective in killing paramecia in the dark; subsequent exposure of these dyed paramecia to light, even after repeated washing, has a deleterious effect. According to the present mechanism for the photodynamic effect, the protein molecule receives electrons from the adsorbed dye molecule when it is activated by light. This local electronic excess produces an indirect *F*-effect affecting the  $c$ -,  $c'$ -, and  $\varepsilon^0$ -values of part or all of the molecule. An oxidation that would not normally occur in the absence of the photo-

\* Professor Blum kindly informed us of the tentative nature of this suggestion made twenty years ago; he does not consider the points raised in this quotation completely settled.

dynamic effect now takes place because of the change of  $\epsilon^0$ -value (of an SH group, for example). Thus, whether an electron-rich adsorbent is a photoactivated dye molecule or an anionic protein denaturant, the same enhancement of chemical reactivity could be achieved by the  $c$ - and  $c'$ -value shifts (see Section 7.4C).



**Figure 13.23. THE PHOTODYNAMIC ACTIVATION, BY VISIBLE LIGHT, OF SQUID NERVE STAINED WITH NEUTRAL RED.** A squid axon stained with neutral red was subjected to local irradiation with visible light of moderate intensity for one second. The recorded intracellular potential began to rise during illumination concomitant with subthreshold oscillations. These increased in amplitude with time. When the threshold was reached, a repetitive action potential followed. The vertical calibration indicates 4mv. The arrow marks the beginning of illumination. (From Arvanitaki and Chalazonitis, 1957.)

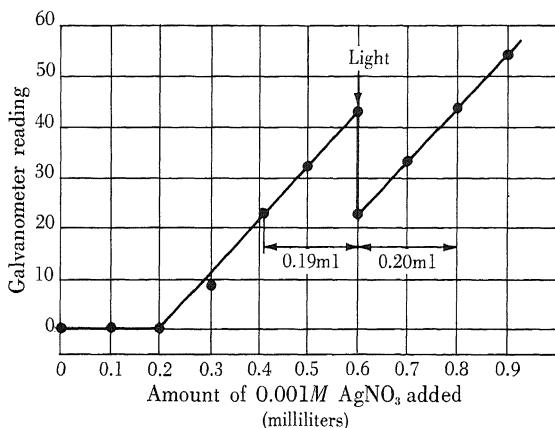
If the above scheme holds, fluorescent dyes adsorbed on proteins and then exposed to white light should produce  $c$ - and  $c'$ -value shifts in proteins and tissues generally. Such  $c$ - and  $c'$ -value shifts may both damage tissues as discussed above and activate the cell so that it undergoes a physiological shift in the  $c$ -value ensemble, producing a normal activity. Such photodynamic stimulation is known in a variety of tissues.\* For illustration, we mention the work of Arvanitaki and Chalazonitis (1957), Figure 13.23. These authors show that a single giant nerve

\* For review see Blum (1941). Examples include isolated heart (Rask and Howell, 1928), smooth muscle (Adler, 1919; Supniewski, 1927), voluntary muscle (Lippay, 1929), nerves (Lillie *et al.*, 1935; Auger and Fessard, 1933), and rabbit aorta (Furchtgott *et al.*, 1961).

axon of the squid *Sepia* becomes sensitive to visible light of moderate intensity if the axon has previously been stained with the dye, neutral red, so that one minute of light exposure leads to the production of repeated action potentials. In the framework of the present theory, this demonstrates that photoactivation of the dye leads to inductive changes in the proteins.

### (3) The mechanism of visual excitation

As discussed by Wald and Brown (1952), the bleaching and resynthesis of the retinal pigment protein complex, rhodopsin, is associated with a reversible shift of



**Figure 13.24. THE AMPEROMETRIC TITRATION OF THE SULFHYDRYL GROUPS OF RHODOPSIN.** The titration was begun in dim red light to prevent bleaching of the pigment. No current flowed until 0.2ml of silver nitrate had been added; this is equivalent to the free sulfhydryl groups initially present. After 0.4ml more of  $\text{AgNO}_3$  had been added, producing a considerable flow of current, the rhodopsin was bleached in white light. The current immediately fell as new SH groups were exposed. Addition of more  $\text{AgNO}_3$  returned the current to its former level and increased it further. The horizontal distances marked on the figure measure the  $\text{Ag}^+$ -ion equivalent of the SH groups liberated by bleaching. This amount corresponds to two SH groups for each retinene<sub>1</sub> molecule. (Figure after Wald and Brown, 1952.)

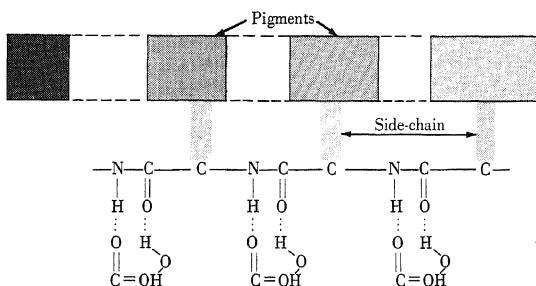
isoelectric point from 4.47 to 4.57 (Broda and Victor, 1940), and the release of sulfhydryl groups. The phenomenon clearly falls within the class of reversible denaturations, the mechanism of which is the indirect *F*-effect.\* Light absorption leading to a local photoreduction brings about a *c*-value change which is transmitted through the indirect *F*-effect as an increase in *c*-value; this is indicated by

\* The suggestion that the bleaching and resynthesis of rhodopsin may be regarded as a reversible denaturation seems to have been made first by Mirsky (1936a).

the increased reactivity of the SH groups (Figure 13.24). These events are primary. Whether the pigment remains associated after such a change or becomes dissociated from the protein depends on the resultant  $c$ - and  $c'$ -values and on their relation to other available competing groups and is, therefore, not central to the excitation phenomenon (K. G. Stern, 1938).

#### (4) Discursive remarks on photosynthesis

The basis for photosynthesis is a reduction of carbon dioxide to carbohydrate and the oxidation of water to oxygen with light supplying the necessary energy



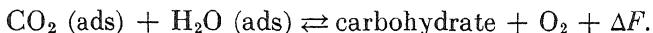
**Figure 13.25.** A POSSIBLE MODEL OF A PHOTOSYNTHESIZING PIGMENT-PROTEIN COMPLEX. In this model, the suggested sites for carbon dioxide and water adsorption in oxidative polymerization are on the polypeptide chain.

(Rabinowitch, 1945). That plant pigments are responsible for the capturing of light energy is universally accepted. The problem to be clarified is how the light energy is injected into the water-carbon dioxide system.

Urea, a hydrogen-bonding agent, does not ionize in the physiological pH range. Nevertheless, it may bring about an increase in the water solubility of native as well as of heat-denatured proteins. The opinion generally held (Section 7.4C) is that urea is adsorbed on the H-bonding sites of the backbone (a mechanism to explain its solubilizing action has already been presented). In some respects, carbon dioxide is similar to urea; it is also essentially not ionized at neutral pH and has been shown to increase the water solubility of proteins appreciably (Mitz, 1957), an action not duplicated by other weak acids such as acetic acid. Like urea, carbon dioxide has a proton-accepting group. Thus it probably has a tendency to form H-bonds with the peptide NH groups of appropriate  $c'$ -value analogue. We have already suggested that the water molecule may occupy these sites (Section 7.4C). Under suitable conditions, therefore, we should expect to find pairs of  $\text{H}_2\text{O}$  and  $\text{CO}_2$  occupying neighboring sites as shown in Figure 13.25. Whether these adjacent proton-donating and proton-accepting sites are those of the peptide

chain or are to be found in some special configuration of amino acids, is only of secondary importance.

The significance of this model is that it provides a mechanism for the reaction,



The advantages of this mechanism—possible within a fixed-charge phase such as a living cell, but not in an ordinary dilute solution of salts and protein—will be seen from the following. The probability of formation of an activated complex, and hence the rate of synthesis, is greatly increased in a fixed-charge system because formation of the complex does not depend on random collision as in free solution. Rather, the reactants are accumulated by adsorption at loci made favorable to the formation of an activated complex by the juxtaposition of specific adsorbing sites for  $\text{CO}_2$  and for  $\text{H}_2\text{O}$ . The probability factor is extremely important for the one-step synthesis of a molecule, like a carbohydrate, containing several monomeric components.

With the carbon dioxide and water appropriately adsorbed, we may again apply the Weiss theory of photooxidation and photoreduction. The capture of light energy by the pigment will lead to a *c*-value change at the adsorption site and, through the *F*-effect, produce *c*-value-analogue changes on the peptide groups. These changes will secondarily alter the ionization potential and hence the reduction potential (see Section 7.2C) of the water and the oxidation potential of carbon dioxide molecule. These alterations permit the occurrence of an oxidation-reduction reaction very unlikely to take place in a free aqueous solution (see Chapter 14 on enzyme mechanisms).

This scheme eliminates the need for transporting the enzyme or the intermediate synthetic products from one site to another because the monomeric elements are located side by side and they can, without transfer, directly polymerize into monosaccharides, disaccharides, or even polysaccharides. The awkwardness of a step-by-step synthesis followed by transport from one synthetic site to another finds a parallel in the problem of protein synthesis. The inadequacy of this type of logistics for protein synthesis has been eloquently discussed by Spiegelman (1957) and Rabinowitch (1945).

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# 14

## ENZYME ACTION

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The maintenance of the living state depends on the continued consumption of food materials for two reasons. The constituent parts of the organism must be built and maintained and energy must be supplied to maintain or return the tissues to their normal metastable state. To achieve the utilization of food materials as well as for the manufacture of essential compounds, living cells are equipped with a large collection of catalysts called *enzymes*. These are capable of bringing about rapid chemical reactions at a relatively low ambient temperature and within a precise framework of balance and control.

Historically, it is interesting to note that Berzelius (1836), who discovered catalysis, included in his examples catalysis by enzymes. With amazing insight he suggested that all substances of living organs are produced under its influence. After a century, this became an accepted truth.

Since Berzelius, a very large number of enzymes have been isolated and carefully studied. However, before discussing the properties of isolated enzymes, we must caution that such enzymes represent semifixed-charge systems which have lost an important part of the structural integrity and functional characteristics they possessed before the true fixed-charge system from which they were derived was degraded. Nevertheless, the great amount of information now available, some of which will be discussed in this chapter, suffices to demonstrate the essential properties that underlie enzymatic behavior in living cells. Thus, while a general qualitative mechanism for the control of enzyme activity in the living cell will be discussed briefly in Sections 14.2 and 14.3, complete understanding of the control of enzyme activity in the living state is one of the challenging problems to confront biologists in the future.

All enzymes are proteins or protein complexes which demonstrate in their action two characteristics distinguishing them from the catalysts found in the nonliving world. First, they are highly specific; that is, an enzyme which avidly attacks one molecule may be totally inert toward another similar molecule. Important as this fact is, we shall not discuss it in detail in this chapter; the discussion of the general theory of proteins (Chapter 7) has provided sufficient information to explain such high specificity. The explanation finds its basis in the concept of the *c*-value ensemble which characterizes all or part of a protein molecule. The major part of the discussion will center on a second fundamental property of enzymes. This is the great variability of activity shown by enzymes in response to changes in factors such as *pH* and cation concentration.

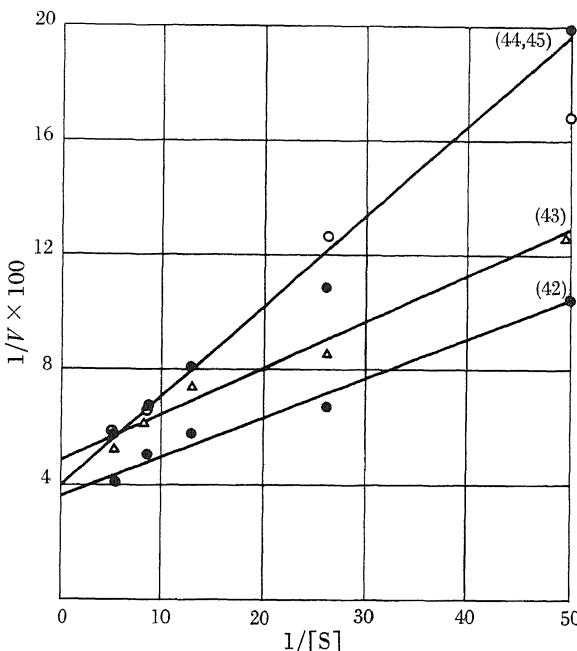
An ion or molecule may modify the activity of an enzyme through a number of different mechanisms. Two relatively direct mechanisms whereby enzyme activity may be modified are the competitive inhibition of Michaelis and Menten and what may be called "competitive activation." The first is familiar to all interested in enzyme chemistry; the second follows naturally from the general theory presented in this work. Other more indirect types of interaction are usually called noncompetitive or uncompetitive interactions. In the following discussion we shall separate all interactions into two major categories: competitive interactions and noncompetitive interactions.

#### 14.1. Competitive Interactions

##### A. COMPETITIVE INHIBITION

Since Michaelis and Menten (1913) proposed their theory of enzyme kinetics (see also Briggs and Haldane, 1925), the concept of competitive inhibition, explicit

from both theoretical and experimental standpoints, has formed the core of our insight into the behavior of enzymes. Of the methods used for the analysis of this type of interaction, which involves direct competition for the enzymatically active site between the substrate and another often similar molecule, an equation



**Figure 14.1. COMPETITIVE AND NONCOMPETITIVE INHIBITION.** Lineweaver and Burk plot [see equation (14-1)] of the competitive inhibitory action of  $\beta$ -glucose (44) and fructose (45) on the action of invertase on sucrose (control, 42) and of the noncompetitive inhibitory action of  $\alpha$ -glucose (43) on the same reaction. Substrate concentration  $[S]$  is given in moles per liter. Initial reaction rate  $V$  is given in terms of relative initial rate of enzyme action.  $\beta$ -glucose, 0.183M; fructose, 0.196M;  $\alpha$ -glucose, 0.175M. (Figure after Lineweaver and Burk, 1934.)

entirely analogous to the one derived from competitive ionic entry into a fixed-charge system [equation (11-24)] was first used by Lineweaver and Burk (1934):

$$\frac{1}{V} = \left( \frac{1}{V_{\max}} \right) \left( K_s + \frac{K_s[I]}{K_i} \right) \left( \frac{1}{[S]} \right) + \frac{1}{V_{\max}} \quad (14-1)$$

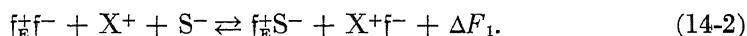
Here  $V$  is the reaction velocity of the enzyme system;  $K_s$  and  $K_i$  are the Michaelis-Menten constants of the enzyme-substrate complex and the enzyme-inhibitor complex, respectively;  $V_{\max}$  is a constant equal to the maximum reaction velocity when all enzyme sites are occupied by the substrate;  $[I]$  and  $[S]$  are the concentrations of the inhibitor and substrate, respectively. Figure 14.1, taken from the

original publication of Lineweaver and Burk, shows the inhibition of invertase, which hydrolytically transforms sucrose into glucose and fructose, by  $\alpha$ -glucose,  $\beta$ -glucose, and fructose. Note that, in the plot of  $1/V$  against  $1/[S]$ , the control curve (42) and the curve showing the action of  $\beta$ -glucose (44) and fructose (45) converge to the same locus on the ordinate, indicating competitive inhibition. On the other hand, the action of  $\alpha$ -glucose (43) is noncompetitive. Before discussing the mechanism of this interaction, we shall present a second form of competitive interaction, activation.

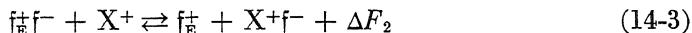
### B. COMPETITIVE ENZYME ACTIVATION

The effects of alkali-metal ions\* on enzymatic activity have often been studied in recent times. Table 14.1 is a collection of the data† known to us on the order of enzyme activation by the monovalent cations when the substrate is ionic. For all of these enzymes the activating effect is highest for the  $K^+$ , the  $Rb^+$ , or the  $NH_4^+$  ion;  $Cs^+$ ,  $Li^+$ , and  $Na^+$  are comparatively less effective.

In Chapter 7, we discussed the importance of salt linkages as the form of ionic association entropically favored when a protein is in solution. If the  $c$ -value of a fixed carboxyl group is near that at which an amino group is preferred, such an anionic group may be expected to form a salt linkage with a nearby amino group. If one member of this salt-linked pair of fixed sites is potentially an enzymatically active site, that particular site will be inactive since the formation of a complex with the counterion that is the substrate of that site is not possible. One can easily imagine how the enzymatic activity of such a site may be increased. Table 14.1 reveals that the substrates of enzymes affected by alkali-metal ions are anionic. To increase the enzyme activity the following reaction must be driven to the right:



This may be achieved through an increase of the free-energy change  $\Delta F_2$  for the half reaction,



where  $\Delta F_1 = \Delta F_2 + \Delta F_3$  and  $\Delta F_3$  is the change of free energy for the other half reaction,



As we have mentioned, the formation of a salt linkage is conditioned by the proper  $c$ -value of the fixed anionic groups. If we postulate that the fixed cationic group  $f_E^+$  behaves much like  $NH_4^+$ , reference to Figure 4.9 will show that its  $c$ -value is about  $-2.5$  to  $-3.0\text{\AA}$ . In this range,  $K^+$ ,  $NH_4^+$ , and  $Rb^+$  are the cations that can

\* The following discussion is applicable to anions as well as to cations although this section was written with reference to monovalent cations.

† These data were collected in 1958; since then, considerably more similar data have been published.

Enzyme	Substrate	Order of activation	Order of inhibition	Source
$\beta$ -Galactosidase (lactase)	lactose	$K^+ > Rb^+ > NH_4^+ > Cs^+$ 6.1      4.7      4.3      1.3 $> Na^+ > Li^+$ 1.2      0.3	—	Cohn and Monod, 1951.
Acetate-activating enzyme system (aceto-CoA kinase)	acetate and CoA	$Rb^+ = NH_4^+ > K^+$ 2.5      2.1 ( $K^+$ , $NH_4^+$ , $Rb^+$ )	$Li^+ > Na^+$ 7.4      7.0 $Na^+, Li^+$	von Korff, 1953.
$\beta$ -Tyrosinase	tyrosine	$NH_4^+, K^+$	$Na^+, Li^+$	Yoshimatsu, 1957.
Tryptophanase apo-enzyme	L-tryptophane	$NH_4^+ > K^+ > Rb^+$	$Na^+, Li^+$ (slight)	Happold and Struyvenberg, 1954.
Yeast aldehyde dehydrogenase	acetaldehyde	$K^+ > Rb^+ > NH_4^+ > Na^+$ 100      97      32      4 $Cs^+ > Li^+$ 4      0	—	Black, 1952.
Phosphotransacetylase	acetyl phosphate	$NH_4^+ > K^{+a}$ $K^+ > NH_4^{+b}$	$Na^+ > Li^+$	Stadtman, 1952.
Pyruvate phosphoferase (pyruvate kinase)	phosphoenolpyruvate	$K^+ > Rb^+ > NH_4^+ > Na^+$ 1.00      0.84      0.72      0 $> Li^+$	—	Kachmar and Boyer, 1953.
Pectinesterase	pectin	$Na^+ > K^+ > Li^+$	—	Lineweaver and Ballou, 1945.
Polygalacturonidase <sup>c</sup>	pectin		—	Matus, 1948.

**Table 14.1. EFFECTS OF ALKALI-METAL IONS ON ENZYME ACTIVITIES.** Collected experimental observations on the effect of alkali-metal ions on enzyme activity. Note the general tendency of Rb, K, and  $NH_4$  salts to have the highest activating effect and the tendency of Na and Li salts to have an inhibiting effect. Numbers under alkali-metal ions indicate relative effectiveness of the particular ion. Sources of data are indicated in the last column.

<sup>a</sup> At concentrations below 0.07M.

<sup>b</sup> At concentrations above 0.07M.

<sup>c</sup> Hofmeister and Schulze Hardy Rule applies to order of activation.

effectively compete with the enzymatic group for the anionic group  $f^-$  of the salt linkage. The  $-\Delta F_2$  for  $K^+$ ,  $NH_4^+$ , and  $Rb^+$  is high and thus these cations may free the enzymatic cation from its salt linkage; the  $-\Delta F_2$  of  $Cs^+$ ,  $Li^+$ , and  $Na^+$  is low and these ions are ineffective.

If  $[S^-]$ ,  $[f_E^+]$ ,  $[X^+]$ , and  $[Y^-]$  represent the concentrations of  $S^-$ ,  $f_E^+$ ,  $X^+$ , and  $Y^-$  respectively, and  $\Delta F_{f_E^+S^-}$ ,  $\Delta F_{f^-f_E^+}$ ,  $\Delta F_{f^-X^+}$ , and  $\Delta F_{f_E^+Y^-}$  are the free energies of association of  $f_E^+S^-$ ,  $f^-f_E^+$ ,  $f^-X^+$ , and  $f_E^+Y^-$  respectively, the rate of enzyme activity  $V$  is proportional to the concentration of the substrate occupying the enzyme site  $f_E^+$ ,

$$V = k \cdot \frac{[f_E^+]_{\text{free}}[S^-] \exp\left(\frac{-\Delta F_{f_E^+S^-}}{RT}\right)}{1 + [S^-] \exp\left(\frac{-\Delta F_{f_E^+S^-}}{RT}\right) + [f^-]_{\text{free}} \exp\left(\frac{-\Delta F_{f^-f_E^+}}{RT}\right) + [Y^-] \exp\left(\frac{-\Delta F_{f_E^+Y^-}}{RT}\right)}. \quad (14-5)$$

A more general equation for  $[f^-]_{\text{free}}$  is

$$[f^-]_{\text{free}} = \frac{[f^-]_{\text{total}}}{1 + \sum_{i=1}^n [p_i^+] \exp(-\Delta F_{p_i^+ f^-}/RT)}. \quad (14-6)$$

Since only the fixed cation  $f_E^+$  and the free cation  $X^+$  compete for  $f^-$ , we can write more specifically,

$$[f^-]_{\text{free}} = \frac{[f^-]_{\text{total}}}{1 + [X^+] \exp(-\Delta F_{X^+ f^-}/RT) + [f_E^+]_{\text{free}} \exp(-\Delta F_{f_E^+ f^-}/RT)}. \quad (14-7)$$

The quantity  $[f_E^+]_{\text{free}}$  is related to  $[f_E^+]_{\text{total}}$  by an analogous equation,

$$[f_E^+]_{\text{free}} = \frac{[f_E^+]_{\text{total}}}{1 + [Y^-] \exp(-\Delta F_{f_E^+ Y^-}/RT) + [f^-]_{\text{free}} \exp(-\Delta F_{f_E^+ f^-}/RT)}. \quad (14-8)$$

For convenience we have designated the above type of activation as competitive activation\* in contrast to the type of inductive (noncompetitive) activation usually characterized by curves showing optima (see below). Typically, an activator ion bears a charge opposite to that of the substrate. We shall construct a few theoretical curves according to equation (14-5). In these computations, instead of solving simultaneous equations involving equations (14-7) and (14-8) to find  $[f_E^+]_{\text{free}}$ , we shall assume a constant value for  $[f_E^+]_{\text{free}}$ . The points we wish to emphasize are not materially changed by this simplifying assumption. We then write

\* Lineweaver and Ballou (1945) proposed a theory for cation activation of pectinesterase in which they suggested that the cation must dissociate an inactive pectin-enzyme complex to release the enzyme.

$$a = [S^-] \exp (-\Delta F_{fES^-}/RT) \quad (14-9)$$

$$b = \exp (-\Delta F_{fEY^-}/RT) \quad (14-10)$$

$$d = [f_E^+]_{\text{free}} \quad (14-11)$$

$$e = [f^-]_{\text{total}} \quad (14-12)$$

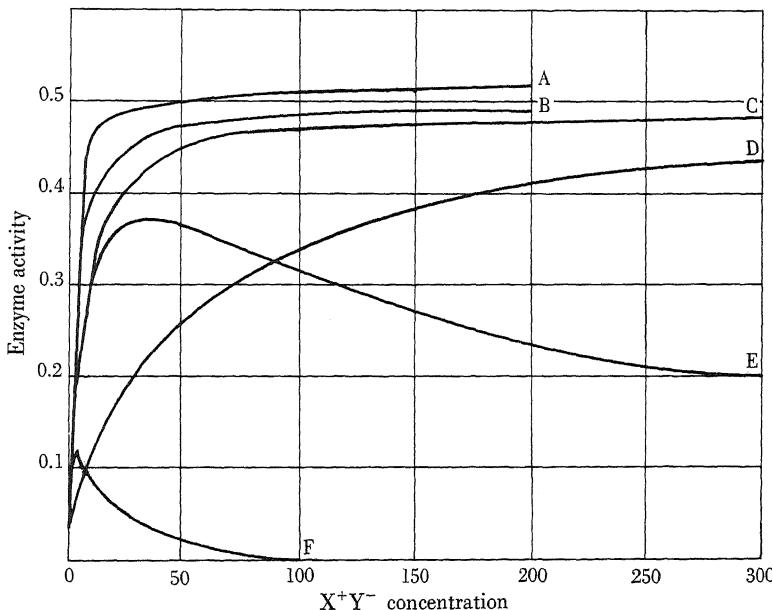
$$g = \exp (-\Delta F_{fEf^-}/RT) \quad (14-13)$$

$$f = \exp (-\Delta F_{X^+f^-}/RT) \quad (14-14)$$

and substitute  $[X^+]$  for  $[Y^-]$  since they are combined as a salt and must be equal in concentration (or in simple proportions). Equation (14-5) can then be expressed in the form,

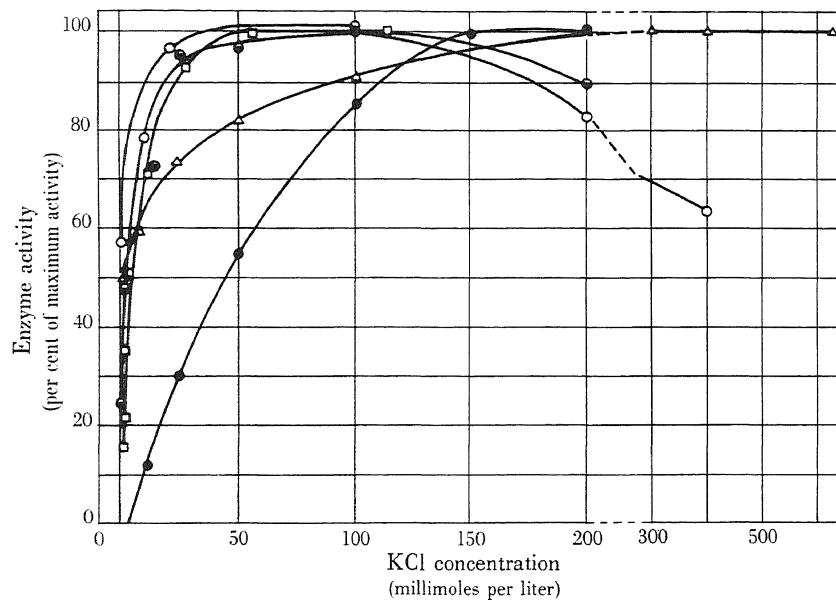
$$V = k \frac{da}{1 + a + \frac{eg}{1 + f[X^+] + dg} + b[X^+]} \quad (14-15)$$

In the following examples, we assume that  $a = e = 1$ ,  $d = 0.01$ . We shall then vary  $g$ ,  $b$ ,  $f$ , and  $[X^+]$  to obtain the theoretical curves shown in Figure 14.2.



**Figure 14.2. COMPETITIVE ACTIVATION: THE THEORETICAL RELATIONSHIP BETWEEN THE CONCENTRATION OF THE ACTIVATING ELECTROLYTE  $X^+Y^-$  AND ENZYME ACTIVITY.** The theoretical curves illustrate the change in the shapes of the curves with variations of the relative affinities of various competing ions. A,  $b = 0$ ,  $g = 100$ ,  $f = 100$ ; B,  $b = 0$ ,  $g = 5$ ,  $f = 1$ ; C,  $b = 0$ ,  $g = 100$ ,  $f = 10$ ; D,  $b = 0$ ,  $g = 100$ ,  $f = 1$ ; E,  $b = 0.01$ ,  $g = 100$ ,  $f = 10$ ; F,  $b = 1$ ,  $g = 100$ ,  $f = 10$ . The units are arbitrary.

The family of curves with  $b = 0$  corresponds to the case in which the anion of the salt ( $\text{Cl}^-$  in  $\text{KCl}$ ) has very little affinity for the enzyme site  $f_E^+$ . Such curves are shown in Figure 14.3 for yeast aldehyde dehydrogenase, pectinesterase, and tryptophanase. When  $\text{Y}^-$  does have substantial affinity for the enzymatic site



**Figure 14.3. THE RELATION BETWEEN KCl CONCENTRATION AND ENZYME ACTIVITY.** Enzyme activity as the per cent of the maximum activity found in the experiment has been calculated from original data. ●—●, pectinesterase, Lineweaver and Ballou (1945); △—△, aldehyde dehydrogenase, Black (1952); ○—○, phosphotransacetylase, Stadtman (1952); □—□, tryptophanase, Happold and Struyvenberg (1954); ○, pyruvate phosphoferase, Kachmar and Boyer (1953).

$f_E^+, b \neq 0$ , the enzyme activity does not progressively increase with the concentration increase of  $X^+Y^-$  ( $\text{KCl}$ , for example). In this case, the curve begins to decline at some salt concentration and an asymmetric curve with an optimum results. Such curves appear in Figure 14.3 for pyruvate kinase and phosphotransacetylase. Other support that the decrease in the activity of these enzymes at very high KCl concentrations is due to competitive inhibition by the anion  $\text{Y}^-$  of the salt used is given in Figures 14.4A and B. Here the salts of chloride and sulfate were studied.

When the more weakly adsorbed sulfate is used (see Table 7.5), the curves approach the theoretical curves for which  $b = 0$ ; when the more strongly adsorbed  $\text{Cl}^-$  ion is used, the curves behave as if  $b \neq 0$ .

We again use sulfates to clarify the action of the various cations on competitive activation. In Figure 14.4B, pyruvate phosphoferase activity is plotted against varying concentrations of alkali-metal sulfates (unpublished; see also Boyer *et al.*, 1943). These curves reveal that the affinity of the fixed anion for alkali-metal ions follows the order  $\text{K}^+ > \text{Rb}^+ > \text{Cs}^+ > \text{Na}^+ > \text{Li}^+$ . Figure 14.4B also shows that  $\text{Na}^+$  and  $\text{Li}^+$  ions do not have inhibitory effects, but are merely weak competitive activators. The inhibitory action usually ascribed to these ions is due to the chloride ion introduced with the  $\text{Na}^+$  ion in  $\text{NaCl}$ .

In concluding this section we point out that it is not possible unequivocally to determine whether any given ionic interaction presented in Table 14.1 represents competitive activation or is partly or totally due to the more indirect mechanism (to be discussed in Section 14.2) merely from the general shape of the curve. Only further research can determine which of these two mechanisms is involved in any particular interaction. For example, a test similar to the Michaelis-Menten equation for direct competitive inhibition between the  $\text{Cl}^-$  ion and the substrate will decide whether competitive activation is involved.

## 14.2. Noncompetitive Interactions

Although the direct competition for sites involved in competitive inhibition and activation involves a fairly simple mechanism, the great variability of control of enzyme action within the living cell must rely on a more subtle and less direct means of control. Such indirect interactions were alluded to in connection with the noncompetitive inhibitions of invertase action by  $\beta$ -glucose. The effects of factors such as *pH* and salt ions on enzyme action are so universal and so varied that competitive interactions seem incapable of explaining them. The present hypothesis explains these effects by the complex indirect interactions that functionally link enzymatically active sites to their neighboring sites through *F*-effects; these interactions are due to the high polarizability, and hence transmissivity, of the polypeptide chain. To be more specific, there are two ways in which the inductive effects can change enzyme activity: they can alter the reactivity of the site and they can alter the affinity of the site for the substrate. Let us consider them in more detail.

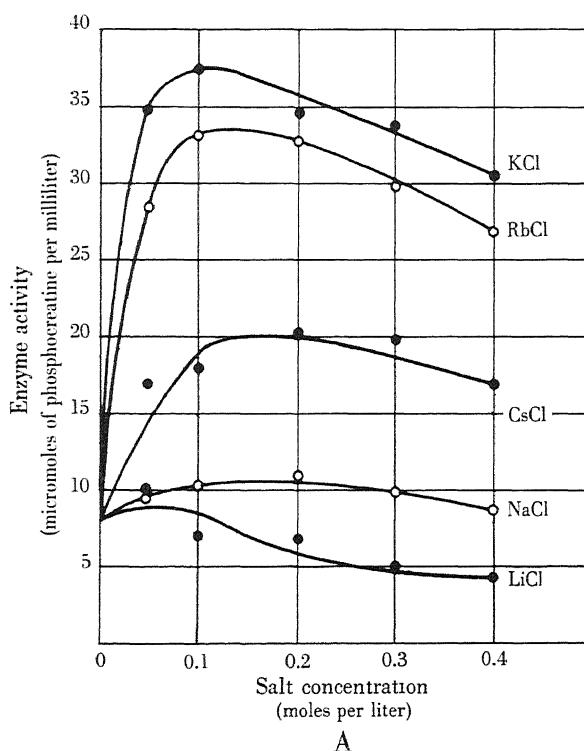
### A. TWO BASIC MECHANISMS FOR INDIRECT INTERACTIONS

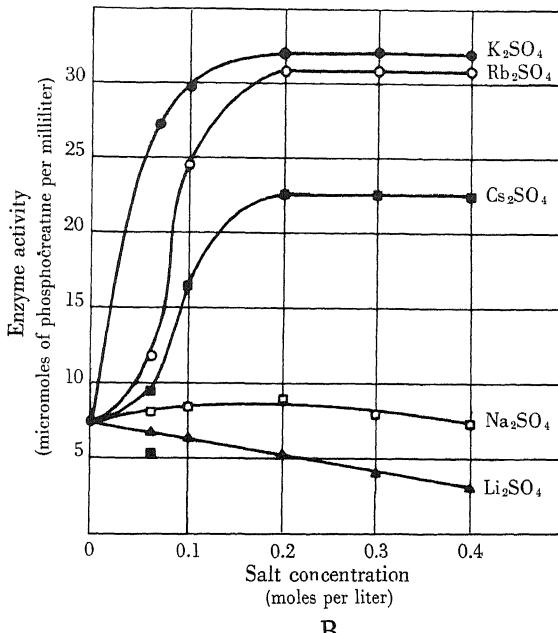
#### (1) The $\delta^\circ$ value versus activation energy

Studies of heterogeneous catalysis and homogeneous acid catalysts have led to the conclusion that catalysts often serve as electron sources or sinks for the formation

of a free radical or an ion of the substrate (Lennard-Jones, 1932; O. Schmidt, 1933; Nyrop, 1937). In metal catalysis, the rate-determining step thus depends on the "work function" and the ionization potential of the metal (see Dowden, 1948, 1950; Dowden and Reynolds, 1950; Schwab, 1943, 1946; for review, see Leach, 1954). For enzymes which contain no conducting electrons and hence have no "work functions" to speak of, the rate-determining factor is the ionization potential of electron-donating enzyme sites or the electron affinity of electron-capturing enzyme sites. Both determine the values of  $\varepsilon^0$ , the effective oxidation-reduction potential of the enzymatic site, and both may be varied by *F*-effects.

Earlier, in the discussion of protein denaturation, we pointed out that in a true fixed-charge system such as a living cell or wool, or even in a semifixed-charge system such as an aqueous solution of egg albumin, acid titration is not the simple dissociation-association reaction described by equation (7-2) but involves, to a greater or lesser degree, specific interaction of the protein with the anions present [equations (7-3) and (7-4)]. In Chapter 7, we showed that the oxidation-reduction potential  $\varepsilon^0$  of one type of oxidizable group, the SH group, is modified by hydrogen-bonding agents (including urea), cations (the H<sup>+</sup> ion, for example), and anions (the trichloracetate ion, for instance). It was pointed out that increasing SH re-





B

**Figure 14.4. THE RELATIONSHIP BETWEEN THE CONCENTRATIONS OF SALTS OF VARIOUS ALKALI-METAL IONS AND THE ENZYMATIC ACTIVITY OF PYRUVATE PHOSPHOFERASE.** A, chloride salts; B, sulfate salts. Rat muscle pyruvate phosphoferase was prepared according to Meyerhof and Kiessling (1935). The reaction mixture contained 0.2ml salt solution at various concentrations, 4mg acetone powder, and 1.0ml substrate buffer mixture containing the following: creatine, 60mM;  $MgSO_4$ , 5mM; ATP, 0.02mM; 3-phosphoglycerate, 17mM; tris buffer, 0.01M, neutralized with acetic acid to  $pH$  7.4. Incubated at  $38^\circ C$  for 20 minutes. TCA was added to stop the reaction and to make up the final volume to 5ml. The deproteinized solution was analyzed for phosphocreatine synthesized. Enzyme activity is expressed as total number of micromoles of phosphocreatine synthesized in the reaction mixture. (Ling and Chen, unpublished.)

activity reflects a local fall of  $\delta^0$  (increased reducing power) while a decrease in reactivity coincides with a decline in the reducing powers of the group (Section 7.4C), a correlation that permitted the interpretation in Section 7.4D of the data of Burk (1943). As an example, Burk showed (confirmed and extended in our laboratory, to be published) that the concentration of urea necessary to bring about a positive nitroprusside reaction increases and then decreases with a progressive increase in  $MgCl_2$  concentration. Since enzyme activity, like SH reactivity, is related to  $\delta^0$ , we may reasonably expect to find enzymes whose activity as a function of  $MgCl_2$  concentration follows a bell-shaped curve. Many examples of this can be found in the literature.

- (2) The *c*-value versus selective substrate adsorption on enzymatically active sites

Although a proper value of  $\varepsilon^0$  may be necessary for the reactivity of a specific enzyme, the possession of this  $\varepsilon^0$  value does not automatically guarantee that the reaction will occur. For this to happen the substrate must first become part of an activated complex; this is an adsorption process and, as such, critically depends on the *c*-value of the enzymatically active site. This also can be modified by the adsorbents and reactants on neighboring sites.

In the theoretical treatment given in Section 4.2B, we demonstrated that an ionic adsorbent may form adsorption complexes with no, one, or more water molecules interspersed between itself and the adsorbing site. In dealing with enzymatic adsorptions, it is probable that, of these configurations, only configuration 0 allows the formation of an activated complex. The rate of an enzymatic process is thus related to the probability of formation of an enzyme-substrate complex of this particular configuration. The statistical probability that a given adsorption will assume configuration 0 depends, of course, upon the *c*-value.\*

Inductive effects brought about by changes of the adsorbent on neighboring sites may thus alter enzymatic activity by changing either the  $\varepsilon^0$  value or the *c*-value of the site. In reality, these two mechanisms cannot be separated, for a change in  $\varepsilon^0$  is accompanied by a change in the *c*-value and conversely. In Chapter 6, we showed how various physiological activities can be theoretically related to adsorbents on neighboring sites (see Figures 6.1 to 6.4). Let us now use experimental results from studies of enzymes to determine whether these patterns apply to enzyme activity.

### 14.3. Comparison of Theoretical Predictions and Experimental Facts

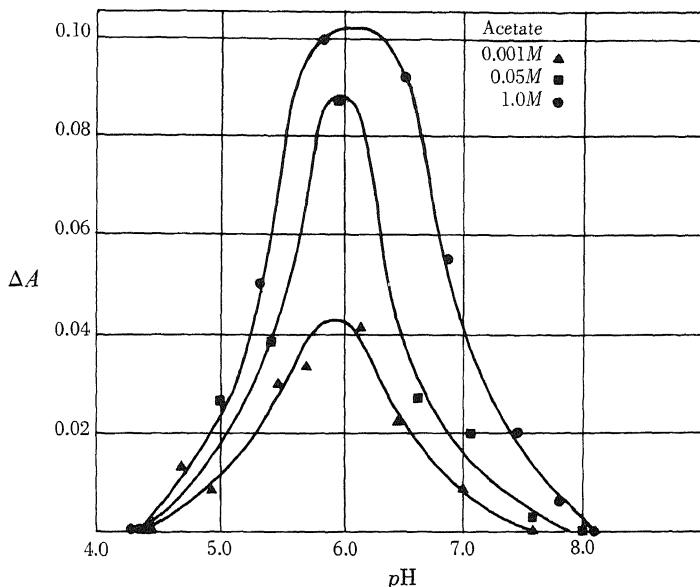
#### A. THE EFFECT OF IONS ON ENZYME ACTIVITY

Virtually all studies of new enzymes provide data showing the effect of  $H^+$ -ion concentration on enzyme activity. Such studies usually reveal a typical bell-shaped curve. In most cases, however, this curve is reproducible only if a more or less arbitrary set of experimental conditions is rigidly followed. For example, variation of the nature of the buffer used generally effects a change in the *pH* curve.

Let us focus our attention on a complex system with two variables: the concentration of the cationic adsorbent, the  $H^+$  ion, and the concentration of an anionic

\* There is competition both among different configurations for the same substrate and between the 0-configuration-substrate complex and other nonsubstrate ions and molecules. Thus the success of the formation of the effective enzyme-substrate complex depends, in part, on the activities of the competing ions and molecules. However, when the activities of all competing components are fixed, the probability that the effective substrate-enzyme complex will be formed must depend on the *c*-value.

adsorbent, the acetate ion. The effects of simultaneous variation of these two variables on the enzymatic activity of 5-adenylic acid deaminase are shown in Figure 14.5. After the abscissa is inverted (since pH, not H<sup>+</sup>-ion concentration, was plotted), the resemblance of these experimental curves to the theoretical



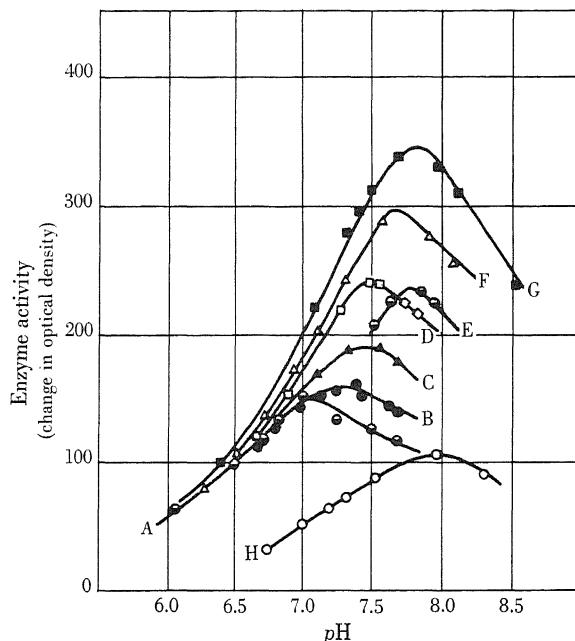
**Figure 14.5. THE EFFECT OF pH AND OF ACETATE CONCENTRATION ON THE ENZYMATI**C ACTIVITY OF 5-ADENYLYC ACID DEAMINASE. 5-adenylyc acid deaminase was prepared from rabbit muscle; its activity was estimated by measuring the initial rate of change of extinction ( $\Delta A$ ) at  $265\text{m}\mu$  of 3ml of an acetate buffer mixture containing  $0.05\text{mM}$  5-adenylic acid and approximately 100 units of adenylic acid deaminase; pH was adjusted with NaOH. A,  $1.0\text{M}$  acetate; B,  $0.05\text{M}$  acetate; C,  $0.001\text{M}$  acetate. Compare these experimental results with the theoretical curves in Figure 6.4. (Unpublished data.)

curves shown in Figure 6.4 may be readily seen.\* Correlations like these offer excellent possibilities for future investigation of the structure of enzymes from studies of their functions, especially if these studies were made in conjunction with methods introduced by Dixon and others (see Dixon and Webb, 1958).

In the above example, when the pH is constant, an increase of enzymatic

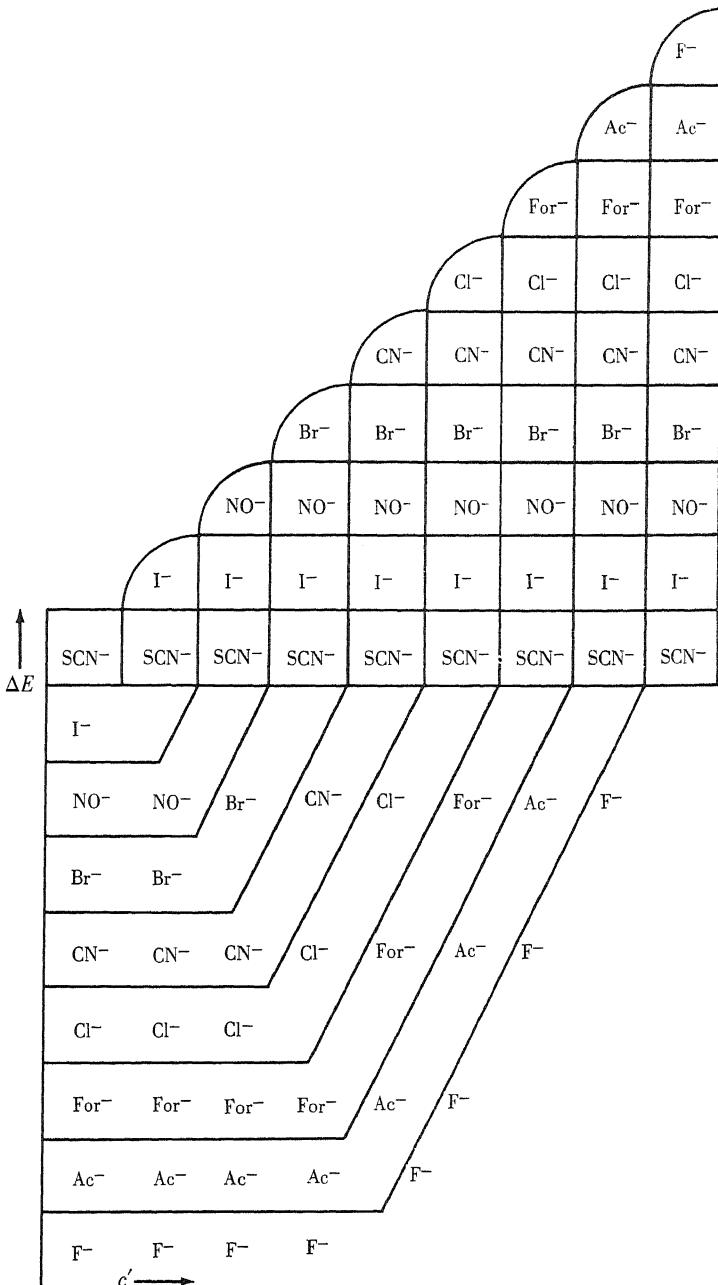
\* These comparisons between theoretical expectations and experimental facts are made in the loosest sense to indicate possible directions for future studies. Thus we have deliberately neglected the effect of substrate ionization which can be studied by known methods (see Dixon and Webb, 1958).

activity follows the adsorption of acetate anion at a neighboring cationic site. Such an adsorption of acetate anion may be brought about by increasing the acetate-ion concentration in the system. Another way to increase the probability of the adsorption of the anion at the neighboring site is to increase the anion's



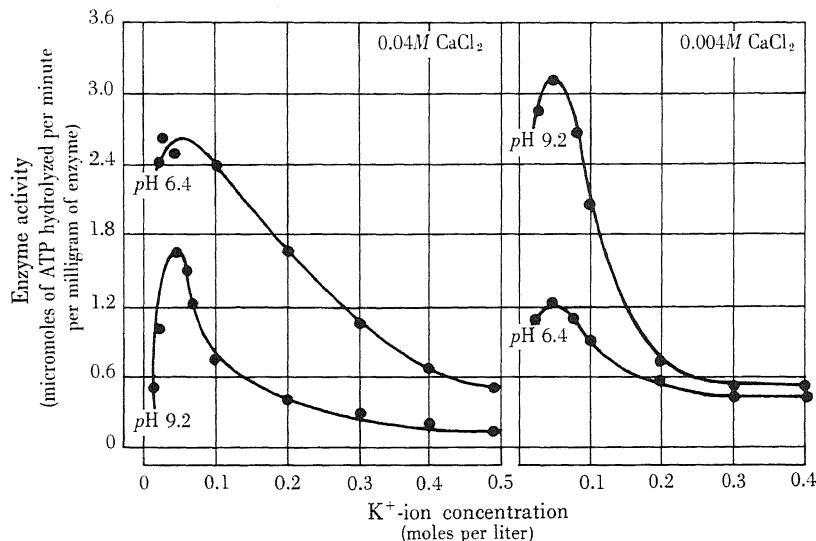
**Figure 14.6. THE EFFECT OF SOME ANIONS AND pH ON THE ENZYMATIC DEHYDRATION OF L-MALATE BY FUMARASE.** The pH of the medium is plotted against the enzyme activity obtained by measuring the initial rate of change in the optical density ( $240\text{m}\mu$ ) of the enzyme-substrate system. The product of the dehydration, fumaric acid, contains a double bond which absorbs strongly at this wavelength. The concentration of L-malate was  $0.0835\text{M}$ . A, in the absence of added anions; B,  $0.03\text{M}$  sodium selenate or  $0.025\text{M}$  sodium sulfate; C,  $0.01\text{M}$  sodium arsenite; D,  $0.01\text{M}$  sodium citrate; E,  $0.2\text{M}$  sodium borate; F,  $0.06\text{M}$  sodium and potassium phosphate; G,  $0.05\text{M}$  sodium arsenate; H,  $0.1\text{M}$  sodium chloride. Compare with Figures 14.5 and 6.4. (Figure after Massey, 1953a.)

free energy of adsorption. This can be achieved by varying the nature of the anion. Such a study has been carried out on fumarase by Massey (1953a); his results are reproduced in Figure 14.6. Note the similarity between the family of curves, A to G, and Figure 14.5. The results shown in Figure 14.6 suggest that a cationic site neighboring the enzymatically active site binds anions in the order citrate > arsenite > sulfate.



**Figure 14.7.** A SCHEME FOR A POSSIBLE RELATIONSHIP BETWEEN THE  $c'$ -VALUE OF FIXED CATIONS AND THE ADSORPTION ENERGIES OF VARIOUS ANIONS. This scheme is made on the assumption that the sequence,  $\text{SCN}^- > \text{I}^- > \text{NO}_3^- > \text{Br}^- > \text{CN}^- > \text{Cl}^- >$  Formate ( $\text{For}^-$ )  $>$  Acetate ( $\text{Ac}^-$ )  $>$   $\text{F}^-$ , corresponds to the lowest  $c'$ -value order. The justification lies in the fact that the sequence of the halide ions:  $\text{I}^- > \text{Br}^- > \text{Cl}^- > \text{F}^-$  is the inverse of that according to their atomic sizes, hence the suggestion that hydration increases with decreasing atomic weight. This relation has been calculated for the analogous situation with the alkali-metal ions,  $\text{Cs}^+$ ,  $\text{Rb}^+$ ,  $\text{K}^+$ ,  $\text{Na}^+$ , and  $\text{Li}^+$  (see Figure 4.9).

At this point, it becomes pertinent to ask whether or not the order of anion affinity exhibits variations. The simplest series we can investigate in this regard is the halide series. However, a theoretical calculation of the  $\Delta E$ -versus- $c'$ -value relation for the halide ions has not been made. Nevertheless, it is clear that the order for the four halide ions:  $I^-$ ,  $Br^-$ ,  $Cl^-$ ,  $F^-$  is the exact counterpart of the cation series,  $Cs^+$ ,  $Rb^+$ ,  $K^+$ ,  $Na^+$ ,  $Li^+$ , in which the heaviest ion would possess the highest adsorption energy. In Section 7.4B on protein denaturation, we demonstrated that the relative adsorption energies of the halide



**Figure 14.8. THE EFFECT OF  $KCl$ ,  $CaCl_2$ , AND  $pH$  ON THE ENZYMATIC ACTIVITY OF MYOSIN ADENOSINETRIPHOSPHATASE.** 25 ml of the reaction mixture contained  $8.0 \mu M$  of ATP and  $2.2 \text{ mg}$  of myosin. Enzyme activity at  $27.5^\circ\text{C}$  was determined by titration of the acid liberated in ATP hydrolysis. (Figure after Mommaerts and Green, 1954.)

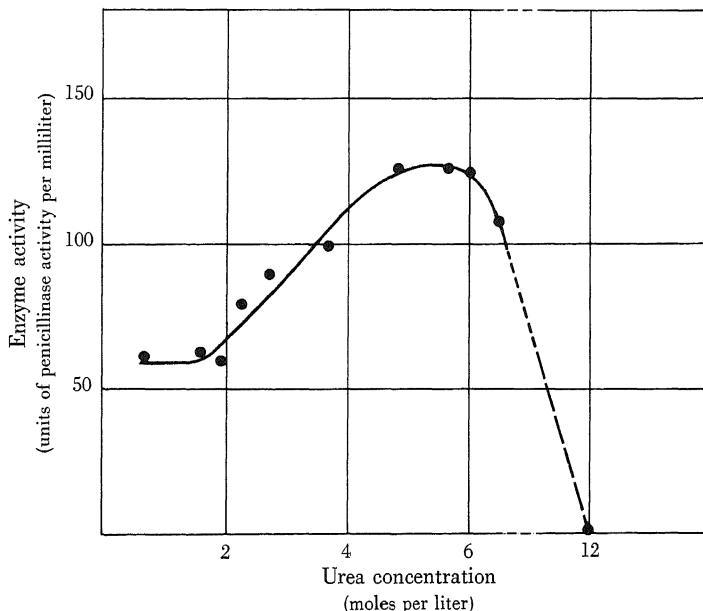
anions on Dowex 1 and 2 follow this order. By analogy with the alkali-metal ions, this corresponds to the order expected at the lowest  $c'$ -value of the cationic site. If we accept this analogy, we may predict an order reversal with increasing  $c'$ -value for the halides and for a number of other anions as well (Figure 14.7). [G. Eisenman *et al.* have made an analysis of the halide-ion orders, similar to their analysis of metal-ion orders. The reader is referred to their work for details (to be published).] At best, however, this prediction can only be approximately correct. Lacking clear theoretical ground for the exact shape of the adsorption curve, we must await future empirical and theoretical studies for its verification and modification. The general paucity of systematic studies of anionic effects permits no conclusion to be drawn as to which rank orders are most favorable or common. For the moment, we need only mention that two orders are frequently observed. One of these anionic orders corresponds to the lowest  $c'$ -value. It is seen in enzymology as the order of anionic inhibition on fumarase activity (Massey, 1953a,b). This is the order mentioned in Section 7.4D, as typical of anionic adsorption on amino groups in

proteins as well as on the cationic exchange resins, Dowex 1 and 2. Another order,  $\text{Cl}^- > \text{CN}^- > \text{Br}^- > \text{NO}_3^- > \text{I}^- > \text{SCN}^- >$  formate  $>$  acetate  $> \text{F}^-$ , is, in general, consistent with that observed for activation of human salivary  $\alpha$ -amylase, that is,  $\text{Cl}^- > \text{Br}^- > \text{NO}_3^- > \text{I}^- > \text{SO}_4^- > \text{HCO}_3^- = \text{SCN}^- = \text{CH}_3\text{CO}_2^-$ . This order is seen also in the effect of anions on the rate of the Hill reaction in photosynthesis,  $\text{Cl}^- > \text{Br}^- > \text{NO}_3^- > \text{I}^- > \text{F}^- > \text{SO}_4^- > \text{HPO}_4^{2-}$  (Warburg and Lutgens, 1946; see also Arnon and Whatley, 1949; Gorham and Clendenning, 1950).

The same general pattern, which resembles Figure 6.4, is found in the curves of Figure 14.8. These show the interactions of KCl and pH on myosin adenosine-triphosphatase. Such results suggest that an enzyme complex consists of three closely situated sites which mutually influence each other through *F*-effects. From our knowledge of proteins and from the viewpoint of the present theory, nothing could be more natural.

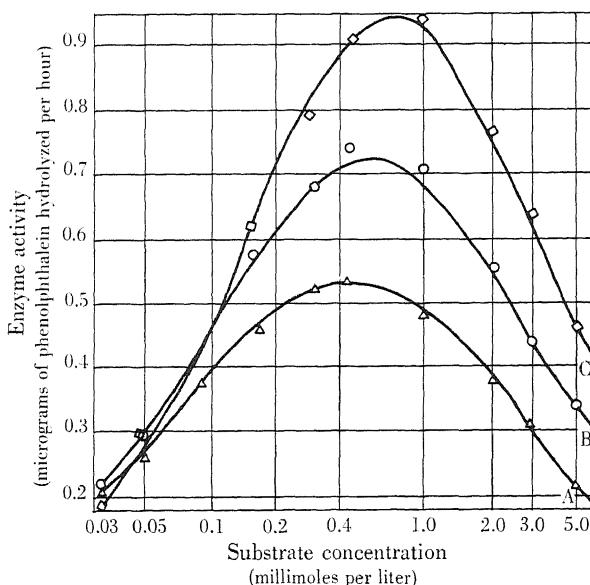
## B. HYDROGEN-BONDING AGENTS

The effect of urea concentration on the  $\gamma$ -type activity of penicillinase is shown in Figure 14.9. The bell-shaped curve obtained with this strong H-bonding agent confirms the belief that the *F*-effect is the underlying mechanism in such inter-



**Figure 14.9. THE EFFECT OF INCREASING UREA CONCENTRATION ON THE  $\gamma$ -TYPE ACTIVITY OF PENICILLINASE.** Penicillinase was prepared from culture supernatant of *Bacillus cereus* strain.  $\gamma$ -type (iodine-sensitive) activity was obtained by subtraction of  $\alpha$ -type activity from total activity. (Figure after Citri *et al.*, 1960.)

actions whether the effects are produced by an adsorbent on an ionic site or on H-bonding sites. Figure 14.10 shows that the same results are seen in the action of



**Figure 14.10. THE EFFECT OF SUBSTRATE CONCENTRATION ON THE ENZYMATIC ACTIVITY OF  $\beta$ -GLUCURONIDASE.** The activity of purified  $\beta$ -glucuronidase from cow adrenal glands was determined by estimating the initial rate of hydrolysis of phenolphthalein glucuronide in an acetate buffer at pH 5.2. The facilitative action of ethylene glycol on enzyme activity is also shown. A, no ethylene glycol; B, 0.4M ethylene glycol; C, 1.6M ethylene glycol. (Figure after Nayyar and Glick, 1956.)

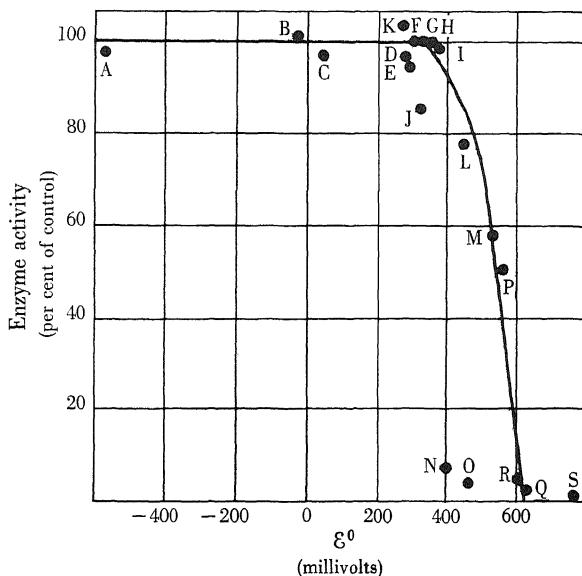
another H-bonding agent, ethylene glycol, on the activity of  $\beta$ -glucuronidase. Both of these figures should be compared with Figure 6.4.

### C. THE EFFECT OF SULFHYDRYL AGENTS ON ENZYME ACTIVITY

The importance of sulfhydryl groups in enzyme activity has been stressed by Hellerman (1937) and by Guzman-Barron (1951). In a large number of DPN-catalyzed oxidation-reduction reactions the hypothesis of direct participation of the SH group seems most reasonable. For other enzyme reactions that are not primarily oxidation-reduction reactions, this point of view is less attractive. The hypothesis to be developed here is directed primarily at these nonoxidation reactions. Reduction of disulfide to sulfhydryl groups entails the injection of electrons into the protein molecule; the reverse reaction, oxidation of sulfhydryl groups, involves the removal of electrons. It is suggested that, in SH enzymes, oxidation of

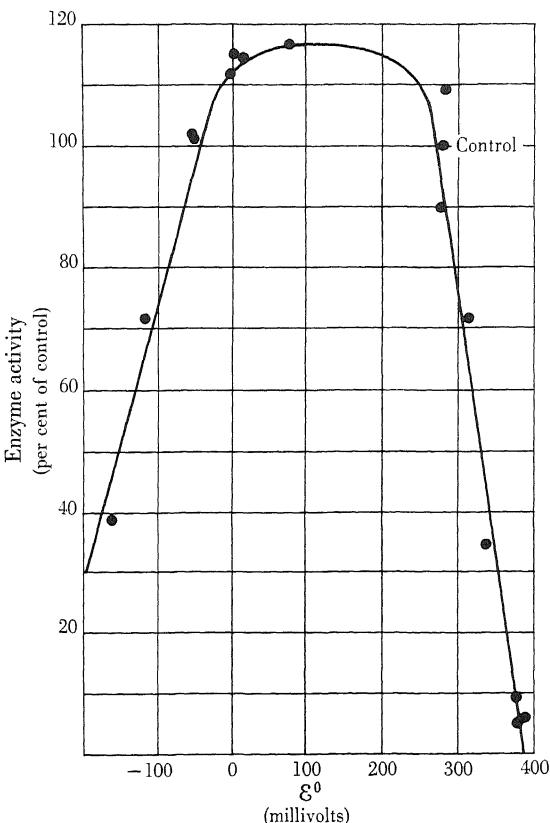
SH or reduction of S—S groups alters enzymatic activity through the direct *F*-effect on the  $\epsilon'$ - or  $\epsilon^0$  value ensembles. Such variation alters enzyme activity by affecting both the reactivity and the affinity of the site for the enzyme.

The statistical distribution of the SH form and the S—S form of a particular cysteine-cystine pair is determined by the oxidation-reduction potential of the



**Figure 14.11. THE RELATIONSHIP BETWEEN THE ENZYMATIC ACTIVITY OF INTESTINAL PHOSPHATASE AND THE  $E^0$  OF THE SYSTEM.** The labeled points refer to the following oxidation-reduction "buffers" used in the system. A,  $H_2 + Pt$  asbestos; B, 0.1 saturated  $H_2S$ ; C, 0.001M  $Na_2S$ ; D, 0.001M  $K_4Fe(CN)_6$ ; E, 0.001M  $Na_2SO_3$ ; F, 0.001M  $Na_2S_2O_3$ ; G, 0.001M  $H_2O_2$ ; H, water; I, 0.001M sodium iodoacetate; J, 0.001M KCNS; K, 0.001M sodium methionate; L, 0.001M  $K_2Cr_2O_7$ ; M, 0.001M  $K_3Fe(CN)_6$ ; N, 0.001M  $CrCl_3$ ; O, 0.001M  $Br_2$ ; P, 0.00001M  $I_2-KI$ ; Q, 0.001M  $I_2-KI$ ; R, 0.00001M  $KMnO_4$ ; S, 0.001M  $KMnO_4$ . (Figure after Sizer, 1942.)

system. If, at the optimum conditions for enzymatic activity, an enzyme molecule possesses one cysteinyl group or only half of a cystine group, a plot of the enzymatic activity against the oxidation-reduction potential of the system would give two flat plateaus separated by one ascending or descending limb. Such a curve was demonstrated by Sizer (1942) for bovine phosphatase activity (Figure 14.11). On the other hand, if at optimum conditions the enzyme possesses two or more cysteinyl or half-cystine residues, the plot of  $E^0$  against enzyme activity could give a bell-shaped curve, as was demonstrated for crystalline urease, shown in Figure 14.12 (Sizer and Tytell, 1941) and for tyrosinase (Figge, 1948).



**Figure 14.12. THE RELATIONSHIP BETWEEN THE ENZYMATIC ACTIVITY OF UREASE AND THE  $E^\circ$  OF THE SYSTEM.** Per cent urease activity is plotted against the oxidation-reduction potential of the digest. The optimum  $E^\circ$  is roughly +130mv. The plotted points represent the urease activity and  $E^\circ$  of digests which contained one of the following: 0.05, 0.025, 0.01, 0.005, 0.0025, 0.0012, 0.0005, 0.00025M Na<sub>2</sub>S (the points on the left-hand side of the curve), or 0.0001, 0.00005, 0.00004, 0.000025, 0.000019, 0.000012, 0.000006M KMnO<sub>4</sub> (the points on the right-hand side of the curve). (Figure after Sizer and Tytell, 1941.)

Thus we see that the effects of ionic, H-bonding, and oxidizing or reducing agents on enzymatic activity all follow the theoretical predictions of the association-induction hypothesis.

#### 14.4. The Problem of Coexistence of Enzymes and Their Substrates in Living Cells

Earlier discussion has led to the conclusion that, within a fixed-charge system, ions and nonelectrolytes are more strongly associated with specific sites than is the case in dilute aqueous solutions. We may thus expect that in a true fixed-

charge system like a living cell there will be no large quantity of free substrate.\* Based on this and other concepts introduced earlier, two different mechanisms may be suggested to maintain within cells both a large amount of enzyme and a high concentration of substrate without their interaction. (1) The substrate and ions of similar charge may be preferentially distributed among the sites so that only nonsubstrate molecules are to be found at enzymatically active sites when the cells are at rest. Activation of cells could then alter the  $c$ -value of the adsorbing

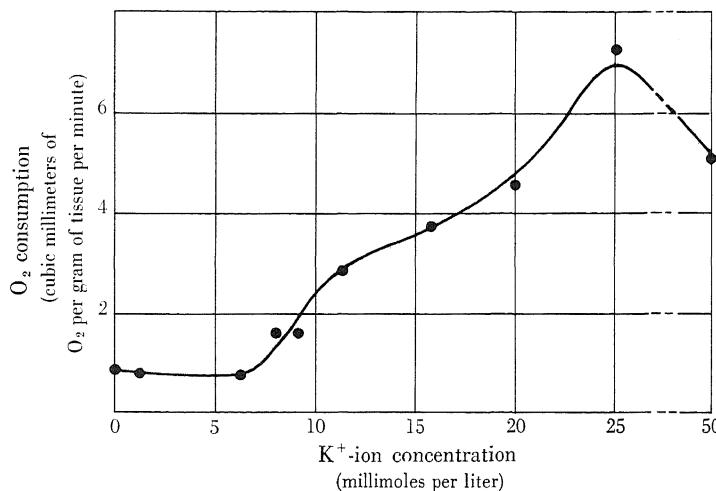
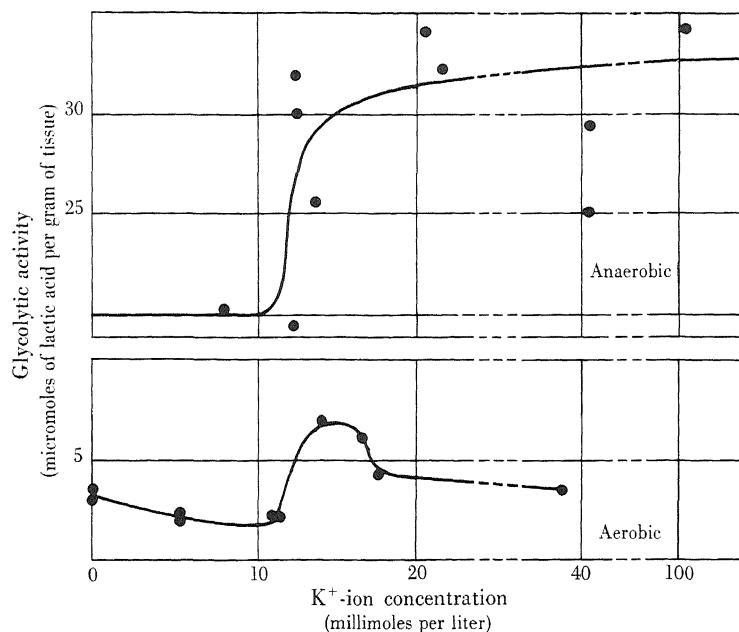


Figure 14.13. OXYGEN CONSUMPTION OF FROG MUSCLES MEASURED ONE HOUR AFTER TREATMENT WITH VARIOUS CONCENTRATIONS OF K<sup>+</sup> ION. (After Hegnauer *et al.*, 1934.)

sites to cause the release of substrate, enzymatically active sites, or both. (2) The substrate could be adsorbed onto potentially active enzyme sites which are inactive when the cell is at rest. Activation of the cell could then bring about a  $c$ -value or an  $\varepsilon^0$  change of this site, activating it enzymatically and causing the formation of an activated complex.

Since the resting cell represents a state of delicate control and restraint of enzymatic activity, it is not surprising that any disturbance, physiological or otherwise, is usually accompanied by an over-all increase of metabolic activity. As an example we mention the generally observed increase of resting heat production and oxygen consumption (and glycolysis) when intact muscle tissues (Hegnauer *et al.*, 1934; see also Solandt, 1936) or brain slices (McIlwain, 1952) are treated with K<sup>+</sup> salts (Figures 14.13 and 14.14). It seems likely that this effect involves the type of competitive activation discussed in Section 14.1B. This effect

\* For other theories based on structured barriers to explain the paradox of coexistence of enzyme and substrates see Conway and O'Malley (1944) and Bacq and Alexander (1955).



**Figure 14.14. AEROBIC AND ANAEROBIC GLYCOLYTIC ACTIVITY OF FROG MUSCLES IN RESPONSE TO TREATMENT WITH VARIOUS CONCENTRATIONS OF  $K^+$  ION.** Period of immersion, 3.5 hr for the anaerobic studies and 5 hr for aerobic; temperature, 22°C. (Figure after Hegenauer *et al.*, 1934.)

is not observed in most other types of tissues (Dickens and Greville, 1935; Needham *et al.*, 1937), suggesting that (as we know independently) a modal  $c$ -value prevails in muscle and nerve-tissue proteins such that the  $K^+$  ion is highly preferred (large  $\Delta F_{x+\bar{f}}$ ); see Table 9.5. This prevailing preference for  $K^+$  ion by resting muscle protoplasm offers a clue for the understanding of another physiological manifestation of the living state, contractile phenomena, discussed in Chapter 15.

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# 15

## REVERSIBLE CONTRACTILE PHENOMENA

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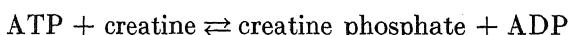
Motility is one of the most easily observed manifestations of the living state. From gross appearances there are many types of motion: ciliary, undulatory, amoeboid, contractile, peristaltic, and so on; nevertheless, all basically involve a reversible lengthening and shortening of the cell or cell components. Because these reversible contractions permit the organism to do work on its environment, the contractile cells must in some way derive energy from the food substances and oxygen taken in by the organism.

The most familiar devices known to early physiologists for the transformation of energy into useful work belong to the category of heat engines. Thermodynamics tells us that the maximum efficiency of such a heat engine is given by

$$\text{Maximum efficiency} = \frac{T_2 - T_1}{T_2} \quad (15-1)$$

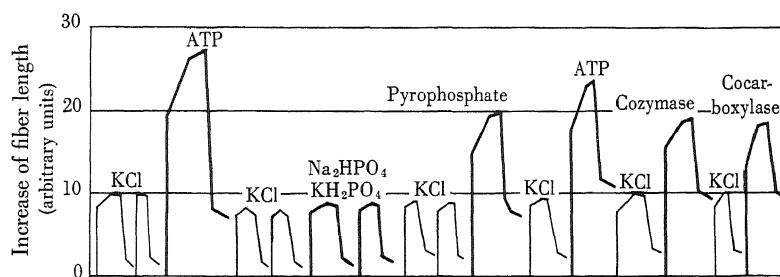
where  $T_1$  is the ambient temperature and  $T_2$  is the highest temperature reached by the engine during the course of its work cycle. The efficiency of muscular work is commonly estimated to be about 30 per cent (see Wright, 1952, p. 431). Since  $T_1$  for, say, warm-blooded animals, is  $273^\circ + 37^\circ = 310^\circ\text{K}$ ,  $T_2$  would have to be as high as  $443^\circ\text{K}$  or  $170^\circ\text{C}$  were these living machines to operate as heat engines. Since such temperatures are inconsistent with the function of these organisms, we must look for other processes of energy transformation.

Because the energy utilized by the living organism originally resides in the chemical bonds of the various foodstuffs (glucose, fat, and so on), it occurred to physiologists and biochemists that the contractile machine might be utilizing chemical energy directly. This concept led to some of the most rewarding experiments in the history of physiology. The studies of physiological work performance were teamed with an inquiry as to how the step-by-step degradation of the carbohydrate molecule (or other food material) makes energy available for the performance of muscular work. In the early studies all the evidence indicated the central role of lactic acid, for this substance is the product of carbohydrate metabolism during muscular exercise (Hill, 1926). Speculation on the crucial role of lactic acid was abruptly halted by the finding of Lundsgaard (1930) that a muscle poisoned with iodoacetate can contract normally without producing lactic acid (Figure 8.3). This revolutionary discovery (see Hill, 1932) caused attention to be focused upon the newly discovered compounds, creatine phosphate, CrP (Eggleton and Eggleton, 1929; Fiske and Subbarow, 1929), and adenosinetriphosphate, ATP (Lohman, 1929). Both of these compounds are found in abundance in resting muscle (Table 9.2); together they comprise about two-thirds of the "free" anions in living muscle cells. While ATP is the immediate product of the metabolic degradation of glucose, the ready conversion of creatine to creatine phosphate through the Lohman reaction (1935),



which involves a negligible free-energy change means that the energy in the phosphate bonds of creatine phosphate as well as in those of ATP could be made available as a ready source of energy for contraction at an intensive phase of muscular activity.

The trend of these developments was climaxed by the discovery of Engelhardt and Lyubimova (1939; see also Engelhardt, 1946) that ATP causes mechanical changes, the lengthening of threads\* made from the extracted muscle protein



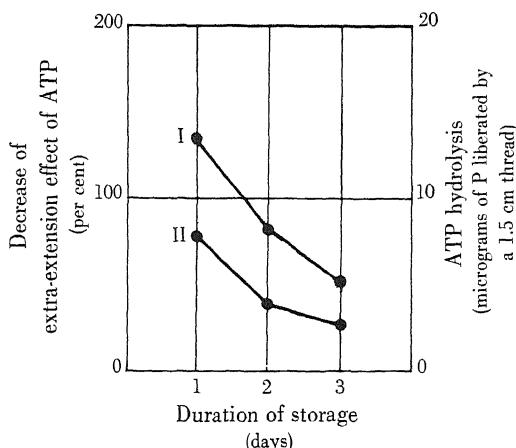
**Figure 15.1. DIMENSIONAL CHANGES OF MYOSIN THREAD CAUSED BY ATP AND RELATED COMPOUNDS.** By extruding myosin (actually an undefined mixture of myosin and actomyosin, see Szent-Györgyi, 1951, p. 33) into a solution of low ionic strength, a gel-like thread is formed which, when mounted on a lever, reversibly responds to the addition of a load (usually 200mg) by lengthening (see also Figure 15.8). This extensibility is found to be greatly increased by the presence of ATP at very low concentrations (5mM). Other related compounds, inorganic pyrophosphate, cozymase, and cocarboxylase, containing the pyrophosphate groups also are effective but to a smaller degree. (Figure after Engelhardt and Lyubimova, 1942; see Engelhardt, 1946.)

myosin (Figure 15.1), and that myosin possesses the specific ability to split ATP enzymatically (Figure 15.2). The significance of the latter discovery lies in the presumption that it is the chemical energy (the high-energy phosphate-bond energy) of ATP which actually energizes the mechanical work of contraction. The question then became: How is the energy thus liberated injected into or captured by the protein system to permit performance of mechanical work?

In Chapter 8, on the function of metabolism, we reviewed evidence demonstrating that a number of biological functions, including muscular contraction, can be entirely normal when no known energy source is present other than that of compounds such as CrP and ATP (see Hill, 1932; Lipmann, 1941). We then showed that one of these functions, selective ionic accumulation, which was sup-

\* Weber (1934) first prepared and investigated the properties of these "myosin" threads.

posed to depend on a constant supply of energy released from the high-energy phosphate bond, does not depend on the actual hydrolytic liberation and utilization of the energy in ATP. Rather, it depends only on the presence of ATP *per se* within the cell. In Chapter 9, we considered evidence showing that ATP serves as a cardinal adsorbent maintaining a certain cooperative state of the muscle protein in which a portion of the fixed anions prefer the K<sup>+</sup> ion to fixed cations. In the



**Figure 15.2. THE CORRELATION OF MECHANICAL RESPONSE OF MYOSIN THREAD AND ITS ENZYMATIC ACTIVITY.** The extensibility of myosin threads decreases with storage at 0°C. Such a change in the mechanical property is shown to be correlated with a similar change of the ATPase activity of the thread. Curve I, extensibility response to ATP; Curve II, enzymatic activity of myosin. (Figure after Engelhardt and Lyubimova, 1942; see Engelhardt, 1946.)

present chapter we extend the concept of the role of cardinal adsorbents in physiological function to the mechanism of muscular contraction. This process, we suggest, consists of a cooperative and reversible transformation of fixed cationic and anionic sites from the state in which they prefer free counterions, to the state in which they prefer each other. We shall devote our major effort to an attempt to put a number of valuable and yet, so far somewhat incoherent, experimental facts into a simple framework. Toward this end, we shall proceed from the simplest model systems such as actomyosin solutions, through somewhat more complex model systems such as glycerol-treated *psoas* muscle fibers, to various intact muscles such as the frog *sartorius* muscle, the cardiac muscle of the turtle, and the smooth muscle of cat gut.

### 15.1. The Transformation of Actin from Globular to Fibrous Form in the Presence of a Critical Salt Concentration

Actin is a protein which normally constitutes about 15 per cent of the total protein of muscle fibers (see Table 3.1). Isolated actin in a salt-free solution or a very dilute salt solution is present in the globular (G-actin) form (see Figure 15.3, background). If HCl is gradually added to the system, a critical concentration is reached at which a short period of latency is followed by an all-or-none transformation into fibrous actin (F-actin). Thus, the globular-fibrous (G-F) transformation of actin has many of the characteristics of the acid denaturation of hemoglobin through the indirect *F*-process (Steinhardt and Zaisser, 1951, 1953) described in Section 7.4C. The G-F transformation of actin may be brought about by HCl or any other alkali-metal salt of the halides (Figures 15.3 and 15.4). The salts are effective in the order  $\text{Li}^+ > \text{Na}^+ > \text{K}^+ > \text{Rb}^+$  and  $\text{F}^- > \text{Cl}^- > \text{Br}^- > \text{I}^-$ , a fact which affirms the contention that the G-F transformation involves an indirect *F*-process in which both cation and anion are *specifically adsorbed at individual sites* rather than merely contributing to the nonspecific ionic strength (see Introduction) of the media.

A significant characteristic of unfolded fibrous actin, which is equivalent to the unfolded "denatured" state of many other proteins, is that, under certain conditions at least, it is highly regular. Thus, if a critical salt concentration is attained through the evaporation of water from an actin-salt solution, a striking phenomenon occurs. From an apparently random distribution of globular proteins and salts, a system of regular fibers begins to appear (Figure 15.3A). When several of these fibers come into contact, a system with "cross striations" is formed (Figure 15.3B), reminiscent of living striated muscle.

The significant point here is that the unfolded state, which in protein denaturation is usually considered a random state, is, in the present case, not random but highly regular. This verifies our contention (Section 7.4B) that denaturation of proteins is controlled by free-energy differences and is not of necessity a unidirectional entropy change toward a more random state.

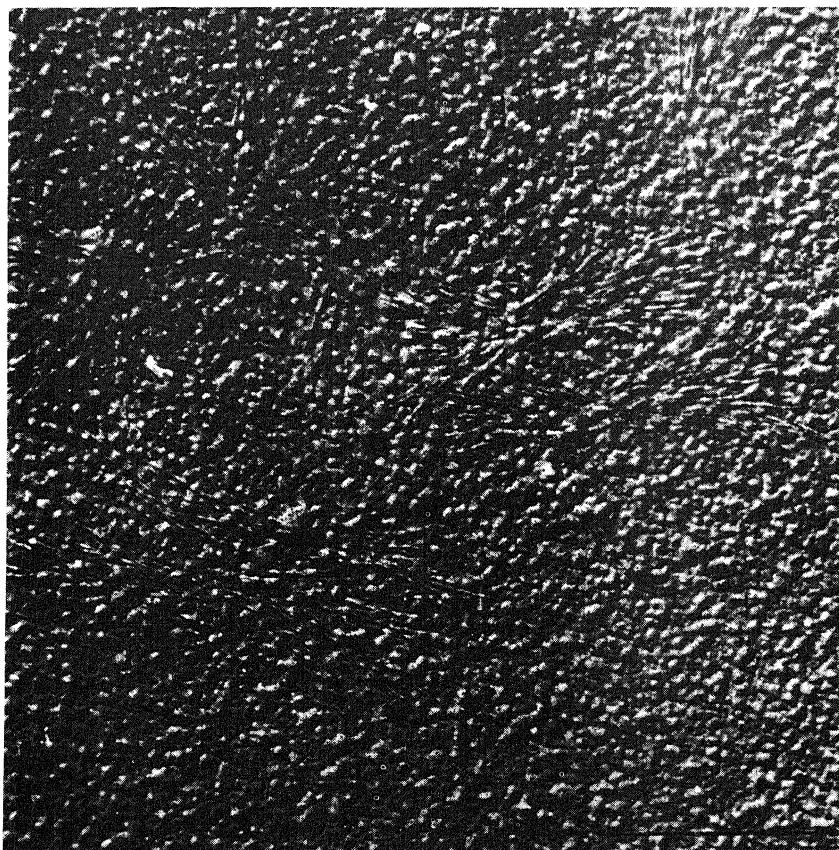
A second point worth mention is that  $\text{Li}^+$  and  $\text{F}^-$  ions are very effective in bringing about the G-F transformation; that is, they are the most strongly adsorbed ions. This indicates that the cardinal sites of the fibrous form of actin have high  $c$ - and  $c'$ -values.

### 15.2. The "Superprecipitation" of Actomyosin

When actin is mixed with the major muscle protein, myosin, the resulting viscous protein system is referred to as actomyosin (Szent-Györgyi, 1951). If KCl is added

in increasing concentration to this salt-free system, a moderate precipitate of the protein occurs, followed by a gradual solution (Figure 15.5, broken line). On the other hand, if ATP is added to a salt-free actomyosin system to a final concentration of  $1mM$ , the highly viscous actomyosin system suddenly dissolves completely. If KCl is then added gradually, a concentration will be reached at which an *all-or-none precipitation* of all protein occurs; this effect was named "superprecipitation" by Szent-Györgyi. Further increase of KCl produces no further effect until another critical concentration is reached, at which point an *all-or-none dissolution* occurs. The entire process is represented in Figure 15.5.

Let us consider how this sequence may be interpreted in terms of the present theory. The high viscosity of actomyosin in the absence of salt and ATP suggests that linkages are formed between actin and myosin (Figure 15.6A). This system adsorbs ATP with avidity (see Szent-Györgyi, 1951; Nanninga and Mommaerts, 1957) so that at a  $1mM$  ATP concentration, enough negatively charged quadri-



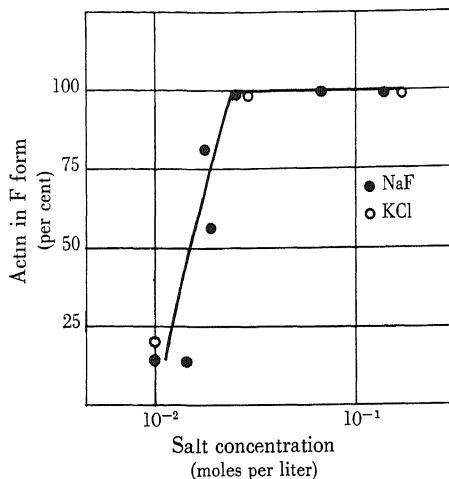
A



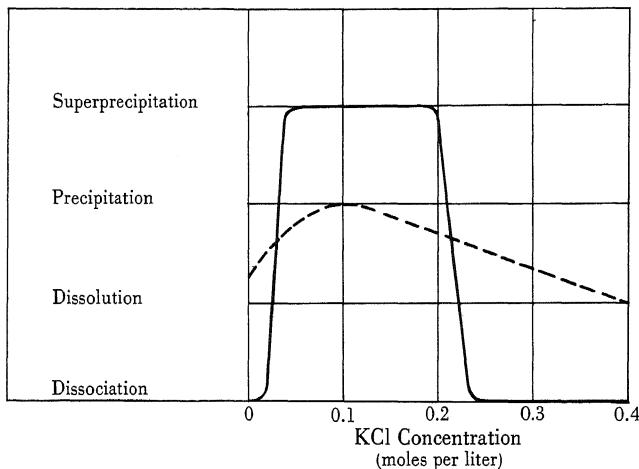
B

**Figure 15.3. THE TRANSFORMATION OF GLOBULAR TO FIBROUS ACTIN.** The G-F transformation was carried out directly on a glass slide by allowing water to evaporate from a drop of G-actin in dilute salt solution. A, the point at which a critical salt concentration is first reached may be seen as a focus at which sheaves of F-actin are formed. Further progress of the process leads to the formation of a large number of fibers which show some degree of long-range order revealed as cross striations. In some cases, B, this cross striation is very conspicuous. (Figure from Rosza *et al.*, 1949.)

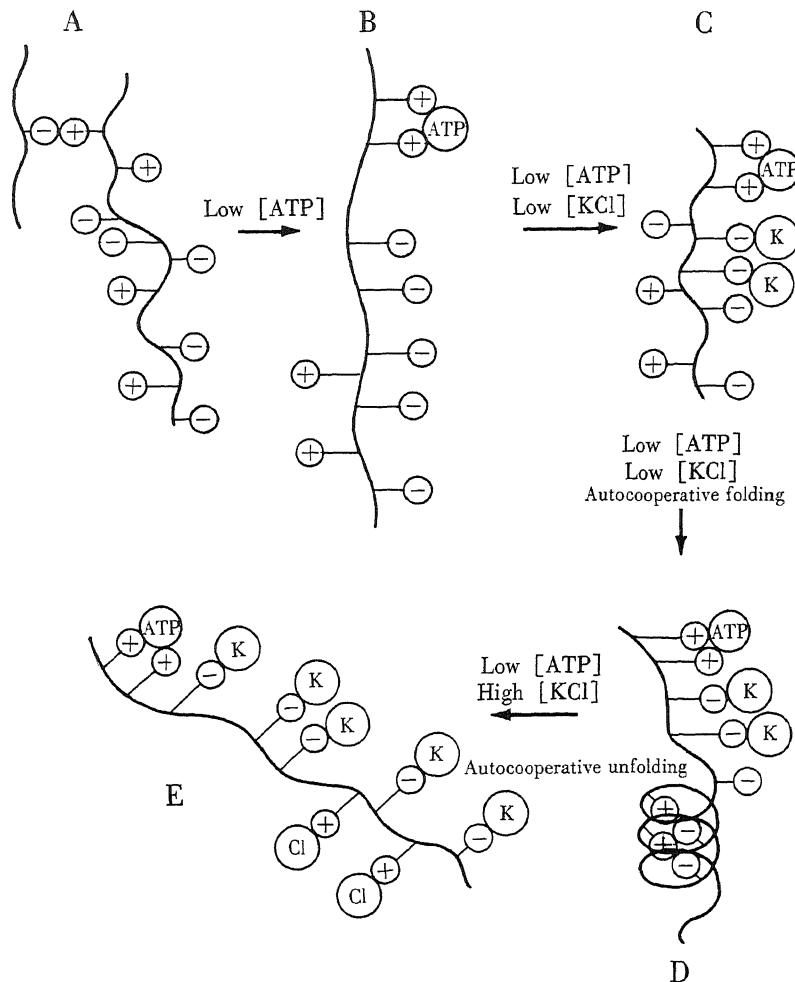
valent ATP has been adsorbed to cause the dispersion of the loose protein net by electrostatic repulsion leading to solubilization (Figure 15.6B). These net negative charges also cause the individual molecules to assume the extended configuration, for the tendency of individual cations and anionic sites to form salt linkages is not sufficient to offset the gross electrostatic repulsion in the salt-free medium. If KCl is added gradually, at some moderate concentration the positively charged K<sup>+</sup> ions begin to associate with the protein and perhaps ATP anionic groups until



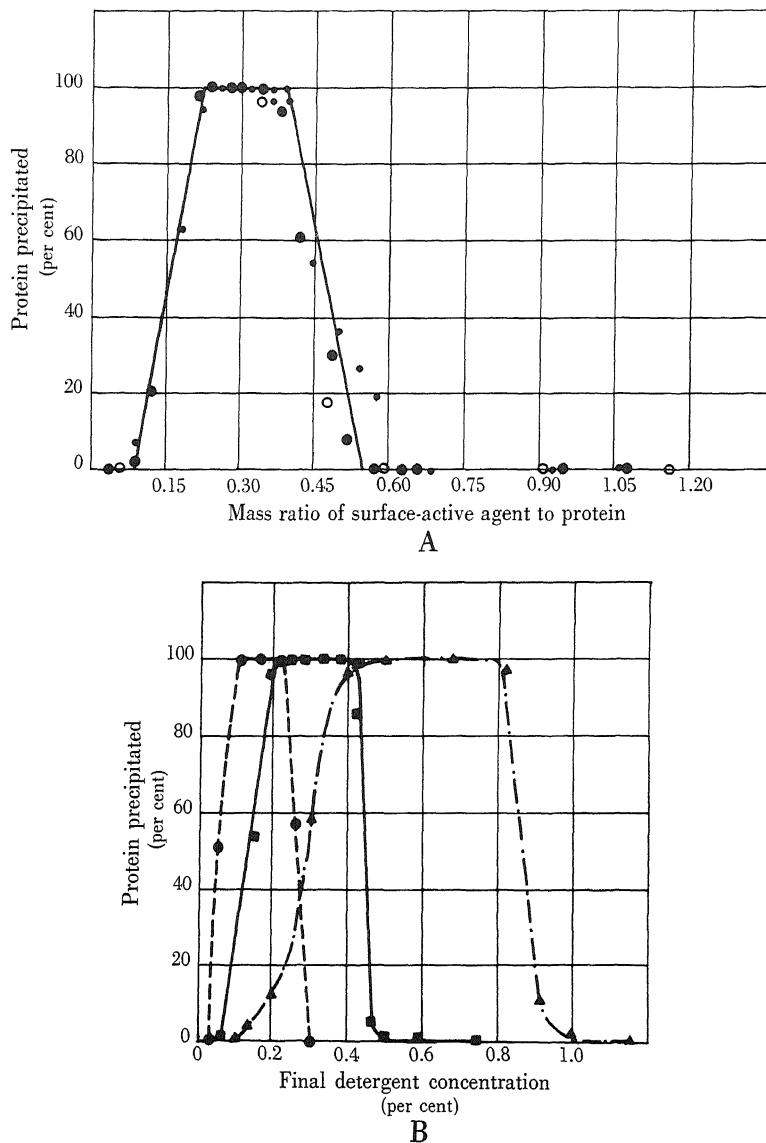
**Figure 15.4. THE PROMOTION OF TRANSFORMATION FROM GLOBULAR TO FIBROUS ACTIN BY SALTS.** Data indicate a steep rate of transformation within a narrow concentration range of salt; this is characteristic of autocooperative changes. Compare with Figure 15.5 and, in particular, with the detailed explanation given under Figure 15.6. (Figure after Straub, 1943.)



**Figure 15.5. SUPERPRECIPITATION OF ACTOMYOSIN BY KCl IN THE PRESENCE AND IN THE ABSENCE OF ATP.** In this diagram the extensive precipitation of actomyosin in the presence of ATP (solid line) involves a high degree of dehydration and is termed "superprecipitation," in contrast to ordinary precipitation in which the proteins are acted on by salts in the absence of ATP (dotted line). (Figure after Szent-Györgyi, 1951.)



**Figure 15.6. THE INTERPRETATION OF SUPERPRECIPITATION PHENOMENA IN TERMS OF THE PRESENT THEORY.** A viscous state of the protein in the absence of ATP, A, indicates the existence of occasional intermolecular ligands; this changes to one of low viscosity by adsorption of quadrivalent  $\text{ATP}^{4-}$ . The protein molecule then exists in more or less extended form and is kept from entangling with other protein molecules by its gross electrostatic charge at the very low ionic strength where there is little ion-cloud shielding effect, B. Introduction of a small amount of salt,  $\text{KCl}$ , leads to adsorption of some  $\text{K}^+$  ion and the effective elimination of the net electrostatic charges that keep the chain in the extended form, C. This is followed by an autocooperative folding, leading to superprecipitation, D. Further addition of  $\text{KCl}$  leads to the type of autocooperative unfolding, E, shown in Figure 7.13.



**Figure 15.7.** PRECIPITATION PHENOMENA SIMILAR TO THE SUPERPRECIPITATION OF ACTOMYOSIN SHOWN IN THE INTERACTION OF OTHER PROTEINS WITH SALT IONS AND DETERGENTS. A, the interaction of bovine serum albumin with an anionic detergent, Lorol (a straight-chain alcohol sulfate) pH 4.4. The abscissa represents the ratio of weights of detergent to protein in a unit volume of solution. Albumin-concentration levels in milligrams per milliliter are: ●, 4.2512; ●, 8.4612; and ○, 17.1890. (Figure after Glassman, 1950.) B, interaction of horse serum albumin and sodium dodecyl sulfate at pH 4.5. Protein concentrations in milligrams per milliliter: ●, 4.80; ■, 9.80; ▲, 19.72. (Figure after Putnam and Neurath, 1944.)

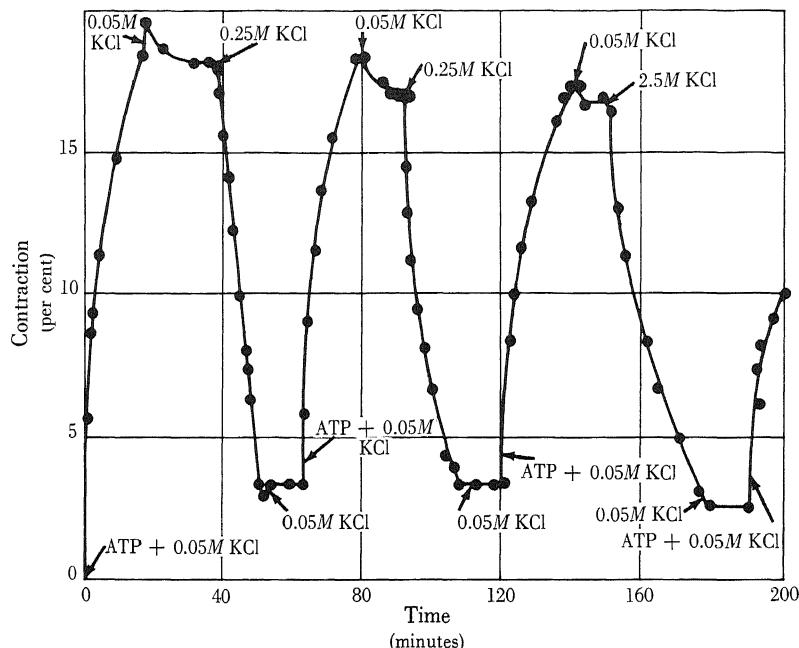
a concentration is reached at which the electrostatic repulsion is effectively weakened and the autocooperative gangs of sites fold into a superprecipitated state through the operation of the indirect *F*-process (Figure 15.6C). This cooperative state, like all other cooperative states, resists further change until another critical concentration of KCl is reached at which the dissociation of cardinal salt linkages by K<sup>+</sup> and Cl<sup>-</sup> ions leads to the cooperative unfolding of the gangs (Figure 15.6D) and solubilization, a phenomenon we have discussed in detail in the case of the hemoglobin denaturation.

In this discussion, we have made no particular reference to the high-energy phosphate-bond energy of ATP, but have stressed the function of ATP as a negatively charged cardinal adsorbent as well as the cooperative all-or-none changes in physicochemical properties characteristic of some proteins. To demonstrate the general applicability of this point of view, we have reproduced figures from Glassman (Figure 15.7A) and from Putnam and Neurath (Figure 15.7B). These figures show a similar type of superprecipitation and solubilization in bovine serum albumin, where the H<sup>+</sup> ion serves as the analogue of the anionic ATP and an anionic detergent serves as the analogue of the K<sup>+</sup> ion in the actomyosin-ATP-KCl system. The all-or-none response is almost identical in both systems; however, in the albumin system, none of the reacting entities contains an easily hydrolyzable phosphate bond.

### 15.3. The Mechanical Properties of Oriented Actomyosin Threads—The Transformation of Adsorption Energy into Macroscopic Mechanical Work

Since the primary action of ATP on actomyosin systems is due to an *F*-effect induced through its adsorption and is independent of the energy content of a hydrolyzable bond in the molecule, we may expect that a number of agents, manifestly lacking in the ability to deliver metabolic energy, may, nevertheless, cause dimensional changes similar to those produced by ATP. The shortening of a loaded actomyosin thread (Hayashi type, see Figure 15.8 and Hayashi, 1952) in the presence of various agents shows clearly that this is the case. In this system, histamine produces a 4.5 per cent contraction at a concentration of  $1.16 \times 10^{-7} M$ ; veratridine, a 4.43 per cent contraction at  $1.5 \times 10^{-7} M$ ; and CaCl<sub>2</sub>, a 4.35 per cent contraction at  $2 \times 10^{-6} M$ , and a 19.06 per cent contraction at  $2 M$  (Table 15.1).\* ATP itself produces a 12.8 per cent contraction at  $10^{-2} M$  concentration (Robb *et al.*, 1955). This demonstration of the shortening of actomyosin threads

\* Other examples are the shortening of glycerinated muscle fibers in the presence of CuCl<sub>2</sub> and K<sub>2</sub>HgI<sub>4</sub> (Varga, 1951), thiocyanate and iodide ion (Laki and Bowen, 1955), as well as changes in the viscosity of actomyosin brought about by guanidine and arginine (Mommaerts, 1945).



**Figure 15.8. THE REVERSIBLE CONTRACTION OF (HAYASHI TYPE) ACTOMYOSIN THREADS AGAINST A LOAD.** Actomyosin threads were prepared by rolling films of actomyosin. Isotonic contraction against a 11.7mg load is brought about by ATP in the presence of low KCl concentration, elongation, in the presence of high KCl concentration (with or without ATP). (Figure after Hayashi and Rosenbluth, 1952.)

against a load in response to ions and molecules which cannot deliver metabolic energy offers an unequivocal demonstration of the conversion of adsorption energy to macroscopic work, a fundamental premise of the present thesis.

#### 15.4. The Behavior of Glycerol-Extracted Muscle Fibers and Actomyosin Solutions

##### A. CONTRACTION AND RELAXATION

If we represent the length of the  $y$  dimension of a protein fixed-charge system (such as a glycerinated *psoas* fiber) by  $l_1^y$  when the gang is in the extended ( $\text{f-K}^+ \nwarrow \text{f}^+ \text{Y}^-$ ) form, and  $l_2^y$  when it is in the salt-linkage ( $\text{f}^+ \text{f}^-$ ) form,  $L_y$ , the  $y$  over-all macroscopic dimension of the whole system, may be represented by the equation,

$$L_y = N_y[(l_1^y) - (l_1^y - l_2^y)X_{\text{salt lk}}^{\alpha}] \quad (15-2)$$

Test substance	Concentration, <sup>a</sup> wt/vol	Degree of shortening, % initial length
Acetyl B methylcholine	1-100,000	4.92
	1-1,000,000	4.96
	1-2,500,000	5.55
	1-5,000,000	4.45
	1-10,000,000	4.95
	1-25,000,000	2.0
	1-50,000,000	3.0
Alcohol	1-50	4.89
	1-100	7.71
	1-200	5.99
Calcium chloride	1-5	19.06
	1-500,000	5.56
	1-1,000,000	6.05
	1-5,000,000	4.35
Epinephrine	1-100,000	3.05
	1-1,000,000	5.0
Histamine	1-41,400	2.53
	1-16,560,000	4.07
	1-60,000,000	4.5
Potassium chloride	1-370	3.1
Thyronin	1-100,000	3.0
Thyroxine (prep. 1)	1-100,000	4.17
Thyroxine (prep. 2)	1-100,000	3.77
Distilled water	100%	5.83
Veratridine	1-12,000	4.4
	1-50,000	2.5
	1-100,000	4.3
	1-500,000	3.7
	1-1,000,000	3.67
	1-10,000,000	4.43

**Table 15.1. THE SHORTENING OF MUSCLE MODEL AGAINST A LOAD IN RESPONSE TO DRUGS AND CHEMICALS THAT DO NOT CONTAIN HIGH-ENERGY PHOSPHATE BONDS.** Threads of actomyosin made by compressing a monomolecular layer of the protein (Hayashi, 1952) shorten against a glass weight (16.6mg). The amount of shortening is the average value of readings made 15 minutes after introduction into the test solution. (Table from Robb *et al.*, 1955.)

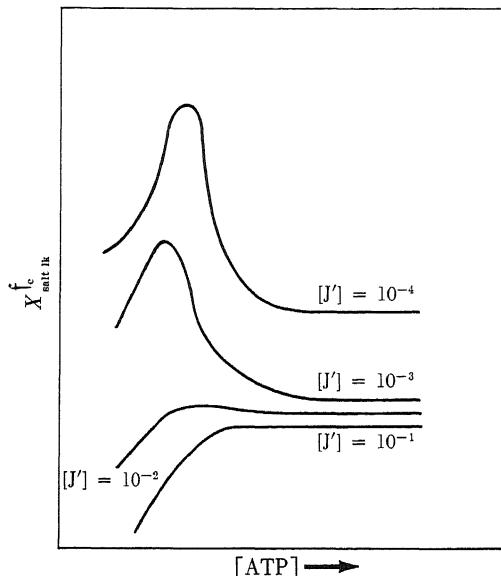
<sup>a</sup> 1-100,000 indicates 10 mg/l.

where  $N_y$  is the total number of gangs serially arranged in the  $y$  dimension, and  $X_{\text{salt lk}}^g$  is the *mole fraction of gangs* in the salt-linkage configurations (see J. J. Blum *et al.*, 1957, for an equation of similar form). Equation (15-2) may be simplified to

$$L_y = \text{const}_1 + \text{const}_2 X_{\text{salt lk}}^g. \quad (15-3)$$

Referring to Section 7.4C on protein denaturation, we may recall that the con-

figuration of a cooperative gang is determined in an all-or-none fashion by adsorption at the cardinal site. Thus the over-all dimension of the system, instead of being expressed in terms of mole fraction of gangs in one or the alternative state, may simply be expressed in terms of the mole fraction of cardinal adsorption. In the present case, the alternative adsorbents at the critical\* site are a free cation



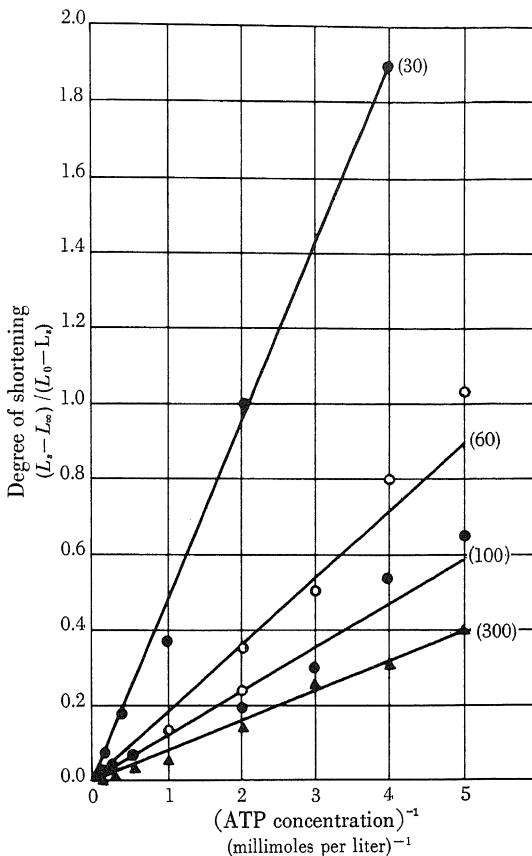
**Figure 15.9. THE ACTION OF ATP AND RELAXING FACTORS.** The model is similar to Figure 6.3. The cardinal adsorbent G is, in the present case, specifically ATP.  $J'$  then represents the relaxing factor. An optimal concentration of ATP brings about an optimum contraction (as illustrated by the critical adsorption  $X_{salt\ lk}^{f_c}$ ); addition of a relaxing factor can reverse this effect, bringing about relaxation.

(leading to the unfolded state), or a fixed cation (leading to the folded state). In Figure 7.13, for example, the critical site is site d which adsorbs onto site 8 in the folded state. We may thus write

$$L_y = \text{const}_1 + \text{const}_3 X_{salt\ lk}^{f_c} \quad (15-4)$$

in which  $X_{salt\ lk}^{f_c}$  represents the mole fraction of critical sites in salt-linkage form. Whether this critical site actually forms a salt linkage in a constant ionic environ-

\* To distinguish between their important but differing roles in the present model, we shall refer to the site adsorbing ATP as the cardinal site and the cardinal reversible salt linkage as the critical site.

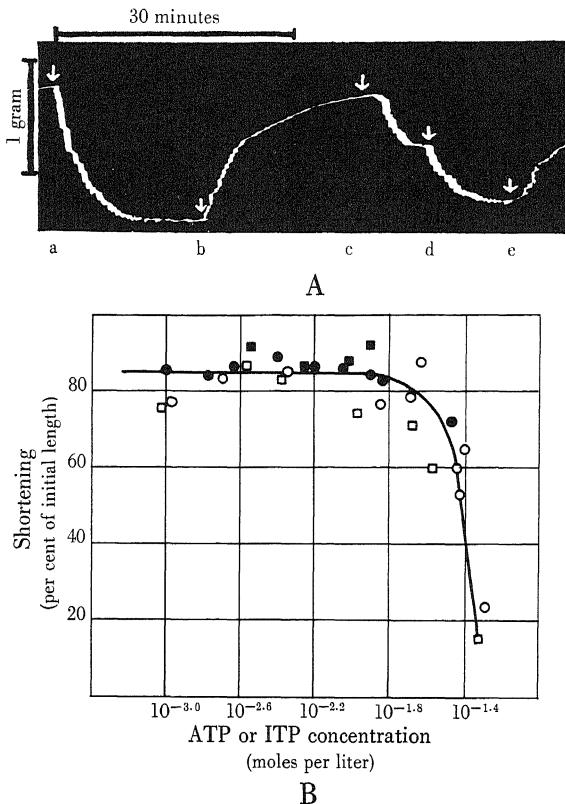


**Figure 15.10.** INHIBITION OF THE ATP-INDUCED SHORTENING OF GLYCEROL-TREATED MUSCLE FIBERS BY KCl.  $L_s$ ,  $L_\infty$ , and  $L_0$ , refer to length of glycerol-extracted rabbit *psoas* muscle-fiber bundle when the concentration of ATP is that given by the abscissa, very large, and zero respectively.  $(L_s - L_\infty)/(L_0 - L_s)$  refers to the extent of shortening produced by a certain concentration (S) of ATP; (300) represents a KCl concentration of 300 millimoles per liter of solution. (Figure after J. J. Blum *et al.*, 1957.)

ment is a function of the adsorbent on the neighboring sites, for example, site 9, in Figure 7.13.

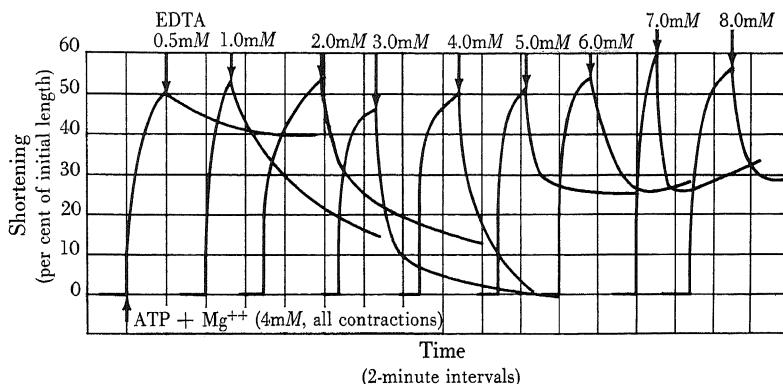
Let us redraw Figure 6.3 in a form more suitable to the present discussion\* where G is represented by ATP, and  $X_{\text{salt}}^{\text{f}_0}$  by  $X_{\text{salt lk}}^{\text{f}_0}$  (Figure 15.9); an increase along the ordinate will lead to shortening; a decrease, relaxation. In the presence of a

\* Site 2 in the theoretical model is anionic; in the case of myosin it would have to be cationic to react with ATP, but this difference is of no significance for the argument. Similarly, the relative positions of the three sites may be varied without changing the basic shapes of the theoretical curves.



**Figure 15.11. CONTRACTION AND RELAXATION OF VARIOUS CELLULAR SYSTEMS PRODUCED BY LOW AND HIGH ATP CONCENTRATION.** Aa, contraction of glycerine-treated muscle fibers (rabbit *psoas*) produced by 0.2 per cent ATP; b, relaxation produced by 1.5 per cent ATP; c, preparation was washed in saline causing contraction; d, additional contraction produced by 0.2 per cent ATP; e, relaxation caused by 1.5 per cent ATP; tension downward. (Figure from Bozler, 1951.) B shows similar dependence on nucleotide concentration of the per cent of shortening of glycerol-extracted muscle fibers (rabbit *psoas*). ●●● refer to ATP; ■■■, ITP. Ionic strength,  $0.12\mu$ , pH 6.9. Similarly, for glycerol-extracted amnion fibroblasts, ○○○ refers to ATP; □□□, ITP. Ionic strength,  $0.12\mu$ , pH 7.2. (Figure after Weber, 1955, after Hoffmann-Berling, 1954, and Portzehl, 1954.)

constant low concentration ( $10^{-3}$  to  $10^{-4}$ ) of  $J'$  (another reactant which adsorbs onto another neighboring site) an increase of ATP in small increments will cause progressive shortening. Such an effect has been observed by Bowen (1954) and Blum *et al.* (1957); see Figure 15.10. Further increase of ATP concentration leads to a reversal of the direction of the response; that is, a high ATP concentration causes relaxation, a phenomenon which has also been repeatedly observed (Bozler, 1952; Hoffmann-Berling, 1954; Portzehl, 1954) (Figure 15.11). In our hypothetical model the muscle shortens maximally at a certain optimal ATP concentration.

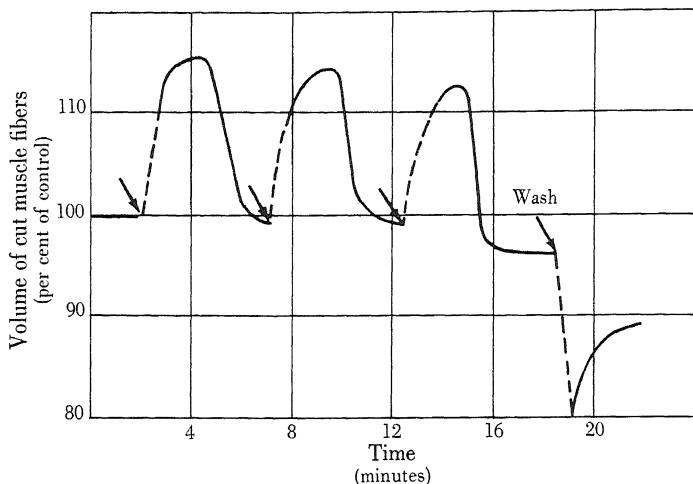


**Figure 15.12. DEPENDENCE OF THE EXTENT OF RELAXATION OF ATP-SHORTENED MUSCLE FIBERS ON THE CONCENTRATION OF ETHYLENEDIAMINETETRAACETATE.** Glycerol-extracted muscle fibers were treated with ATP and  $Mg^{++}$ , each 4 mM/l at all times. The EDTA concentration for each relaxation is indicated in millimoles per liter above the curve. (Figure after Watanabe and Sleator, 1957.)

If we choose to maintain this concentration of ATP constant, the model predicts that an increase of  $J'$  from  $10^{-4}$  to  $10^{-1}$  will reverse the direction of the dimensional change and lead to a marked relaxation (Figures 15.12, 15.13). A number of adsorbents are known to bring about such a relaxing effect. These include ethylenediaminetetraacetic acid (EDTA) and pyrophosphate; they have been called "relaxing factors." While the possibility has not been ruled out that some relaxing factors may act by directly changing the effective concentrations of ATP, phosphocreatine kinase, for example (Bozler, 1954; Goodall and Szent-Györgyi, 1953; Lorand, 1955); others, such as EDTA\* must produce relaxation by direct adsorption onto the protein as demonstrated by Watanabe and Sleator in 1957.

\* EDTA brings about complete relaxation at a concentration of half of that of the  $Mg^{++}$  ion. This indicates that EDTA cannot act by binding and thus removing the  $Mg^{++}$  essential for contraction, since a strong contraction can be produced at a  $Mg^{++}$ -ion concentration half of that usually employed (Watanabe and Sleator, 1957).

We suggest that adsorbed EDTA and similar agents act on the contractile system through the *F*-effect; in conjunction with ATP they produce contraction or relaxation as theoretically depicted in Figure 15.9.



**Figure 15.13. REVERSIBLE VOLUME INCREASE (BEFORE WASHING) AND IRREVERSIBLE SHRINKAGE OF CUT MUSCLE FIBERS (AFTER WASHING) IN RESPONSE TO ATP.** Fiber volume expressed as a per cent of the control volume following uniform centrifugation; 0.76mg ATP was added at each arrow. (Figure after Marsh, 1952.)

## B. ENZYME-ACTIVITY CHANGES

Michaelis and Menten (1913) introduced an equation which relates the rate of enzyme activity to the concentration of the enzyme-substrate complex,

$$V = \text{const } X_s^{\text{fE}} \quad (15-5)$$

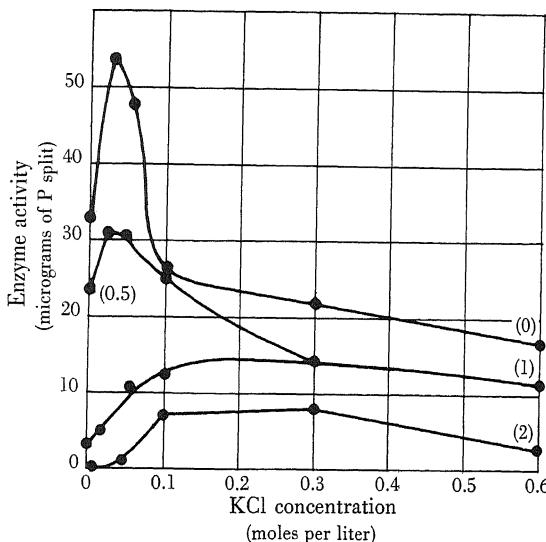
where  $V$ , the rate of enzyme action under specified conditions, is equal to the product of a constant and  $X_s^{\text{fE}}$ , the mole fraction of substrate occupying the enzymatically active site. For the rate of hydrolysis of ATP, this equation is

$$V = \kappa \cdot X_{\text{ATP}}^{\text{fE}} \quad (15-6)$$

where  $\kappa$  is the constant specific to this reaction.

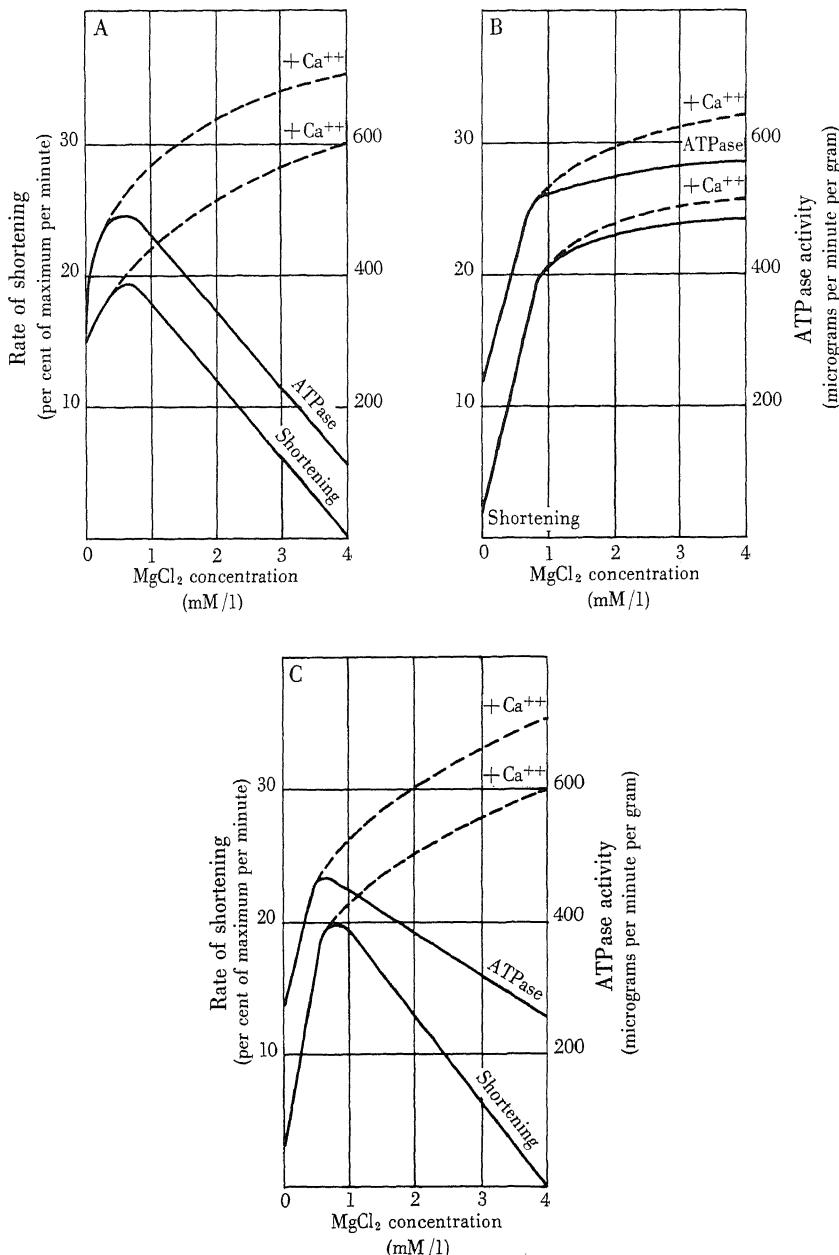
In Chapter 14, we presented our theoretical interpretation of enzyme activity in general; in the course of this presentation we considered several examples of the operation of the *F*-effect in a protein molecule containing an enzymatically active site separating two nonenzymatic sites as in our theoretical model (Figure

6.3). This pattern has also been observed experimentally by Bowen and Kerwin (1955), for the activity of myosin ATPase at different concentrations of uranyl chloride and KCl. Their curves, reproduced in Figure 15.14 are almost identical in shape with the theoretical curves based on the action of the *F*-effect in the three-site model system of Figure 6.3.



**Figure 15.14. THE EFFECT OF KCl CONCENTRATION ON THE INHIBITION OF ATPase ACTIVITY OF ACTOMYOSIN IN THE PRESENCE OF URANYL CHLORIDE.** The actomyosin (myosin B of Szent-Györgyi) was isolated from rabbit muscle. ATPase activity was studied at 26°C at pH 7.5. The concentration of ATP was 0.001M and of actomyosin, 0.05 mg/ml. The ordinate is given in micrograms of P liberated per milligram of myosin per test of 10 minutes duration. The numbers (0), (0.5), (1), (2), refer to concentrations of UO<sub>2</sub>Cl<sub>2</sub> in millimoles per liter. (Figure after Bowen and Kerwin, 1955.)

It has now been shown that both ATPase activity and the contraction-relaxation process are potentially capable of behaving in the complex manner predicted by Figure 6.3. The examples quoted, however, have been taken from somewhat different systems (myosin for ATPase and actomyosin or glycerinated fibers for contraction-relaxation) acted upon by different agents. A system exists in which the two phenomena, ATPase activity and contraction-relaxation, covary in response to the same adsorbents. In 1952, Marsh studied the dimensional and enzyme-activity changes in freshly broken muscle fibers. These fibers undergo a reversible swelling when ATP is added. However, if the broken fibers are first washed, a marked contraction occurs with the addition of ATP (Figure 15.13).



**Figure 15.15. THE PARALLEL CHANGE OF MECHANICAL BEHAVIOR AND ENZYME ACTIVITY IN GLYCEROL-EXTRACTED MUSCLE FIBERS.** Comparison of effects of  $Mg^{++}$ -ion concentration with and without 0.2mM  $CaCl_2$  on the ATPase activity and the rate of shortening of A, briefly washed fibers; B, extensively washed fibers; C, extensively washed fibers with a dialyzed factor. The rate of shortening is given as a per cent of the maximum shortening per minute and is the average rate for the first two minutes-of shortening. ATPase activity is given in micrograms of P liberated per minute per gram of fibers. (Figure after Bendall, 1953.)

It was found that the swelling effect depends on another factor which is removable by washing. Present in the original muscle juice, this factor was known as the Marsh-Bendall factor.\* In the presence of this factor, relaxation follows ATP addition and the fibers have a low ATPase activity; systems which have lost this factor respond to ATP by immediate contraction and demonstrate a tenfold increase in their ATPase activity (see Figure 15.15).

In the model system considered above, the  $\varepsilon^0$  value optimum for ATPase activity (or the  $c$ -,  $c'$ -value optimum) of the enzymatically active sites coincides with the  $c$ - and  $c'$ -values for the gang of polar groups involved in the formation of salt linkages. This relation is reminiscent of the similar covariance in protein denaturation where the all-or-none unfolding due to salt-linkage dissolution of a gang of polar groups parallels the change of the  $\varepsilon^0$  value of isolated sulphydryl groups leading to their unmasking. It should be cautioned that, as for SH-group unmasking, since this covariance between dimensional change and enzymatic activity involves the cooperation of more than one or two factors, we may expect that when other factors are altered, the covariation may disappear. This has, indeed, already been found in a number of cases (Morales, 1956; Feuer, 1954).

### 15.5. A Theoretical Model of the Contractile System of Living Muscles

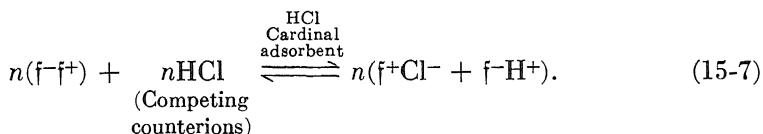
In this, the difficult and subtle task of presenting a model of muscular contraction as it occurs in the intact cell, we shall limit ourselves to a discussion of the reversible contraction-relaxation process and its control by cardinal adsorbents, since these are direct extensions of the present thesis.

Proteins are basically linear polymers. That they exhibit three-dimensional variation in size and shape has frequently been interpreted as a consequence of changes in the balance between macroscopic factors such as mutually repelling net electrical charges, which favor expansion, and random entropic coiling, which favors contraction. In model systems, at very low ionic strength, these factors play important roles (see Section 15.3). In Chapter 2, we showed theoretically why almost all fixed ions are coupled with counterions (additional experimental evidence has been presented in Chapters 9, 10, and 11). For this reason and because of the complete reversibility of the contractile process, which is not consistent with random coiling, we suggest that the dimensional changes of muscle cells are due to the reversible formation and dissolution of intermolecular and intramolecular bonds (for earlier views of the author, see Ling, 1952). Two types of bonds found in protein systems are readily reversible and are also abundant enough to fill this role; these are the salt linkages and the backbone hydrogen bonds. We shall limit

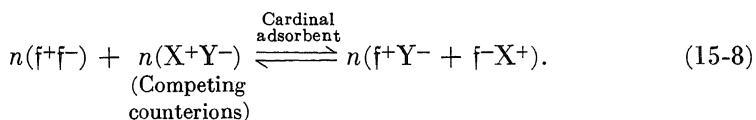
\* This factor, at one time thought to be myokinase (Bendall, 1954), proved not to be myokinase (see Bendall, 1958).

our discussion to the salt linkages because the basic principles involved are directly applicable to the H-bond system, a matter already considered in Section 7.4 in our comparison of urea denaturation of proteins with acid denaturation of proteins.

The fundamental contractile mechanism is thought to involve an indirect F-process (refer to the model shown in Figures 5.3 and 7.13) as in Steinhardt and Zaiser's experiment on acid denaturation of hemoglobin (1951, 1953). In that system, HCl served the dual function of the cardinal adsorbent (the four moles initially adsorbed) and the competing counterion (the 36 moles taken up subsequent to denaturation). The reversible process involving a gang of  $n$  pairs of sites may be written



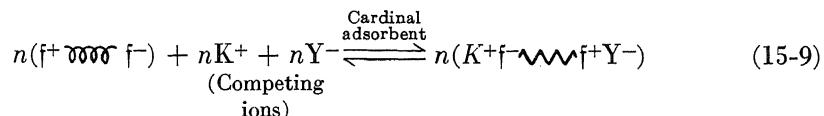
This all-or-none process is thus brought about by varying the cardinal adsorbent and the competing counterions simultaneously. More generally, the competing counterions and the cardinal adsorbents are not the same; in this case we can write the more general equation,



In Section 15.5A, we shall discuss muscular contraction, as it relates to this equation, in two parts: first, the reversible contractile process itself, which involves competition between counterions and, second, the cardinal adsorbents that control the contractile process.

#### A. THE CONTRACTILE PROCESS

In a living cell, there is little variation of the intracellular composition during each contraction cycle; therefore, the competing counterions in equation (15-8) cannot be varied by migration to or from the external medium. They must be part of the constant internal cellular content. In our theoretical model we suggest that these competing counterions are the  $K^+$  ion and an anion  $Y^-$ , most probably a fixed anionic group of high  $c$ -value on a different protein chain. Thus, the process may be described by the reaction,



where  $n$  refers to the number of pairs of sites in the gang.

We emphasize that, if such a reaction underlies all contractile phenomena, whether reversible as normal physiological contraction or irreversible as a response to a noxious agent, a shortening of the muscle tissue should always be accompanied by a release of  $K^+$  ion. We shall now present data to show that this is the case.

### (1) Normal contractions

(a) *Cardiac muscle.* Wilde and O'Brien (1953; also Wilde *et al.*, 1956) injected  $K^{42}$  into turtles. After equilibration, the excised cardiac ventricle was perfused with a nonradioactive Ringer's solution through the single coronary artery. The outflow from the cut ends of the coronary veins was continually monitored for radioactivity. The results showed that a distinct increase of  $K^{42}$  outflow accompanied each contraction of the ventricle (Figure 15.16).

(b) *Voluntary muscle.* Following the earlier work of Fenn (1936) and others, E. H. Wood *et al.* (1940) determined the  $K^+$ -ion content of blood samples returning from the perfused *gastrocnemius* muscle of a dog. Stimuli applied to the sciatic nerve elicited contraction of the muscle which was accompanied by a reversible loss of  $K^+$  ion (Figure 15.17).

Hardt and Fleckenstein (1949), in an extensive series of studies, extended these results to the lower vertebrates. They observed a close parallel between shortening and  $K^+$ -ion release from the muscles of frogs (Figures 15.18 and 15.19). The following is a free translation of portions of the authors' summary relevant to the present discussion.

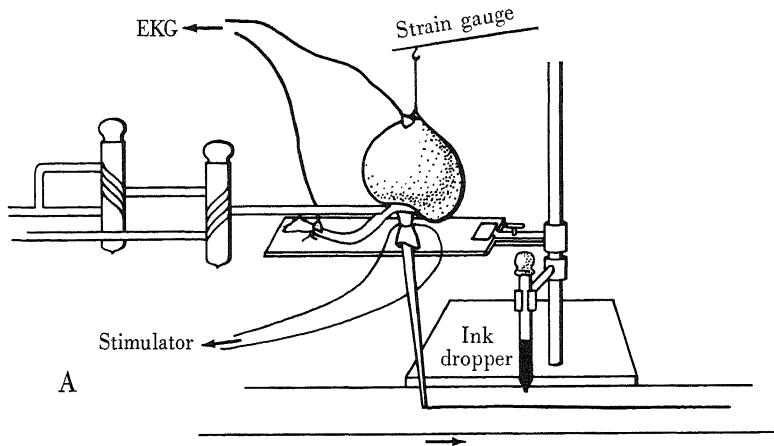
(i) The shortening wave coincides with the wave of  $K^+$ -ion liberation; (ii) as the amount of shortening increases, there is a regular increase in the amount of  $K^+$  ion liberated; (iii) local anesthetics inhibit both shortening and  $K^+$ -ion release; (iv) anodic polarization with direct current counteracts both the  $K^+$ -ion release and the shortening brought about by many substances.

Creese *et al.* (1958) found a substantial increase (23 per cent) in the rate of efflux of  $K^+$  ion from rat diaphragm in response to two stimuli per second (Figure 15.20).

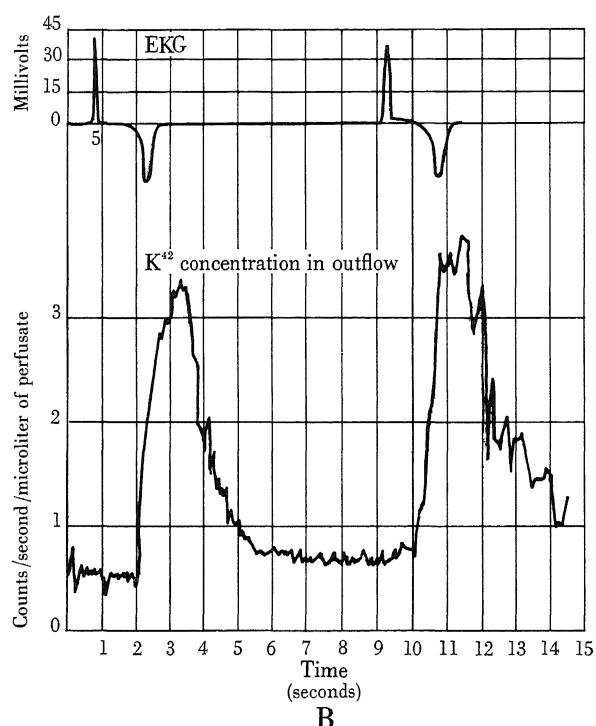
(c) *Smooth muscle.* Lembeck and Strobach (1955) studied the smooth muscle of cat small intestine and observed that, in response to a variety of agents, contraction of smooth muscle is regularly accompanied by increase of  $K^+$ -ion release.

### (2) Rigor of striated muscles

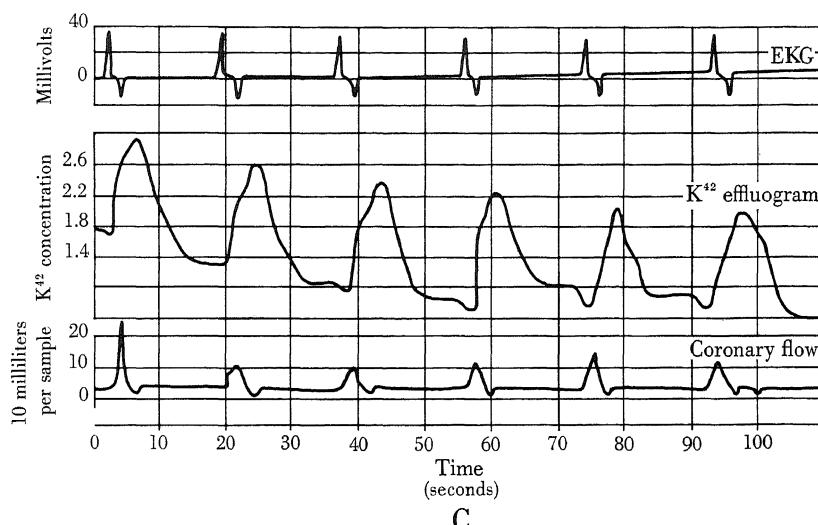
While the above-quoted instances are expressions of physiologically normal phenomena or, at least, phenomena that are totally reversible, the covariance of shortening and  $K^+$ -ion release is not limited to such examples. It obtains also in an irreversible shortening, usually referred to as *rigor*. The correlation between



A



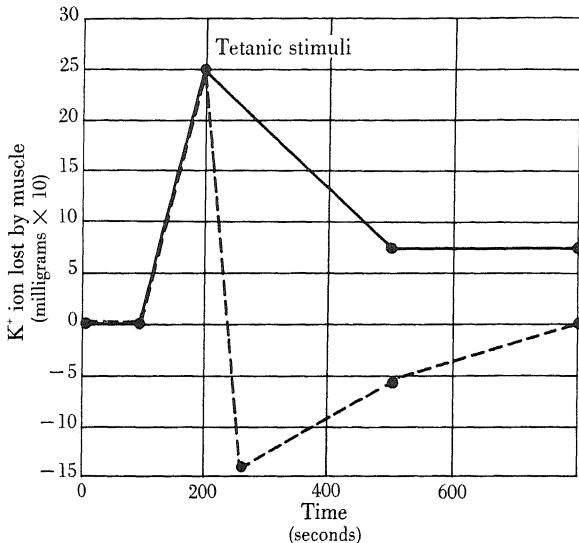
B



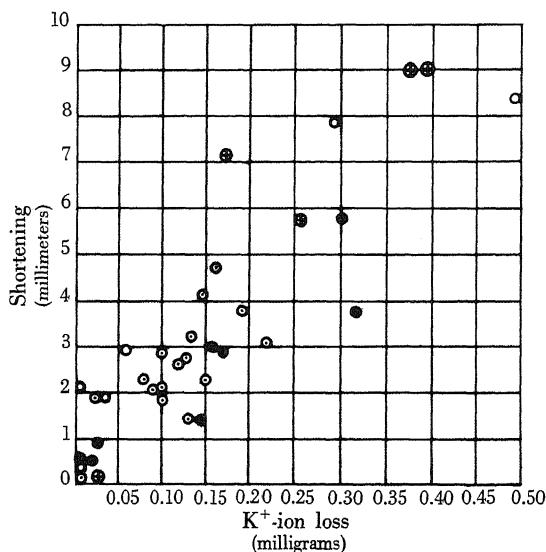
**Figure 15.16. THE INCREASED K<sup>+</sup>-ION EFFLUX ACCOMPANYING CONTRACTION OF THE TURTLE HEART.** A turtle heart previously equilibrated with highly radioactive K<sup>42</sup> was perfused with a nonradioactive Ringer's solution through its coronary vessels. The effluent was continually monitored for radioactivity. The rate of K<sup>42</sup> loss together with the electrocardiogram recording is plotted against time. A, the experimental apparatus; B, efflux during two contraction cycles; the first contraction was elicited by electric stimulus, the second was spontaneous. C, a record of six successive electrically elicited contraction cycles. (Figure after Wilde *et al.*, 1956.)

intracellular K<sup>+</sup> ion, ATP concentration, and shortening of voluntary muscles entering into rigor may be seen by comparing Figures 9.19 and 15.21 where the K<sup>+</sup>-ion concentration and the degree of contraction of the muscle cell are shown to vary with the ATP concentration. Supportive evidence is obtained from studies of the effect of temperature on the rate of K<sup>+</sup>-ion loss, muscle contraction, and ATP depletion (Figures 15.22, 15.23, and 15.24); in all, a similar relation prevails. The evidence clearly indicates that the K<sup>+</sup>-ion content, as well as shortening, is correlated with the concentration of ATP present and not with the rate at which ATP is degraded. This implies that ATP performs its appropriate function simply by its presence or absence as an adsorbent; energy changes associated with modifications in the molecule are mediated through differences in the energies of adsorption of ATP and competing anions.

While it appears that, with loss of ATP, muscles develop irreversible shortening, careful experimentation has revealed no significant change in the levels of ATP or CrP in normal muscle during reversible contractions (Fleckenstein, 1956; Mommaerts, 1955). By itself, this may merely indicate that the rate of resynthesis of these compounds matches the rate of their destruction. This seems probable if we consider that an earlier parallel

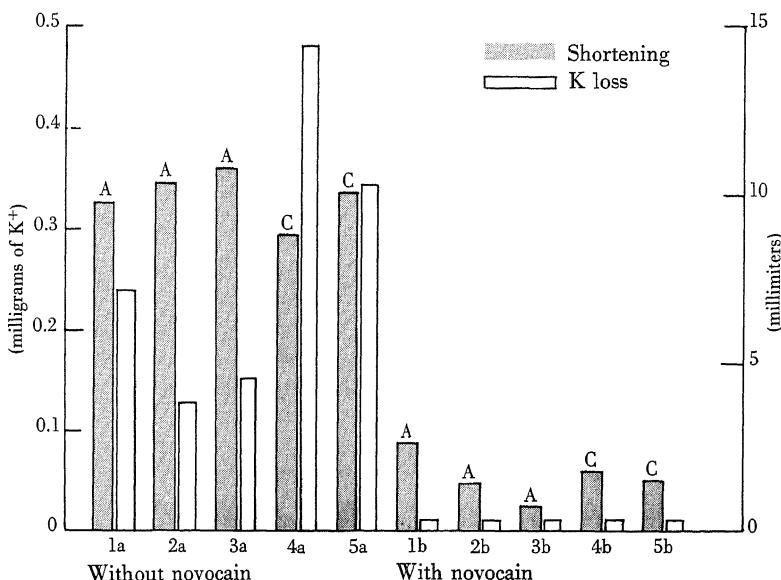


**Figure 15.17. THE LOSS OF  $K^+$  ION DURING STIMULATION AND ITS GAIN IMMEDIATELY FOLLOWING STIMULATION IN DOG *GASTROCNEMIUS* MUSCLE.** The  $K^+$ -ion gain and loss was determined by analyzing the  $K^+$ -ion content of blood circulating through the *gastrocnemius* muscle in the heart-lung *gastrocnemius* preparation. Stimulation was indirectly applied through the sciatic nerve. The solid line indicates  $K^+$ -ion loss by muscle; the broken line, rate of  $K^+$ -ion loss. (Figure after E. H. Wood *et al.*, 1940.)



**Figure 15.18. CORRELATION OF SHORTENING OF FROG *GASTROCNEMIUS* MUSCLE AND  $K^+$ -ION LOSS.** Contracture brought about by the action of lactic acid (○), sodium hydroxide (●), heat (○), and chloroform (+). (Figure after Hardt and Fleckenstein, 1949.)

finding, that there is no change in the rate of turnover of the terminal phosphate of ATP under the same conditions (Sacks, 1953, p. 182; Fleckenstein, 1956), proved to be in error. This error arose from the assumption that the cell interior offers no effective barrier to the diffusion of  $P^{32}$ -labeled phosphate ions. Fleckenstein *et al.* (1960) concluded that there are "powerful diffusion barriers" in the cytoplasm for these ions; his finding might be anticipated from the present thesis that the cytoplasm represents a fixed-charge

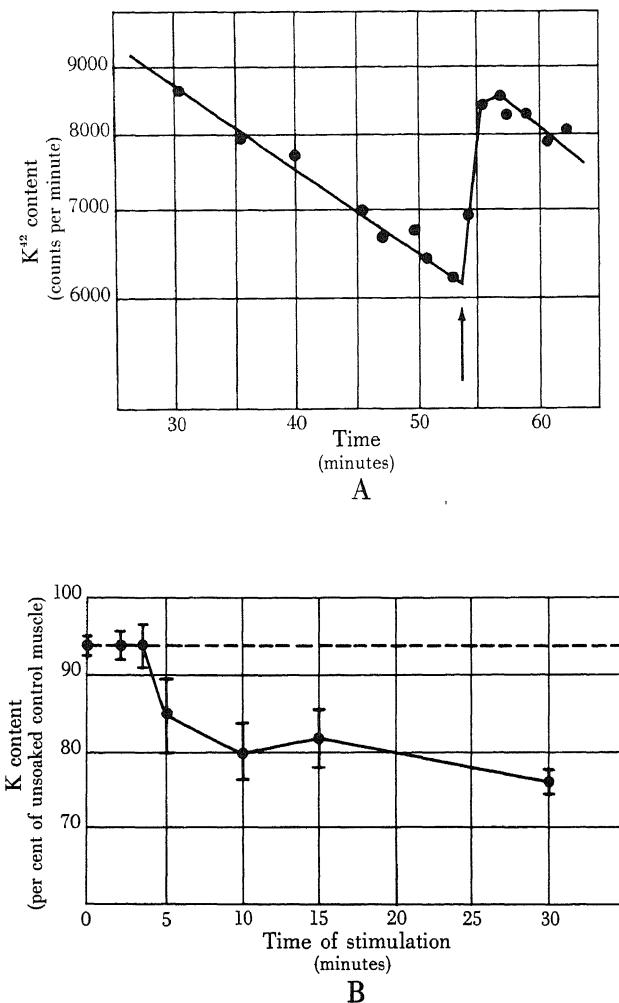


**Figure 15.19. THE PARALLEL BETWEEN K<sup>+</sup>-ION LOSS AND SHORTENING OF FROG GASTROCNEMIUS MUSCLE IN RESPONSE TO DRUGS.** Here the parallel is not limited to the positive action of caffeine and Avertin (tribromoethanol), but extends also to the protective action of Novocain (procaine) against these drugs. Shortening in millimeters; K<sup>+</sup>-ion loss in milligrams. A, Avertin; C, caffeine, both at a concentration of  $5 \times 10^{-3}$ ; Novocain (procaine),  $10^{-2}$ . (Figure after Hardt and Fleckenstein, 1949.)

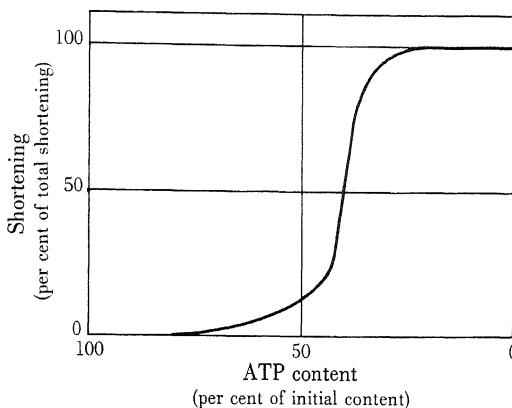
system. Using O<sup>18</sup>-labeled water, these authors showed that the turnover of the terminal phosphate of ATP is, in fact, intensified by muscular activity.\*

The problem of the energy source for the normal muscular-contraction cycle will be considered in detail in a future publication. Here we may merely point out that the basic mechanism as we now envision it is reminiscent of the mechanism by which acetylcholine creates a reversible electrical potential change. The contractile cycle begins with a change in the surface of the cell during excitation (see Sections 13.1 and 15.5B); this leads, by the *F*-effect, to the release of a metabolic product *M* (at present, ATP is the most likely

\* Considering the nature of the fixed-charge system, it is possible that the diffusion of water may, in itself, be the rate-limiting factor in attempts to measure very rapid chemical reactions occurring at fixed sites.



**Figure 15.20. THE INCREASE OF  $K^+$ -ION EFFLUX FROM STIMULATED RAT DIAPHRAGM MUSCLE.** A, loss of radioactive  $K^{42}$  from diaphragm muscle previously equilibrated with the isotope was monitored during continuous washing with a nonradioactive Ringer's solution. The arrow marks the onset of electrical stimulation which elicited an immediate increase in the rate of outward  $K^{42}$  flux. B, The effect of stimulation on the potassium content of rat hemidiaphragm. The value at the zero line is the average of 10 muscles which were soaked and not stimulated. Other points are the average of four muscles. The limits are  $2 \times S.E.$  (Figure after Creese *et al.*, 1958.)

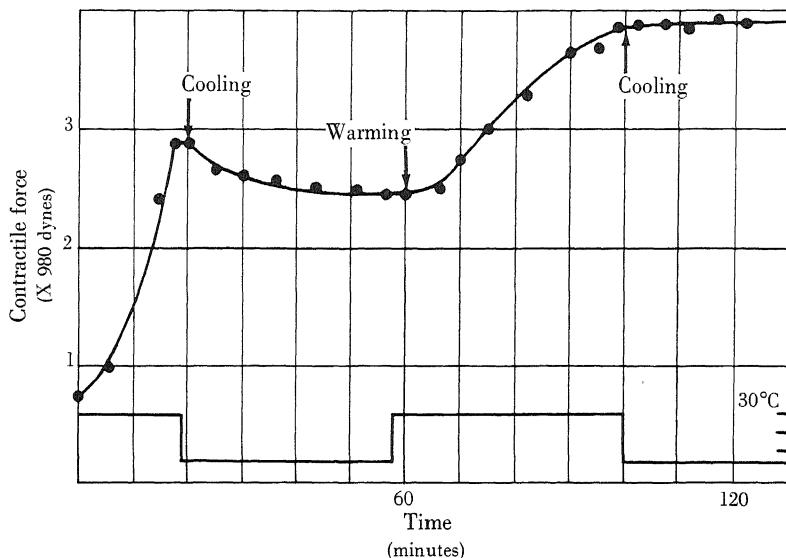


**Figure 15.21. A PLOT OF THE SHORTENING OF MUSCLES AGAINST THEIR ATP CONTENT.** Shortening studies and ATP analyses were performed on different fiber bundles from the same *psoas* muscles of rabbits. The ultimate pH of the muscle was 5.80. Note the general resemblance of this curve to Figure 9.19, except that the last 25 per cent or so of ATP appears to have no effect on shortening although it is the last 20 per cent or so of ATP in frog muscle that critically determines the  $K^+$ -ion accumulation and shortening. However, Bendall analyzed ATP with the assumption that a seven-minute hydrolysis in dilute acid dephosphorylates no compound other than ATP; there is good reason to suspect that a considerable amount of hexosephosphates and ADP would accumulate near the end of the rigor development. If so, the 25 per cent ineffectual ATP content shown above may be only apparent. (Figure after Bendall, 1951.)

candidate for this role), from a site where it is adsorbed when the cell is at rest. Adsorption of  $M$  onto a different cardinal site, probably on another protein molecule, triggers an all-or-none change in the adsorbents on an autocooperative gang of alternately cationic and anionic sites (Figure 15.6). In this exchange salt linkages are formed, leading to shortening of the muscle. The cardinal site, at least under the new conditions, is enzymatically active and rapidly splits  $M$  into two or more products which are not preferred at the cardinal site and are desorbed. The sites of the gang then revert to their original preference for free ions and lengthening occurs. In this cycle, metabolic energy is utilized to resynthesize the split products. Once resynthesized,  $M$  reoccupies the site of adsorption it occupied when the cell was at rest.

We have seen that there is, with few exceptions, a correlation between shortening of muscle tissue and release of  $K^+$  ion as is demanded by equation (15-9). The prevalent interpretation of this observation is based on the postulate of Hodgkin and Huxley (1952a) that there is a period of increased permeability to the  $K^+$  ion during the passage of the action potential. If an increase in permeability is the true cause of  $K^+$ -ion release, then increased  $K^+$ -ion exchange should be demonstrable in the inward as well as in the outward  $K^+$ -ion flow; there is no evidence of increase in the inward permeability of muscle under identical condi-

tions (Noonan *et al.*, 1941; Creese *et al.*, 1958); see Table 15.2. This precludes a change of permeability constant as a cause of  $K^+$ -ion liberation. This outward movement of  $K^+$  ion cannot be accounted for by a transient displacement of  $K^+$  ion by the entry of  $Na^+$  ion because the loss of  $K^+$  ion is not accompanied by significant increase in the intracellular  $Na^+$ -ion content. This observation of

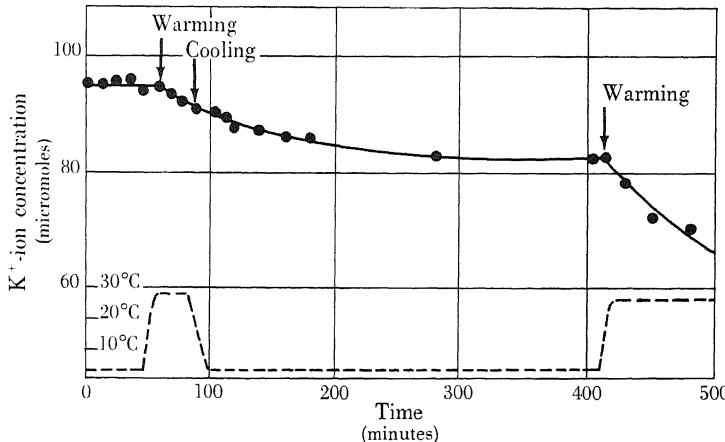


**Figure 15.22. EFFECT OF TEMPERATURE ON THE CONTRACTILE FORCE DEVELOPED BY A FROG SARTORIUS MUSCLE POISONED WITH IODOACETATE.** The contractile force was recorded with an isometric lever calibrated against known weights. The lower curve indicates the variation of temperature; the upper, the variation in contractile force. Muscle weight, 140 milligrams. The concentration of iodoacetate used was five millimoles per liter. A curve essentially like this is obtained when isotonic shortening is plotted against time. (Figure after Ling, 1952.)

Creese, derived from studies of mammalian muscle, has been confirmed by our comparable studies on frog voluntary muscles,\* the results of which are shown in Table 15.3. Stimulation of these muscles at five stimuli per second for 15 minutes leads to a consistent reduction of about 10 per cent in the intracellular  $K^+$ -ion

\* Early work on ionic changes during contraction was performed on intact animals. Such studies led to the conclusion that there was a large exchange of intracellular  $K^+$  ion for external  $Na^+$  ion (for review see Fenn, 1940). Later work of Noonan, Fenn, and Haege (1941) proved that the earlier conclusion of a large increase of inward  $K^+$ -ion permeability was erroneous; the observed changes arose primarily from vascular responses in the intact animals studied. Judging from the observation of Creese and our own work (Table 15.3), vascular changes might also be responsible for the earlier observations of  $Na^+$ -ion-concentration increase.

concentration, accompanied only by an insignificant gain and loss of  $\text{Na}^+$  ion in all experiments except A in which the stimulated muscle was found to be in contracture at the end of the experiment. Even here, there was only a partial exchange of  $\text{K}^+$  ion for  $\text{Na}^+$  ion.\*

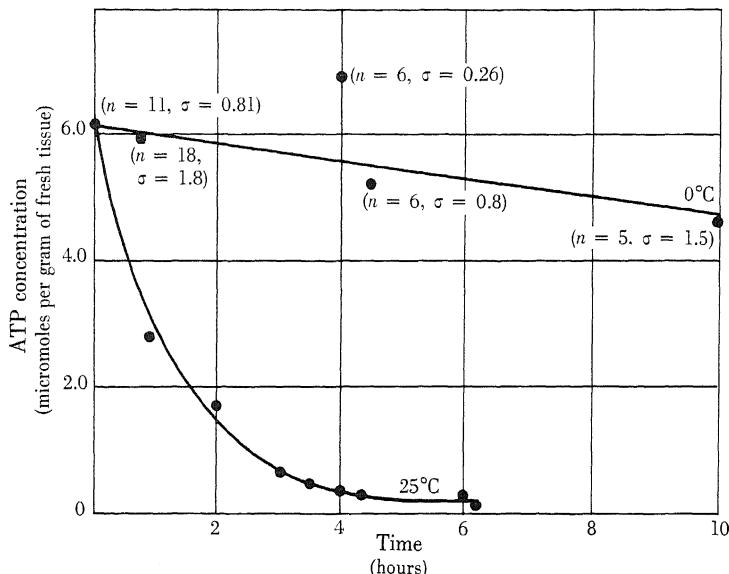


**Figure 15.23. EFFECT OF TEMPERATURE ON THE LOSS OF  $\text{K}^+$  ION FROM FROG MUSCLES POISONED WITH IODOACETATE.** Potassium content in micromoles was found for the group of small muscles weighing in total 1.15 grams. Muscles were kept in Ringer's solution containing 5mM iodoacetate and 1mM sodium cyanide. The potassium content of aliquots of the bathing solution was determined by flame photometry. Warming caused rapid potassium loss; cooling greatly slowed this after a delay. Such a delay may be only apparent because time would be needed for the potassium already lost, or in the process of being lost, upon warming, to diffuse out through the extracellular space into the bathing solution. (Figure after Ling, 1952.)

It is now clear that the loss of  $\text{K}^+$  ion from muscle during contraction can be the result of neither a change in surface permeability to the  $\text{K}^+$  ion nor an increased  $\text{Na}^+$ -ion permeability leading to a  $\text{K}^+-\text{Na}^+$  ion exchange. Still another explanation possible is that a portion of the intracellular  $\text{K}^+$  ion previously "bound" within the muscle is suddenly made available for outward diffusion. Such a phenomenon can readily be explained by the principle of the variability of the preferred counterion. Thus, through an excitatory process, the counterion

\* In order to preserve macroscopic electric neutrality, this  $\text{K}^+$ -ion loss, uncompensated by an equivalent  $\text{Na}^+$ -ion gain, must be accompanied by a parallel loss of intracellular anions. Recent findings of Abood, Koketsu, and Koyama (1961) suggest that these anions include creatine phosphate and ATP (see Ling, 1952, p. 761, for other significance of these findings). This footnote was added to proof on March 30, 1962.

on a certain cardinal site is altered and an indirect *F*-process occurs during which the fixed anionic sites change their preference for the  $K^+$  ion to a preference for the fixed cations; salt-linkage formation follows and the released  $K^+$  ion is, at



**Figure 15.24. THE EFFECT OF TEMPERATURE ON THE ATP CONTENT OF IAA-POISONED FROG VOLUNTARY MUSCLES.** The data for 25°C were obtained from a single set of experiments; those for 0°C represent data collected over several years. The  $n$ , in brackets, indicates the number of individual determinations represented by each point;  $\sigma$ , the standard deviation (see also Samson *et al.*, 1959 for similar observations on cerebral ATP).

least temporarily, free to exchange with external  $K^+$  ion or to diffuse out of the muscle.

#### B. CONTROL OF THE CONTRACTILE PROCESS BY CARDINAL ADSORBENTS

In discussing our equation of contraction [equation (15-9)], we pointed out how the autocooperative changes underlying the contraction cycle as described in the preceding section are controlled, in an all-or-none fashion, by adsorption onto cardinal sites. Judging from the results of experiments determining the effects of anions and cations on intact muscle cells, some of these cardinal sites appear to be cytologically located close to the cell surface.

## (1) Monovalent anions as cardinal adsorbents

The work of Chao (1935) and of Kahn and Sandow (1950, 1951) has focused attention on the important effect of anions in increasing the twitch tension of muscles without affecting the action potential. The effect of these anions is very rapid and has been interpreted as taking place near the cell surface (A. V. Hill and MacPherson, 1954; see also Figure 15.25). The magnitude of the effects of

Animal	Muscle type	Control and experimen- tal muscles	Duration of stimulation, min	No. of stimuli per sec	$K^{42}$ uptake, %	Source
Frog	<i>sartorius</i>	control (1)	—	—	9.4	Noonan <i>et al.</i> , 1941
		experimental (1)	60	1.1	11.3	
	<i>semilendinosus</i>	control (1)	—	—	13.2	
		experimental (1)	60	1.1	12.4	
	<i>sartorius</i>	control (1)	—	—	13.5	
		experimental (1)	90	1.0	14.0	
	<i>semilendinosus</i>	control (1)	—	—	12.4	
		experimental (1)	90	1.0	12.7	
	<i>peroneus</i>	control (1)	—	—	7.2	
		experimental (1)	90	1.0	6.7	
Rat	diaphragm	control (6)	—	—	10.7 ± 0.56	Creese <i>et al.</i> , 1958
		experimental (7)	5	5.0	11.0 ± 0.88	
	diaphragm	control (7)	—	—	20.6 ± 2.13	
		experimental (7)	10	5.0	20.4 ± 1.36	

**Table 15.2. THE LACK OF CHANGE IN THE RATE OF  $K^{42}$  ENTRY INTO STIMULATED MUSCLES.** The  $K^{42}$  uptake is expressed as a per cent of intracellular  $K^+$ -ion content. Data from Creese *et al.* include the mean values as well as their standard errors. The numbers of individual experiments are indicated in parentheses. (Data from Noonan *et al.*, 1941; Creese *et al.*, 1958.)

the ions studied follows the familiar order,\* SCN > I > NO<sub>3</sub> > Br > Cl (Figure 15.26), which appeared for the adsorption energies of these monovalent anions onto the amino-type cationic sites (Section 7.4D). This leads us to suggest that one type of cardinal site controlling the all-or-none cooperative contractile process is an arginine or lysine side chain on a protein close to the cell surface and that successful adsorption of a free anion onto this site following excitation enhances the autocooperative changes of the gang of sites of the same or closely associated protein molecules to the shortened state. From this we may begin to

\* Chao, 1935; Kahn and Sandow, 1951; Hill and MacPherson, 1954; Hodgkin and Horowicz, 1960.

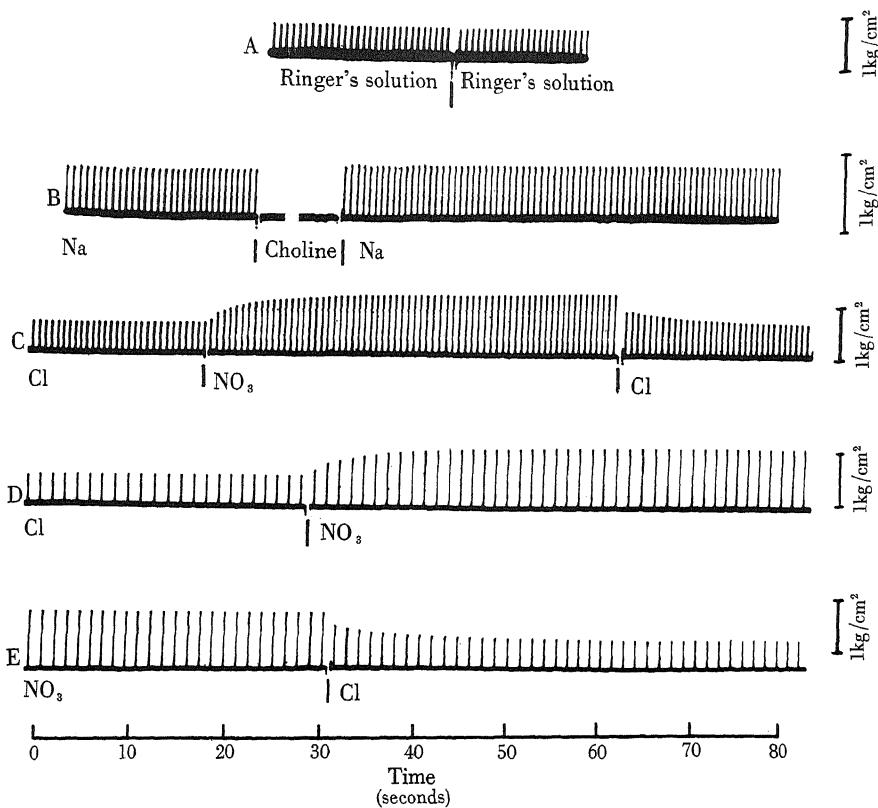
Experiment	Muscle type	Muscle weight, mg		[K] <sub>in</sub> , $\mu\text{M/g}$	$\Delta[\text{K}]_{\text{in}}$ , $\mu\text{M/g}$	[Na] <sub>in</sub> , $\mu\text{M/g}$	$\Delta[\text{Na}]_{\text{in}}$ , $\mu\text{M/g}$
A	1	57.4	control	98.34	-25.97	16.50	+12.31
	1	58.6	experimental	72.37		28.81	
B	2	63.6	control	95.04	-7.21	14.56	-0.04
	2	65.4	experimental	87.83		14.52	
C	3	56.3	control	94.24	-12.70	14.78	+1.02
	3	60.0	experimental	81.54		15.80	
D	1	36.2	control	99.93	-8.99	19.06	-4.23
	1	38.4	experimental	90.94		14.83	
E	2	50.2	control	96.09	-12.42	16.70	+0.22
	2	50.0	experimental	83.67		16.92	
F	3	44.0	control	90.72	-16.85	17.11	+3.30
	3	47.0	experimental	73.87		20.41	

**Table 15.3. ION EXCHANGE IN STIMULATED FROG VOLUNTARY MUSCLES.** Frog muscles (1, *sartorius*; 2, *semitendinosus*; 3, *tibialis anticus longus*) were first equilibrated at room temperature for one hour in a Ringer's-bicarbonate solution bubbled with 5% CO<sub>2</sub> + 95% O<sub>2</sub> in which one half of the normal NaCl content had been substituted by sucrose. Transferred to fresh solution of the same composition, the experimental muscles were stimulated at five stimuli per second for 15 minutes. The K<sup>+</sup>- and Na<sup>+</sup>-ion contents of both experimental and control muscles were analyzed by flame photometry. [K]<sub>in</sub> refers to intracellular K<sup>+</sup>-ion concentration expressed in micromoles of potassium per gram fresh weight of muscles obtained following the experimental period.  $\Delta[\text{K}]_{\text{in}}$  refers to changes of intracellular K<sup>+</sup>-ion concentration. All muscles remained normal in appearance except the experimental muscle in A which was found to be in contracture at the end of the experiment.

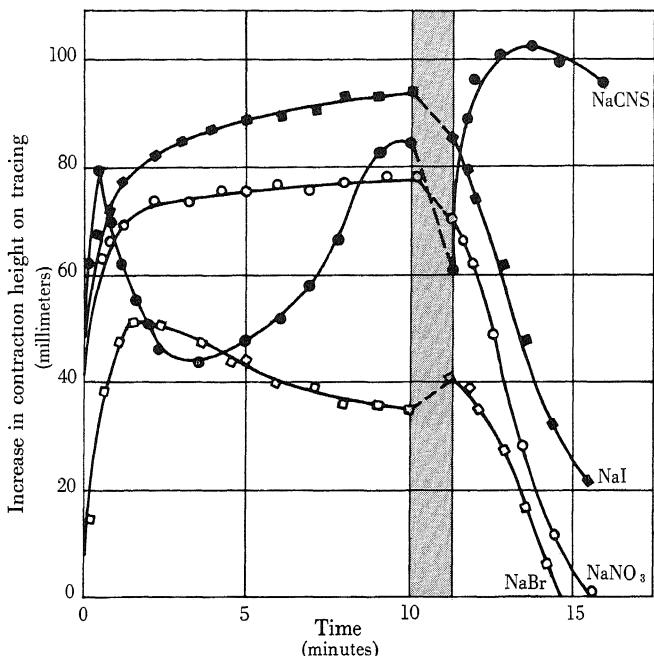
understand the role of the anion in determining the contractile strength. Thus, the greater the adsorption energy of the anion, the greater the statistical number of gangs entering the contractile state would be; the greater the number of gangs in the shortened state, the greater the twitch tension would be.

## (2) The sodium ion as a cardinal adsorbent

The sodium ion affects muscle contractility in at least two ways. First, it creates the action potential which is propagated along the surface of the muscle fiber (this effect was examined in Chapters 10 and 13). A second role is more direct. Axelsson and Bülbring (1959; see also Axelsson, 1960) have shown that the action potential and the contractile process may be dissociated in the smooth muscle from guinea pig *taenia coli*. Thus lithium, choline, hydrazinium, or lithium-ethane sulfonate can, at least for a limited period of time, effectively substitute for the Na<sup>+</sup> ion or for NaCl in maintaining spontaneous action potentials. Yet under these condi-



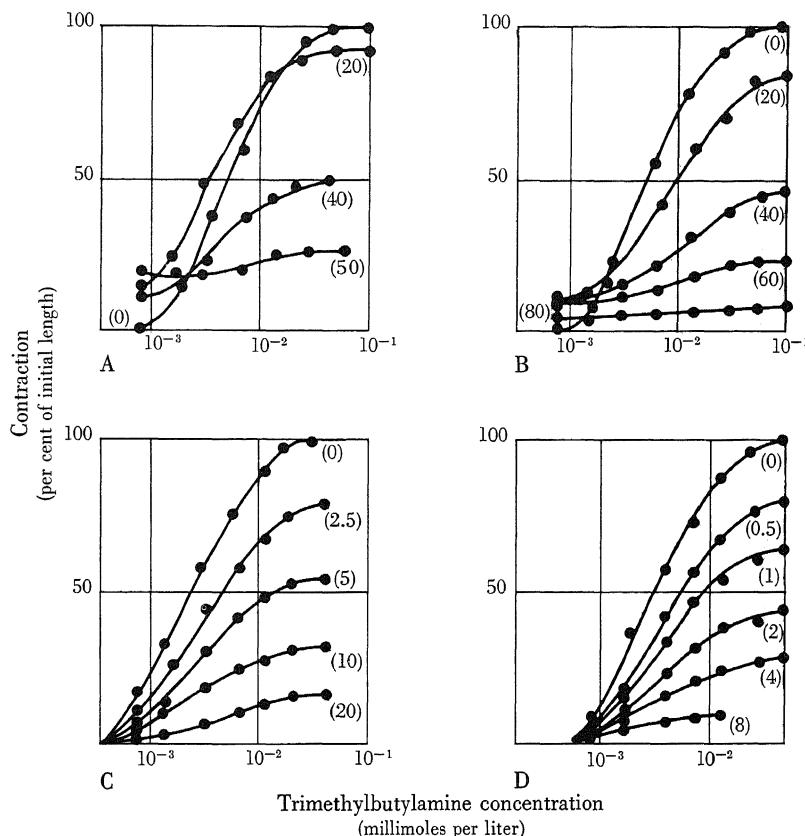
**Figure 15.25. THE EFFECT OF SUDDEN CHANGES IN SOLUTION ON THE TWITCH TENSION OF SINGLE MUSCLE FIBERS.** Solutions were changed by forcing 2.0ml through the chamber containing the fiber in about 0.4sec; the gaps in the record were caused by the rapid flow. A, the effect of control flush in which the solution already in the chamber (Ringer's solution) was replaced with the same solution; stimulation frequency, 1.56/sec. B, the effect of replacing Ringer's solution containing 120mM Na<sup>+</sup> ion with similar solution containing 115mM choline and 5mM Na<sup>+</sup> ion; frequency, 1.56/sec. C, the effect of replacing Ringer's solution containing 121mM Cl<sup>-</sup> ion with similar solution containing 117.5mM NO<sub>3</sub><sup>-</sup> ion and 3.6mM Cl<sup>-</sup> ion; stimulation frequency, 1.56/sec. D and E, consecutive records similar to C but with stimulation frequency of 0.78/sec. The records were taken in the order, C, D, E, B, A; shortly before C, the tetanus tension was 2.9 kg/cm<sup>2</sup>. The fiber diameter was 122 $\mu$ ; the fiber was stretched to 1.8 times slack length; temperature, 19°C. Note the instantaneous effect of changes of Na<sup>+</sup>-ion concentration, B, in contrast to the somewhat slower but still extremely rapid response to changes of monovalent anions, compared with the anionic rate of ion exchange between intracellular and extracellular phases. (The time constant for Cl<sup>-</sup>-ion exchange was found to be 15 to 55 hours for squid axons, Tasaki *et al.*, 1961.) (Figure from Hodgkin and Horowicz, 1960.)



**Figure 15.26. THE EFFECT OF ANIONS ON THE CONTRACTION HEIGHT OF FROG *SARTORIUS* MUSCLES.** Nerve-free portions of these muscles were stimulated electrically using a block-type fluid electrode at a uniform submaximal stimulus. After the muscles had been immersed in the experimental solution for 10 minutes, the solutions were replaced by a normal Ringer's solution and stimulation was applied once more (the shaded area indicates time for changing solutions). The base line indicates the level of contraction in normal Ringer's solution before contact with the experimental solutions. (Figure after Chao, 1935.)

tions no tension develops. These authors concluded that the  $\text{Na}^+$  ion plays an essential part in the activation of the contractile mechanism by the action potential. We suggest that the surface anionic sites whose adsorption of  $\text{Na}^+$  ion during activation leads to the creation of the action potential may, themselves, be the cardinal sites for contraction-relaxation phenomena. Adsorption of  $\text{Na}^+$  ion onto these sites leads to the cooperative process of shortening, according to equation (15-9).\*

\* It has long been suspected that the  $\text{Ca}^{++}$  ion may play an important role in the excitation of contractile responses. It has been suggested that release of  $\text{Ca}^{++}$  triggers contractions (Heilbrunn, 1952; Heilbrunn and Wiercinski, 1947; Morales and Botts, 1956; Frank, 1960). Although we agree with these authors in assigning an important role to the  $\text{Ca}^{++}$  ion, the proposed mechanism is different. We suggest that  $\text{Ca}^{++}$  is a cardinal adsorbent whose adsorption onto the cardinal sites is a necessary condition for the preservation of the proper *c*-value ensemble for the all-or-none contractile cycle. There is considerable evidence in agreement with this view. Thus, recently Frank has shown that depletion of  $\text{Ca}^{++}$  inhibits the contraction of muscle induced under ordinary circumstances by high concentrations of KCl (1960).



**Figure 15.27. THE SHORTENING OF FROG *RECTUS ABDOMINIS* MUSCLE IN RESPONSE TO COMBINED ACTION OF TRIMETHYLBUTYLAMINE WITH HIGHER TRIMETHYLALKYLAMINES.** The numbers in the graph refer to concentration of the higher trimethylalkylamines in  $\gamma$  per 10 milliliters of solution. The higher amines are: A, trimethyloctylamine; B, trimethyl-nonylamine; C, trimethyldecylamine; D, trimethyldodecylamine. (Figure after Ariëns *et al.*, 1954.)

From the actions of anions and the  $\text{Na}^+$  ion, we may begin to understand better the control of cellular contraction as well as other responses of cells by physiological and pharmacological agents. For example, a large variety of drug-interaction patterns ably elucidated by Ariëns and co-workers for frog *rectus abdominis* muscle (Figure 15.27) show various types of interesting curves similar to those given in Chapter 6 (Figures 6.2 to 6.4). Such patterns are conceivable only if the sites reacting with the drug molecules are close to or on the cell surfaces; otherwise, the reaction pattern would be hopelessly complicated by the divergent diffusion coefficients of the different drugs studied. To achieve quick responses to external and internal exigencies, it would be most logical to locate the controlling sites of muscle and other effector cells close to the mainstream of general information,

whether this is the blood vascular system or the nerve endings. The effect on the mechanism within the cell could then proceed in functional unity.

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# 16

## THE ACTION OF HORMONES AND DRUGS

- 16.1. The General Effect of Hormones on the *c*-Value Ensemble 458
- 16.2. The Action of Some Pharmacological Agents 461
  - A. The apparent paradox of digitalis action 462
  - B. Histamine liberators and trigger action 466
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The polypeptide chain permits interaction among neighboring sites on the protein molecule; this intimate interaction, in turn, provides the foundation for the cooperative changes which we have postulated are basic to all major physiological activities. Cooperative changes of state are, however, quite commonly observed in the inanimate world (see Temperley, 1956). The uniquely characteristic feature of the living state is that cooperative changes are controlled by events occurring at a relatively small number of specific sites, the sites we have called cardinal. The behavior of the living system as a unit is achieved by coordinated control of functional adsorbents at these cardinal sites. An example of such control has already been presented in the chapter on excitation and inhibition in which we discussed the actions of acetylcholine and curare. In our discussion of the effect of the polypeptide, poly-L-lysine, we noted that an extremely small amount of this polypeptide is needed to produce a profound effect on cells. Its action finds a close

parallel in the action of hormones; many of these are also polypeptides which act at very low concentrations.

In the present chapter we shall attempt briefly to do two things. First, we shall relate, in a general way, all hormonal action to the association-induction hypothesis. Second, we shall show, by three examples, how the complex patterns of action of pharmacological agents follow naturally from the present theory.

### 16.1. The General Effect of Hormones on the *c*-Value Ensemble

Hormones, like other bioregulants, are capable of inducing profound cellular responses when only a very small number of molecules is present. Here, the biological amplifier and transmitter action are strikingly exemplified. That hormones can be adsorbed onto proteins is well established.\* The present theory suggests that the hormones affect their target cells through *F*-effects created by their adsorption onto the cell proteins and the consequent cellular *c*-value-ensemble change. If hormones interact with the proteins of the target organs, and if the same proteins also function as the fixed-charge systems for ionic accumulation, a shift of the labile K<sup>+</sup>-ion-Na<sup>+</sup>-ion balance in the intracellular ionic distribution is to be expected as a result of hormone adsorption. In Table 16.1, the effects of a number of hormones on the concentration of cellular K<sup>+</sup> ions are tabulated to show that this is indeed the case. These data indicate that a K<sup>+</sup>-ion shift is generally observed as a consequence of hormone action. Such a shift bespeaks a *c*-value variation in response to hormones.

In Figures 16.1 and 16.2, results of experiments specifically designed to test the above hypothesis are presented. Virgin rats were given 1.0 microgram of estradiol in cottonseed oil while control rats, the litter mates of the experimentals, received only cottonseed oil. After 28 and 48 hours, the rats were sacrificed. The partition ratios of the previously injected radioactive isotopes, Na<sup>24</sup>, K<sup>42</sup>, Rb<sup>86</sup>, and Cs<sup>137</sup>, between the uterine tissue and the plasma were compared. The data showed an unequivocal increase of the modal *c*-value from a primary preference for Cs<sup>+</sup> to a preference for Rb<sup>+</sup>, thus supporting the hypothesis that hormones function through their *F*-effects (Ling and Ochsenfeld, unpublished work).

S. B. Horowitz (1959, also unpublished work) studied the effect of insulin on the relative uptake of four alkali-metal ions in isolated frog muscles. The results, shown in Figure 16.2, revealed a marked increase of Rb<sup>+</sup>-ion accumulation at the expense of the Na<sup>+</sup> ion. Since the ratio of the muscle dry weight to wet weight changed very little, no significant alteration of the extracellular space could have

\* Some examples are as follows, Insulin: Chayen and Smith, 1954; Stadie, 1951; testosterone: Levedahl and Perlmutter, 1956; Trevorrow, 1939; estrogen: Szego and Wolcott, 1953; progesterone, desoxycorticosterone, and cortisone: Westphal, 1957; other steroids: Sandberg *et al.*, 1957.

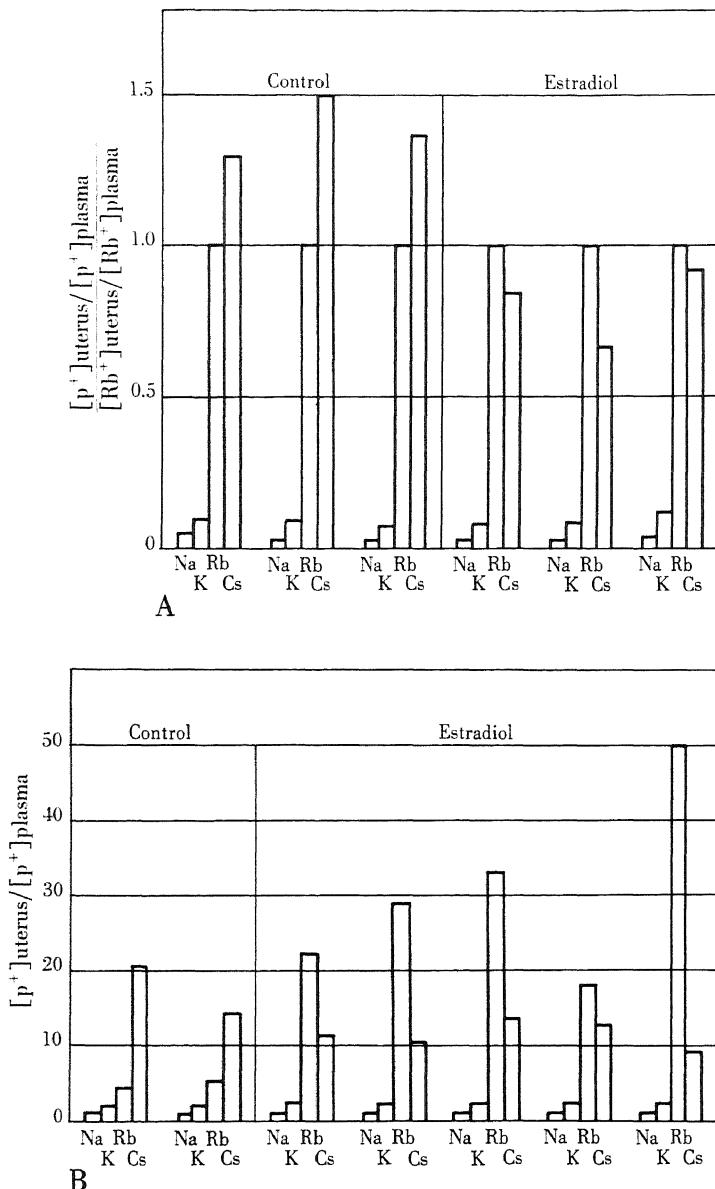
occurred. The  $\text{Rb}^+ - \text{Na}^+$  shift must, therefore, reflect a change of the preference for these two ions; such a change of alkali-metal-ion preference can be used to support the concept that a  $c$ -value-ensemble change of the fixed ionic groups is produced by insulin.

The work of Cafruny and co-workers (1957) demonstrated that the protein SH groups in kidney tubule cells show an increase or a decrease of reactivity in response to hypophysectomy and to the administration of growth hormone, thyroxin, thyrotropin, desoxycorticosterone, and pitressin (Table 16.2). Similarly, Ungar and Kadis (1959) showed an increase in the titratable SH groups of muscle protein in response to insulin. Both groups of experiments are consistent with the concept that a change in the  $\delta^\circ$  value of SH groups is brought about by hormone

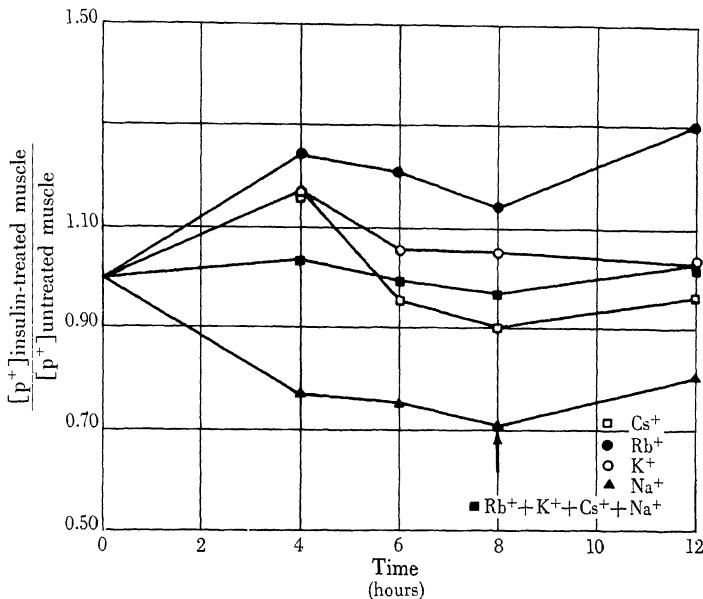
Hormone	Tissue	Cellular $\text{K}^+$ ion increased or decreased	Reference
Adrenocorticotropin (ACTH)	skeletal muscle	decreased	Gris, 1953
	heart muscle	decreased	
	liver	decreased	
	skeletal muscle	decreased	Eliel <i>et al.</i> , 1951
	lymphoid tumor	decreased	
	erythrocytes	decreased	
Growth	skeletal muscle	increased	Batts <i>et al.</i> , 1954
Pitressin	into extracellular fluid	decreased	Friedman <i>et al.</i> , 1956
Cortisone	skeletal muscle	decreased	Einbinder <i>et al.</i> , 1954
Desoxycorticosterone (DOCA)	skeletal muscle	decreased	Darrow <i>et al.</i> , 1948
			Muntwyler <i>et al.</i> , 1950
			Gardner <i>et al.</i> , 1954
Thyroxine	skeletal muscle	decreased	Scopinaro <i>et al.</i> , 1954a
	ventricular muscle	decreased	
	auricular muscle	increased	
Parathyroid	skeletal muscle	decreased	Scopinaro <i>et al.</i> , 1954b
Insulin	skeletal muscle	increased	Kamminga <i>et al.</i> , 1950
Epinephrine	skeletal muscle	increased	S. B. Horowitz, 1959
	liver	decreased	Goffart and Brown, 1947
			Ling, 1952
Estrogen	uterine muscle	increased	D'Silva, 1934, 1937
Relaxin	pubic symphysis	decreased	Marenzi and Gerschman, 1936
Histamine	intestine	decreased	Horvath, 1954
			Talbot <i>et al.</i> , 1940
			Engel <i>et al.</i> , 1954a,b
			Parrot <i>et al.</i> , 1953

Table 16.1. THE GENERAL EFFECT OF HORMONES ON THE CELLULAR  $\text{K}^+$ -ION CONTENT.

<sup>a</sup> Restores normal  $\text{K}^+$ -ion concentration in hypophysectomized animals.



**Figure 16.1. THE EFFECT OF ESTRADIOL ON THE UPTAKE OF  $\text{Na}^{24}$ ,  $\text{K}^{42}$ ,  $\text{Rb}^{86}$ , AND  $\text{Cs}^{137}$  BY RAT UTERUS.** Virgin rats were injected subcutaneously with  $1.0\mu\text{g}$  of estradiol in cottonseed oil simultaneously with the intraperitoneal injection of  $\text{Na}^{24}$ ,  $\text{K}^{42}$ ,  $\text{Rb}^{86}$ , and  $\text{Cs}^{137}$ . Controls were injected with cottonseed oil and the isotopes. The concentrations of the isotopes in the uterus and in the blood plasma were determined at the specified time using a multichannel pulse-height analyzer. A, Rats sacrificed after 28 hours. B, Rats sacrificed after 48 hours. Note that the ordinates of the two graphs are not the same. (Ling and Ochsenfeld, unpublished work.)



**Figure 16.2. THE EFFECT OF INSULIN ON THE DISTRIBUTION OF  $\text{Na}^{24}$ ,  $\text{K}^{42}$ ,  $\text{Rb}^{86}$ , AND  $\text{Cs}^{137}$  BETWEEN FROG *SARTORIUS* MUSCLE AND RINGER'S SOLUTION (25°C).** Paired muscles were maintained overnight at 4°C in an excess of normal Ringer's solution containing 2.5 mM/l of  $\text{K}^+$ ,  $\text{Cs}^+$ , and  $\text{Rb}^+$  ions with  $\text{Na}^{24}$ ,  $\text{K}^{42}$ ,  $\text{Rb}^{86}$ , and  $\text{Cs}^{137}$  present as tracers. The muscles and solutions were then brought to 25°C and incubated for one hour at this temperature. They were then placed in a fresh Ringer's solution of identical composition for another two hours. At this time, one member of each pair was exposed to 0.2 units of insulin per milliliter. At specified intervals following the introduction of insulin, muscles were removed from solution and analyzed for their isotope contents with the aid of a multichannel pulse-height analyzer. Data are presented as the ratios of the concentration of isotope in insulin-treated muscle to that in an untreated muscle. Corrected for 12 per cent extracellular space. (Figure after S. B. Horowitz, 1959, and unpublished work.)

action and confirms the conclusion (Section 7.4C) that the  $\varepsilon^0$  value and the  $c$ -value covary with the adsorption of bioregulants.

## 16.2. The Action of Some Pharmacological Agents

It should be apparent from the preceding text that pharmacological agents which are effective at low concentrations must act, according to the present hypothesis, through their adsorption on proteins and their influence on the  $c$ -,  $c'$ -, and  $\varepsilon^0$ -value ensembles of the reacting tissue. The action of these bioregulants is, in all respects, comparable to that of hormones. We shall discuss only a few of the innumerable

Treatment	Substitution therapy	Proximal tubules		Loops of Henle Descending limbs
		Convoluted portions	Straight portions	
Control	none	0.552 ± 0.010	0.407 ± 0.001	0.343
Hypophysectomy	none	0.534 ± 0.020	0.268 ± 0.007	0.332
Hypophysectomy	growth hormone	0.579 ± 0.031	0.363 ± 0.005	0.334
Hypophysectomy	pitressin	0.359 ± 0.019	0.259 ± 0.012	—
Hypophysectomy	thyroxine	0.502 ± 0.020	0.328 ± 0.013	—
Hypophysectomy	thyrotropin	0.470 ± 0.011	0.392 ± 0.013	—
Hypophysectomy	DOCA	0.503 ± 0.011	0.476 ± 0.008	—

Treatment	Substitution therapy	Loops of Henle	Distal convoluted tubules	Medullary collecting ducts
		Ascending limbs		
Control	none	0.383	0.547 ± 0.016	0.264 ± 0.003
Hypophysectomy	none	0.357	0.550 ± 0.005	0.176 ± 0.013
Hypophysectomy	growth hormone	0.358	0.543 ± 0.006	0.168 ± 0.012
Hypophysectomy	pitressin	—	0.371 ± 0.020	0.128 ± 0.018
Hypophysectomy	thyroxine	—	0.554 ± 0.007	0.146 ± 0.025
Hypophysectomy	thyrotropin	—	0.540 ± 0.001	0.143 ± 0.021
Hypophysectomy	DOCA	—	0.531 ± 0.015	0.142 ± 0.021

Table 16.2. THE EFFECT OF SUBSTITUTION THERAPY AFTER HYPOPHYSECTOMY ON THE REACTIVITY OF PROTEIN-BOUND SULFHYDRYL GROUPS IN THE CYTOPLASM OF RAT KIDNEY CELLS. The extent of reaction of the sulfhydryl groups of tissue proteins was determined by histochemical staining methods on trichloroacetate-fixed tissue sections and expressed in terms of average values plus or minus standard error in arbitrary units of optical extinction in a spectrophotometer. The controls were normal animals receiving saline injections; experimental animals were used seven days after hypophysectomy. DOCA is desoxycorticosterone acetate. (Table from Cafruny *et al.*, 1957.)

drugs capable of profoundly affecting cellular function to illustrate that their modes of action fall into the pattern of all bioregulants and are interpretable in terms of *F*-effects.

#### A. THE APPARENT PARADOX OF DIGITALIS\* ACTION

Digitalis and other cardiac glycosides are known to lower the  $K^+$ -ion concentration and to increase the  $Na^+$ -ion concentration in normal heart muscle (*vide infra*), in

\* In this and subsequent discussions, the term digitalis is often used to designate the entire group of cardiac glycosides.

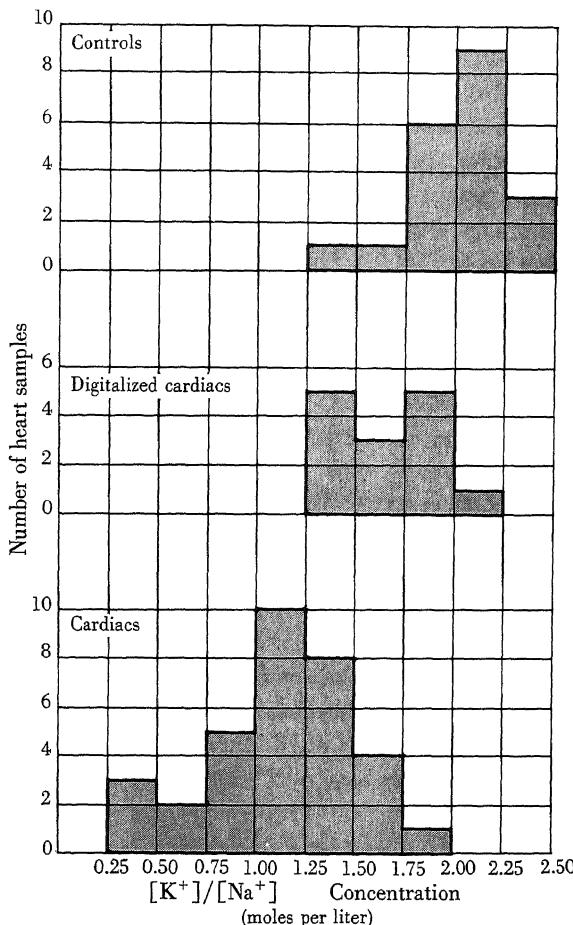


Figure 16.3. THE EFFECT OF DIGITALIS ON THE  $K^+-Na^+$  RATIO OF THE LEFT VENTRICULAR HEART MUSCLE IN CIRCULATORY HEART FAILURE. The frequency distribution of the  $K^+-Na^+$  ion ratio in normal hearts is compared with a similar distribution from specimens from patients in heart failure, untreated and digitalized. (Figure after Clarke and Mosher, 1952.)

voluntary muscle, and in red blood cells (Calhoun and Harrison, 1931; E. H. Wood and Moe, 1938; Cattell and Goodell, 1937; Sherrod, 1947; Holland and Dunn, 1954; and Schatzman, 1953). There is considerable evidence that, in clinical cases of heart failure, there is a loss of  $K^+$  ion from and a gain of  $Na^+$  ion by the cardiac muscle (Harrison *et al.*, 1930; Sinclair-Smith *et al.*, 1949; Iseri *et al.*, 1952). Reports by Clarke and Mosher (1952) have shown that when digitalis is administered to patients with failing hearts, it restores the normal high potassium and low sodium content (Figure 16.3) in apparent contradiction to the effect observed on red blood

cells and on normal cardiac and skeletal muscle. Thus, we find, in the action of digitalis, a common pharmacological phenomenon—a given drug producing opposite effects on different organs or on a particular organ in different states.

In the theoretical treatment of ionic selectivity (Chapter 4) we showed that relative ionic accumulation is a function of the *c*-value. We also demonstrated that an optimal *c*-value exists for the accumulation of a given ion, and that this *c*-value may be produced by the progressive adsorption of a given bioregulant (see Figures 6.2, 6.3, and 6.4). Analysis of the theoretical relations tells us that whether a net increase or a net decrease in the  $K^+-Na^+$  adsorption ratio results from a reaction with a bioregulant depends on at least two factors: the direction and magnitude of the net *c*-value change,  $\sum_j \Delta_{ij}(p_j)$ , which depends chiefly upon the nature of the bioregulant and its concentration, and the initial *c*-value of the active (in this case, anionic) site. We shall discuss the apparent paradox of digitalis action with respect to these two factors.

First, the dosage of digitalis reported by Wood and Moe (1938) to produce a fall in the  $K^+$ -ion concentration of dog heart muscle was high; a smaller dose was shown to cause an increase of  $K^+$  ion in cat heart by P. K. Boyer and Pointdexter (1940). That this difference was due to the existence of an optimum (see Figures 6.2, 6.3, and 6.4, where  $\mathfrak{X}$  represents the  $K^+$  ion) was shown by Hagen (1939). This author showed that about  $4.0\gamma$  of digitalis per gram of wet rabbit heart caused an increase of 6.87 per cent in the intracellular  $K^+$ -ion concentration, while a larger dose,  $24.4\gamma$  per gram, produced a fall of 14.72 per cent (Table 16.3). According to the present theory, the initial increase of  $K^+$  ion preferring anionic sites probably occurs at the expense of salt linkages (Figures 4.9 and 6.3). Consistent with this, Boyer and Pointdexter (1940) observed a significant increase in the water content of heart muscle with the rise of  $K^+$ -ion concentration (for swelling of fixed-charge systems after dissociation of salt linkages, see Sections 9.3C and 9.4). The theoretical curves of Figures 4.10 to 4.13 show that a further increase of *c*-value from the optimum for the  $K^+-Na^+$  ratio by additional adsorption of digitalis would cause a decrease in this ratio, leading to an increase of intracellular  $Na^+$  at the expense of  $K^+$ .

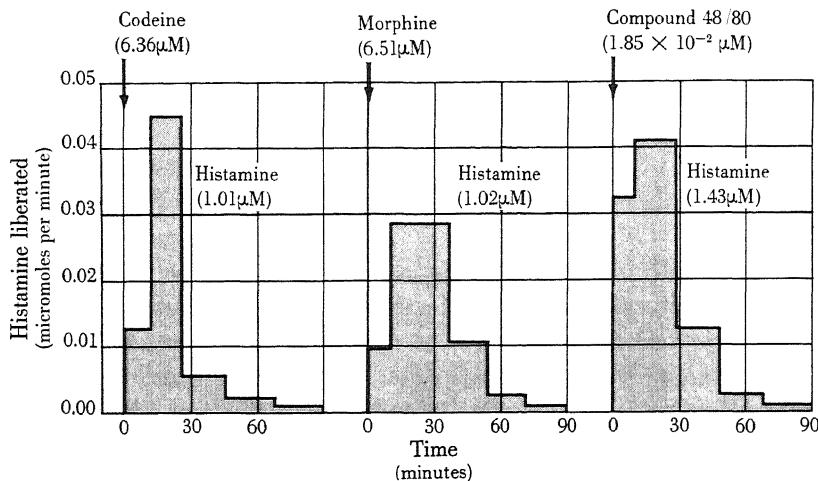
Second, in contrast to the complex situation characterizing the response of heart muscle to digitalis, there have been consistent reports of potassium loss from red blood cells in response to various dosages of digitalis and other cardiac glycosides. Thus, from  $0.1$  to  $10\gamma/ml$  of K-strophanthidin or digitoxin produced a uniform increase of red-cell sodium and a decrease of red-cell potassium (Schatzman, 1953). Regardless of dose, the cardiac glycosides do not bring about a potassium gain in erythrocytes as they do in the normal rabbit heart or the failing human heart. We have just mentioned that changes in the  $K^+-Na^+$  ratio are conditioned by both the magnitude of the *c*-value change, and the initial *c*-value of the fixed anionic group. Thus, if the initial modal *c*-value of red blood cells were con-

	Rab-	Dose,	Before perfusion		After perfusion		Water, % increase	Muscle K, % change
			bit No.	% solids	K, mg per g dry weight	K, mg per g dry weight		
					% solids	increase		
Control	9	0.0			13.92	13.84		-0.58
	11	0.0	20.00	14.29	18.50	14.40	1.50	+0.77
	23	0.0	26.05	12.91	21.45	11.30	4.60	-12.45
	26	0.0	24.06	12.22	22.60	11.70	1.46	-4.26
	35	0.0	21.20	10.58	18.15	10.16	3.05	-3.99
	36	0.0	23.08	9.57	18.87	10.71	4.21	+11.76
	40	0.0	21.53	9.80	18.48	10.68	3.05	+8.98
	47	0.0	24.45	9.94	20.90	9.98	3.55	+0.40
Average					11.65	11.60	2.68	+0.08
Therapeutic doses of digilanid C (3.6 to 4.6 $\gamma$ per g wet heart)	18	3.6	23.72	13.70	19.93	14.52	3.79	+5.98
	29	3.7	22.55	11.30	19.65	12.94	2.90	+14.51
	19	3.8	25.24	11.57	21.21	11.65	4.03	+0.69
	13	4.3	21.65	13.51	18.95	14.50	2.70	+7.33
	25	4.6	24.70	9.90	22.43	10.65	2.27	+7.58
	41	3.2	21.40	11.31	20.28	11.72	1.12	+3.63
	42	3.6	24.48	10.89	20.80	11.37	3.68	+4.41
	43	3.2	24.35	12.31	21.04	12.89	3.31	+4.71
	44	3.6	21.10	9.58	18.70	10.89	2.40	+10.78
	45	4.4	25.29	10.70	20.73	11.94	4.56	+11.59
	46	4.0	24.40	10.33	20.83	10.78	3.57	+4.36
	Average				11.37	12.17	3.12	+6.87
Toxic doses of digilanid C (average dose 24.4 $\gamma$ per gram)	15	14.3		13.30		11.31		-14.96
	16	15.7	24.20	14.62	20.70	12.09	3.50	-17.29
	17	19.2	22.70	15.06	20.35	13.09	2.35	-12.62
	49	46.5	23.74	10.25	18.78	9.20	4.96	-10.24
	50	20.9	24.20	10.37	20.02	7.96	4.18	-23.28
	51	19.2	23.30	12.41	19.98	9.84	3.32	-20.68
	52	35.2	22.51	11.11	19.80	10.67	2.71	-3.96
	Average				12.45	10.59	3.50	-14.72

**Table 16.3. THE EFFECT OF LOW AND HIGH DOSAGES OF DIGILANID C ON THE POTASSIUM CONTENT OF RABBIT HEART MUSCLE.** Digilanid C, one of the precursor glycosides from the leaves of *Digitalis lanata*, was added to the Ringer's solution perfusing the excised rabbit heart. The control and experimental heart muscle specimens were surgically removed from the left ventricle before and one hour after the introduction of the drug, respectively. (Table from Hagen, 1939.)

siderably above that of the normal rabbit heart and the failing human heart, digitoxin administration, in small as well as large doses, would result in a gain of Na<sup>+</sup> ion and a loss of K<sup>+</sup> ion. From the data on the distribution of radioactive alkali-metal ions in tissues, Table 9.5, and from Figure 4.9, we can predict that the modal c-value of the fixed anionic sites in red blood cells is no lower than that

equivalent to our tentative value of  $-3.0\text{\AA}$ . In heart muscle, however, the ion preferences indicate the modal *c*-value is no higher than  $-4.0\text{\AA}$ . The very low ion content found in a failing heart by Clarke and Mosher (1952) indicates that the diseased tissue has an even lower *c*-value than the normal heart (Section 9.3A). On this basis, the paradoxical effects of digitalis become entirely predictable.



**Figure 16.4. HISTAMINE LIBERATION FROM PERFUSED CAT SKIN BY CODEINE, MORPHINE, AND COMPOUND 48/80.** Arrows indicate the times at which the drugs were injected. The total amount of histamine liberated is indicated by the number in each figure. Conversion of the original data to molar concentration was based on the following molecular weights: histamine, 111.15; codeine sulfate, 786.91; morphine sulfate, 758.82; and compound 48/80, 540. (Figure after Feldberg and Paton, 1951.)

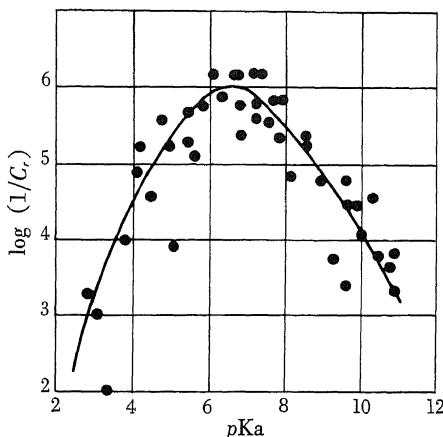
Digitalis action has been discussed in detail to serve as an example of a general explanation for this type of paradoxical physiological response (other examples are thyroxine action on ventricular and on auricular heart muscle; epinephrine action on muscle and on liver, see Table 16.1).

#### B. HISTAMINE LIBERATORS AND TRIGGER ACTION

The action of a number of drugs brings about histamine release from the tissue in which it is bound (for review, see Paton, 1957). Feldberg found that each molecule of one such compound, compound 48/80,\* liberates "explosively" 70 to

\* A condensation product of *p*-methoxyphenylethylmethylamine and formaldehyde, each 48/80 molecule consists of several monomers of phenylethylamine or its derivative.

80 molecules of histamine from the skin (Feldberg and Paton, 1951; Macintosh and Paton, 1949; Feldberg and Talesnik, 1953; Smith, 1953a,b), see Figure 16.4. Clearly, a direct displacement of histamine by the liberator is not possible. The interpretation offered here is that adsorption of the liberator molecule, leads to a *c*-value shift; through the indirect *F*-effect a zipperlike action follows, leading to



**Figure 16.5. THE *IN VITRO* BACTERIOSTATIC ACTION OF SULFONAMIDES AS A FUNCTION OF THEIR ACID DISSOCIATION CONSTANTS.**  $C_r$  is the minimum molar concentration necessary to cause bacteriostasis of *Escherichia coli* in a buffered (pH 7) synthetic medium under standardized conditions. (Figure after Bell and Roblin, 1942.)

the release of a large number of adsorbed histamine molecules through an exchange with other adsorbed entities. The mechanism is properly described by Figure 5.3, J<sup>+</sup> is taken as the 48/80 molecule and A<sup>+</sup> as the histamine originally adsorbed; it must be remembered that the small protein segment with four side chains represents a portion of a much larger gang of sites.

#### C. THE RELATION OF THE BACTERIOSTATIC ACTION OF SULFONAMIDES AND THEIR ACID DISSOCIATION CONSTANTS

Bell and Roblin (1942) studied the minimal concentrations ( $C_r$ ) of a series of sulfonamide derivatives needed to check the growth of the bacteria, *Escherichia coli*, and found a striking relation between this value and the acid dissociation constants  $pK_a$  of these sulfonamides as shown in Figure 16.5. One may recall from the definition of the *c*-value (see also Figures 4.9 to 4.11) that a high  $pK_a$  value of an oxyacid signifies a high *c*-value. The relation shown in Figure 16.5 is

thus a plot of the *c*-value of these drugs versus their bacteriostatic activity. It is interesting to note that Bell and Roblin concluded, first, that the bacteriostatic action bears direct relation to the negativity of the negatively charged SO<sub>2</sub> group, and, second, that the falling limb at very high *pK<sub>a</sub>* values is due to decreased ionization, for the biological activity is exercised by the ionized form alone. In terms of the present thesis, we may draw two conclusions. First, the *c'*-value of the fixed cationic site of the bacteria is high; its affinity for the sulfonamides, therefore, increases with the increase in the *c*-value of the SO<sub>2</sub> group. Second, the bacteriostatic effect is related to the change in the *c'*-value created by the adsorption energy of the adsorbed sulfonamide [see equation (5-2)], probably involving an indirect *F*-process.\* The inactivity of the unionized form may be due to competition between the H<sup>+</sup> ion and the cardinal cationic site for the sulfonamide ion.

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\* Woods (1940) and Fildes (1940) demonstrated the protective action of *p*-aminobenzoic acid against the bacteriostatic action of the sulfonamides. The idea that *p*-aminobenzoic acid is an essential metabolite associated with an enzymatic process and that successful competition by the sulfonamides causes cessation of growth is a reasonable one. We suggest that *p*-aminobenzoic acid acts as a cardinal adsorbent, controlling the *c*-value ensemble of more than one site, to promote normal function, and that this role becomes distorted when certain sulfonamides are absorbed at the cardinal site.

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# 17

## THE MECHANISM OF ANTIGEN-ANTIBODY REACTIONS

- 17.1. The Mechanism of the Mutual Adsorption of Antibody and Antigen 472
- 17.2. The Mechanism of the Adsorption-Desorption of Influenza Virus by Cells 476
- 17.3. The Mechanism of Complement Fixation and Lysis 480

In the course of our discussion of the living state we mentioned both snake venom and tetanus toxin (Section 10.4). Both are biological products immensely destructive to life. In discussing their possible modes of action, we pointed out that their potent function is shared by similar but simpler compounds exemplified by poly-L-lysine, which is capable of intense destructive action in concentrations as low as  $10^{-6}M$ . As dramatic as the action of poly-L-lysine on cells is, it may be prevented or reversed by injection into the system of an equally simple compound, polyaspartic acid (Figure 10.11). Monomers of both lysine and aspartic acid are ineffective in producing these respective actions. It is our opinion that, in this reversal of the effect of poly-L-lysine, we have a prototype of the defense mechanism available to victims of snake bites, toxic bacteria, and so on, a defense mechanism referred to as the *immune response*.

The introduction of many substances (especially of a protein nature) called antigens, into the blood vascular system of an animal elicits the production of certain proteins called antibodies. These antibodies will react with the eliciting antigen to bring about its precipitation and, if it is cellular, agglutination or lysis.

They thus prevent its effect on the tissues of the organism much as polyaspartic acid prevents the effect of poly-L-lysine on red blood cells. In the following discussion we shall consider some aspects of antigen-antibody reactions to which the present hypothesis has obvious applicability.

### 17.1. The Mechanism of the Mutual Adsorption of Antibody and Antigen

The possibility of Coulombic interaction between the polar groups on the antibody and those on the antigen was pointed out by M. Heidelberger and

Form of antigen <sup>a</sup>	Immune sera					
	<i>p</i> -Amino- benzoic acid	<i>o</i> -Amino- benzoic acid	<i>p</i> -Amino- phenyl- arsenic acid	Aniline	<i>p</i> -Nitro- aniline	<i>p</i> -Tolui- dine
<i>p</i> -Aminobenzoic Acid.....	++±	0	0	0	0	0
<i>m</i> -Aminobenzoic acid.....	±	0	0	0	0	0
<i>o</i> -Aminobenzoic acid.....	0	++	0	0	0	0
<i>p</i> -Aminophenylarsenic acid..	0	0	++++	0	0	0
Sulfanilic acid .....	0	0	0	0	0	0
<i>o</i> -Aminocinnamic acid.....	0	0	0	0	0	0
Aniline.....	0	±	0	++±	+	++
<i>p</i> -Nitroaniline .....	0	±	0	+	±	±
<i>p</i> -Toluidine.....	0			±	+	++±
<i>m</i> -Toluidine.....	0	±	±	++	+	±

**Table 17.1. SPECIFICITY IN ANTISERA TO ARTIFICIAL ANTIGENS SYNTHESIZED BY COUPLING PROTEINS WITH ACIDS AND BASES.** The data illustrate the prominent influence of charged acidic groups in comparison with other constituents. In this and the following tables, increasing degrees of precipitation are expressed by the symbols: 0 (none), ± (trace), ±, +, +±, ++, . . . (Table from Landsteiner and van der Scheer, 1927; see Landsteiner, 1945.)

<sup>a</sup> Concentration of antigens was 0.01 per cent.

Kendall as early as 1929. Landsteiner (1945) also considered salt linkages important, offering evidence to support this idea. *Prominently important evidence lies in the observation that the substitution of charged acid groups into the antigen molecule modifies antigenic specificity while, in contrast, substitution of groups like methyl, halogen, methoxyl, and nitro, which bear no net charge, has only weak effects or none at all.* It has been observed that not only does the introduction of carboxyl, sulfonic, or arsenic groups profoundly alter the antigenic specificity of the original

protein, but that the antibody elicited by this artificially modified antigen will also combine specifically with the single monomeric acid bearing the particular acid group (Table 17.1). Landsteiner and van der Scheer (1932) tested antisera against antigens made from peptides by nitrobenzoylating the peptide, reducing it to an amino compound, and coupling this with protein. The results, given in Table 17.2, were interpreted as showing that specificity is determined primarily by the amino acid carrying the free carboxyl group and, to a much lower degree

Immune Seras	Antigens <sup>a</sup> from			
	Glycylglycine	Glycylleucine	Leucylglycine	Leucylleucine
Glycylglycine.....	++ <sup>+</sup>	0	0	0
Glycylleucine I.....	0	++ <sup>+</sup>	0	<sup>+</sup> -
Glycylleucine II.....	+	+++	0	+
Leucylglycine I.....	+	0	+++	0
Leucylglycine II.....	++	0	+++	<sup>+</sup> -
Leucylleucine.....	0	+	0	++

**Table 17.2. THE REACTION OF ANTISERA FOR PEPTIDE-AZOPROTEINS WITH HOMOLOGOUS AND HETEROLOGOUS ANTIGENS.** The data show distinct specificity in reactions with the homologous antisera; they also show overlapping reactions for cases in which the *terminal* amino acid (that is, the charge-bearing residue) was the same as in the immunizing antigen. (Table from Landsteiner and van der Scheer, 1932; see Landsteiner, 1945.)

<sup>a</sup> Concentration of antigens was 0.01 per cent.

by amino-acid residues which carry no charge. When these observations are viewed side by side with the demonstration of Haurowitz *et al.* (1942) that similar effects follow the substitution of positively charged quaternary ammonium groups (Table 17.3), they strongly suggest that salt linkages are the main type of bond formed between the antigen and the antibody (see also Chow and Goebel, 1935; Goebel and Hotchkiss, 1937).

Despite this experimental evidence, current opinion tends to stress van der Waals' forces as the chief source of the antigen-antibody binding energy (Boyd, 1956, p. 272). Perhaps the greatest objection to salt linkages is the assumption that they are nonspecific. It is argued that any positively charged amino group should be capable of reacting with any negatively charged carboxyl group and, hence, no specificity may be expected except the gross type due to the presence or absence of ionized polar groups. We shall demonstrate that the objection of non-specificity disappears if we adopt the present theory of proteins and, in particular, the theoretical derivation showing the variability of preferred counterions as a function of *c*- and *c'*-values.

In Section 7.1, we stressed that the *c*- and *c'*-values of the individual polar groups of proteins, be they carboxyl,  $\epsilon$ -amino, or guanidyl groups, necessarily differ. Thus, the nominally similar carboxyl groups of glutamic-acid residues in the same or on different protein molecules may be expected to react differently when confronted with a given  $\epsilon$ -amino group. One pair may form a high-energy salt linkage, another pair, a low-energy linkage, another may form no salt linkage

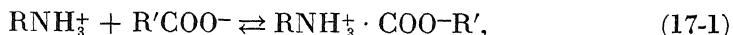
Immune serum	Test antigens		Weight of precipitates, mg					Antigen content of first precipitate, mg
	Name	Amount, mg	1st	2nd	3rd	4th	Total	
Basic ammonium-azo-sheep-serum globulin	sheep-serum globulin	3.0	5.174	trace	—	—	5.17	—
basic ammonium-azo-sheep-serum globulin	basic ammonium-azo ovalbumin	0.8	3.512	0.972	0.902	0.146	5.53	0.44
basic ammonium-azo-sheep-serum globulin	basic ammonium-azo-sheep-serum globulin	1.2	1.498	1.333	0.604	0.286	3.72	0.72

**Table 17.3. SPECIFICITY OF ANTIGENS PREPARED BY COUPLING POSITIVELY CHARGED QUATERNARY AMMONIUM GROUPS TO SHEEP-SERUM GLOBULIN.** The antigen was prepared by coupling sheep-serum globulin with diazotized *m*-aminophenyltrimethylammonium chloride. The immune serum to this antigen contains three kinds of antibodies: one precipitated by sheep-serum globulin, one by ovalbumin that has been coupled to the same basic quaternary ammonium group, and one by the homologous azo-sheep-serum globulin. The extent of the immune reaction is indicated by the weights of the precipitates formed between the antigen and antibodies, and their antigen contents. The amount of immune serum used was 7.5ml. (Table from Haurowitz *et al.*, 1942.)

at all; this may be portrayed by *c*-value-versus- $\Delta E$  plots equivalent to those shown in Figures 4.9 to 4.11. This variability of the preferred counterion in relation to salt-linkage formation has already been discussed under protein denaturation (Section 7.4).

Let us substitute a number of carboxyl groups into one protein molecule, and an equal number of sulfonic-acid groups into another as Landsteiner did in his experiments (1945). When introduced into the protein, the sulfonic as well as the carboxyl groups can be expected to retain different *c*-values so that the *c*-value of the carboxyl group remains higher than the *c*-value of the sulfonate group. Our theoretical expectation is that a positively charged  $\epsilon$ -amino group on an antibody formed in response to the carboxyl antigen will have a higher *c'*-value than a similar  $\epsilon$ -amino group on an antibody formed in response to the sulfonate antigen and will not react with the latter.

Furthermore, it must be made clear that the present interpretation of specificity in salt-linkage formation demands that there be a great diversity in the heats of interaction among the closely related, but distinctly specific, antigen-antibody reactions. This heterogeneity of  $\Delta H$  values is due not only to  $c$ - and  $c'$ -value variations among the interacting groups. It is due also to the fact that, because the reactants are semifixed-charge systems, the reaction is not simply



but a statistical mixture of the reactions described by equations equivalent to equations (7-3) and (7-4), involving the influence of competing ions. That there

Isoagglutinin	Agglutinogen	$-\Delta H$ , kcal/mole
$\beta(\text{A}_1\text{O})$	B	16 $\pm$ 2
$\beta(\text{A}_1\text{A}_1)$	B	6.5 $\pm$ 1.1
$\beta(\text{A}_2\text{O})$	B	9
$\beta(\text{OO})$	B	1.7 $\pm$ 0.4
$\alpha(\text{BO})$	A <sub>1</sub>	19 $\pm$ 3
$\alpha_1(\text{BO})$	A <sub>1</sub>	33 $\pm$ 2.5

**Table 17.4. THE HEAT OF COMBINATION OF VARIOUS TYPES OF ISOHEMAGGLUTININS WITH AGGLUTINOGENS.** Agglutinins from individuals of different blood groups and subgroups as indicated in parentheses. (Table from Wurmser and Filitti-Wurmser, 1957.)

is, in fact, a great heterogeneity of  $\Delta H$  values was beautifully demonstrated by Mme. Filitti-Wurmser *et al.* (1954) for anti-B agglutinins from individuals of different blood groups and subgroups. (Table 17.4; also Section 7.1).\*

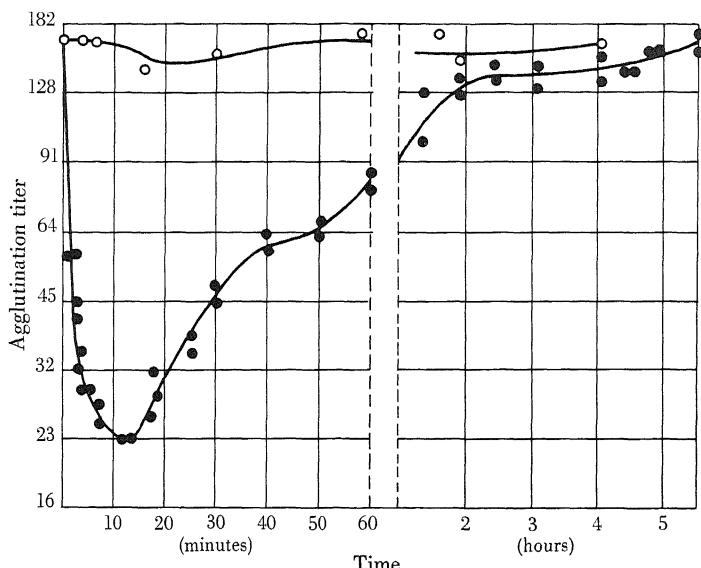
While the present theory of protein behavior stresses the importance of charged groups, theoretical analysis has led us to attribute the characteristic of specificity of preferred bond partners to all groups which may interact electrostatically. The variability of preferred counterions is thus not limited to the formation of salt linkages, but may be extended to include the formation of H-bonds, for instance, between the NH and C=O groups of the backbone. If H-bonds can also be specific, depending upon their  $c$ - and  $c'$ -value analogues, the specific antibody reactions with antigens such as dextran, a linear glucose polymer (Kabat and Berg, 1953), and glycogen (M. Heidelberger *et al.*, 1954) can be explained. None

\* The suggestion of Mme. Filitti-Wurmser of a reversible denaturation initiated by the combination of antigen with anti-B agglutinins of group O serum, and giving rise to a large entropy gain is in accord with another aspect of antigen-antibody reactions to be discussed in the next section.

of these contains polar groups bearing net charges, but all are richly endowed with H-bonding groups.

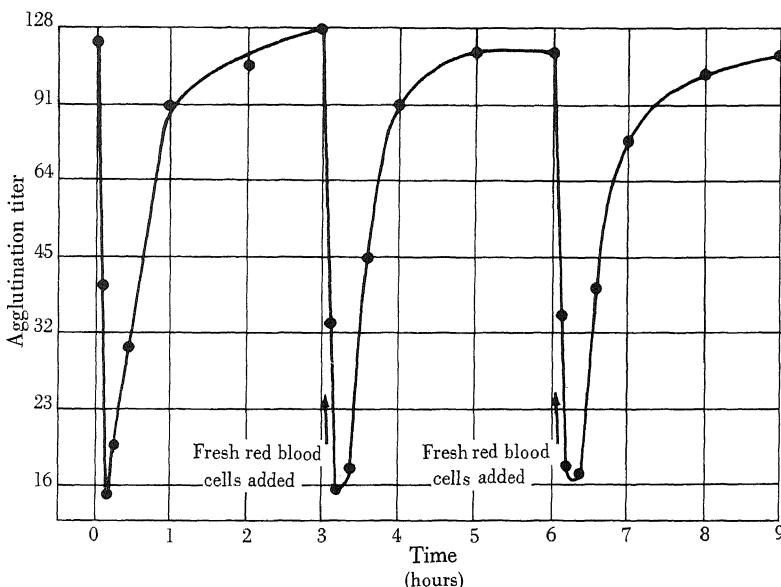
### 17.2. The Mechanism of the Adsorption-Desorption of Influenza Virus by Cells

Certain viruses, such as influenza A and B, react with chicken red blood cells at room temperature in an interesting manner. In the course of a few minutes, almost all become attached to the red cells; this is followed by their spontaneous desorption, the latter process being completed within a few hours; see Figure 17.1 (Hirst, 1942). The net result appears to be almost complete recovery of the



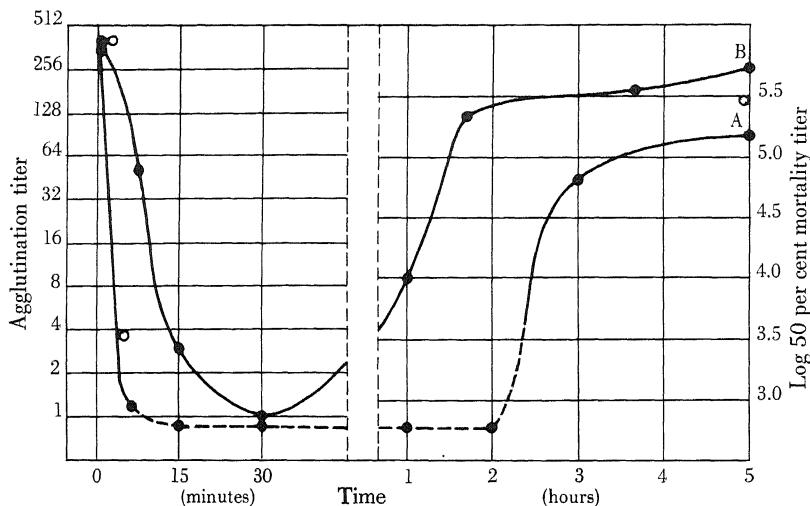
**Figure 17.1. THE ADSORPTION AND RELEASE OF INFLUENZA VIRUS BY CHICKEN RED BLOOD CELLS (24°C).** 50ml of a suspension of Lee (influenza B) virus and 50ml of a 3 per cent suspension of chicken red cells were thoroughly mixed at room temperature. Samples of five milliliters were removed at intervals; the red cells in the samples were quickly sedimented at low centrifugal speed, and the supernatant fluids were pipetted off and tested in duplicate for agglutination titer using a standard red-blood-cell suspension. Dots indicate the titer of virus in the supernatant after addition of fresh red blood cells; open circle, the titer of virus in the supernatant after addition of red cells previously treated with influenza B virus for four hours. The results show that the virus is quickly taken up by fresh red blood cells and then spontaneously desorbs during the ensuing three to four hours. Cells which have been exposed to the virus are no longer capable of taking it up from a fresh suspension of virus. The titer is expressed as the reciprocal of the final dilution of the original virus suspension. (Figure after Hirst, 1942.)

virus in the unchanged form (Figure 17.2), with, however, profound changes in the red cells with respect to their electrophoretic mobility and immunological properties (Figure 17.1; also Boyd, 1956). Such an adsorption-desorption phenomenon is by no means limited to this example. Thus, Figure 17.3, also taken from



**Figure 17.2. THE EFFECT ON INFLUENZA VIRUS OF ADSORPTION ONTO AND DESORPTION FROM RED BLOOD CELLS (24°C).** Packed red blood cells (1.5ml) were added to 100ml of allantoic fluid containing Lee (influenza B) virus. Samples were removed periodically and the virus titer of the supernatant determined as for Figure 17.1. After three hours, all the cells were removed from the remaining virus suspension and fresh packed cells were added in sufficient quantity that the final concentration was 1.5 per cent. The supernatant was again sampled for three hours, the second lot of cells was removed and the procedure repeated a third time. The results show that the activity of the virus in the solution is only slightly decreased by the process of adsorption and desorption. The titer is expressed as for Figure 17.1. Fresh cells added at arrows. (Figure after Hirst, 1942.)

Hirst (1943), shows a similar pattern in the reactions of both influenza virus A strain PR8 and influenza virus B with mammalian lung cells (see also Burnet and Anderson, 1947). Table 17.5 shows that there is only uptake and no release of virus A by ground cell suspensions of ferret lung, ferret liver, and rabbit liver. On the other hand, these tissues show a marked uptake and a significant release of influenza B virus. From the point of view of the present theory of proteins the adsorption-desorption cycle is another striking demonstration of the indirect *F*-effect involving a pronounced reciprocating *c*-value change.

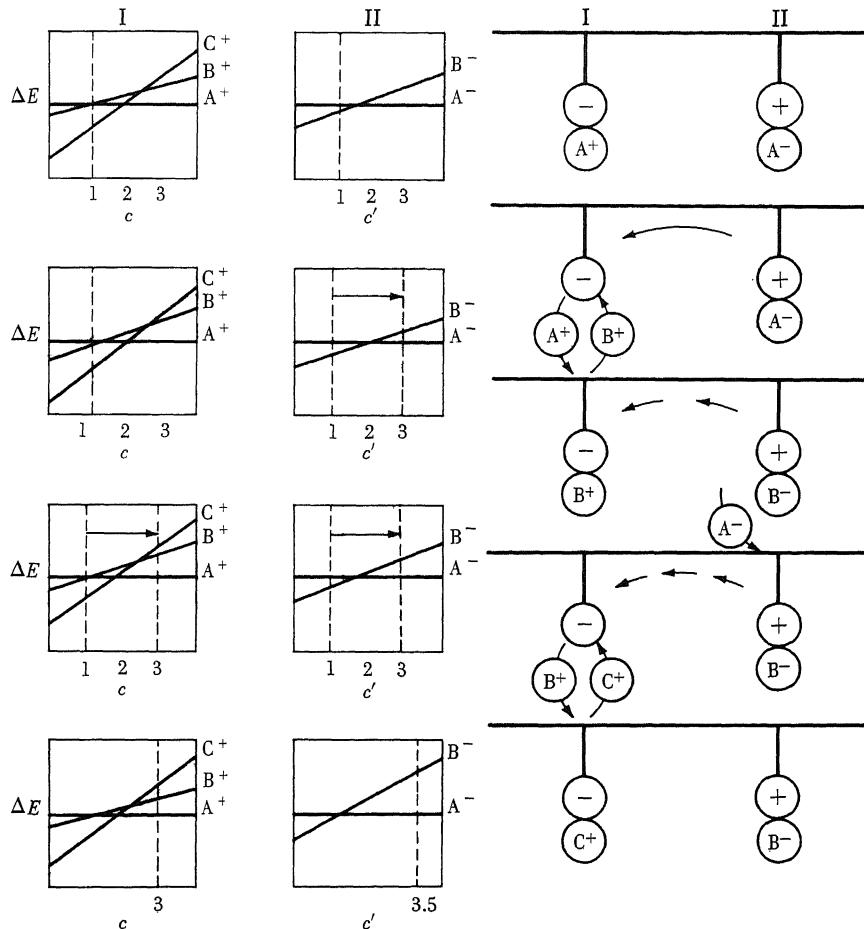


**Figure 17.3. THE RAPID ADSORPTION AND SPONTANEOUS RELEASE OF INFLUENZA VIRUS BY FERRET LUNG EPITHELIAL CELLS.** Suspensions of PR8 strain of influenza A virus and Lee strain of influenza B virus were introduced into excised, intact, and perfused ferret lungs through the tracheal opening. Samples of the suspension were then taken out periodically and assayed by both the red-cell agglutination method (agglutination titer) and by the dose necessary to produce a 50 per cent death rate on mice receiving the virus preparations (log 50 per cent mortality titer). The broken line connects points at which the agglutination titer was too low to be measured. The return to original titer at five hours, however, indicates only partial release (about  $\frac{1}{4}$ ) of original virus due to decrease of total volume after removal of successive aliquots. Curve A, PR8 agglutination; curve B, Lee agglutination; O, PR8, mouse mortality titer. (Figure after Hirst, 1943.)

		Time of combination of virus and cell suspension, min						
		0	1	15	30	60	120	300
Virus		Agglutination titer						
Ferret lung	A	538	182	147	97	97	120	84
	B	52	28	23	21	24	37	42
Ferret liver	A	362	39	12	7	4	3.5	4
	B	632	315	158	97	79	112	169
Rabbit liver	A	164	<2	<2	<2	<2	<2	<2
	B	338	84	60	56	60	64	97

**Table 17.5. ADSORPTION AND RELEASE OF INFLUENZA VIRUS BY LUNGS OF LIVING FERRETS.** Concentrated virus suspensions were introduced intranasally into live ferrets and the virus titer assayed on washings of excised lung preparation. (Table from Hirst, 1943.)

In Figure 17.4, we have drawn two interacting sites, I and II, which represent a sample taken from a gang of many sites. Initially, the  $c$ - and  $c'$ -values of sites I and II are 1 and 1, respectively. From the diagram of the  $c$ -versus- $\Delta E$  and  $c'$ -versus- $\Delta E$  curves shown on the left, sites I and II are paired with counterions to  $A^+$  and  $A^-$ , respectively. Now the virus  $B^+$  is introduced. Having a higher  $\Delta E$ , it displaces  $A^+$  at site I.



**Figure 17.4. THEORETICAL MECHANISM INVOLVED IN THE ADSORPTION-DESORPTION CYCLE OF INFLUENZA VIRUS BY CELLS.** Sites I and II represent two among an auto-cooperative gang of sites with the initial  $c$ - and  $c'$ -values both having the arbitrary value of 1 Å. Introduction of  $B^+$ , the equivalent of the influenza virus, leads to displacement of  $A^+$  at site I and secondary  $c'$ -value increase at site II; this  $c'$ -value leads to displacement of  $A^-$  by  $B^-$ , which, in turn, causes a  $c$ -value increase of site III (not drawn) and the chainlike indirect  $F$ -effect involving the whole gang and a reciprocating further  $c$ -value increase at site I and the consequent adsorption of  $G^+$ , displacing  $B^+$ , the adsorbed virus.

from site I, creating a  $c'$ -value increase at site II. This increase of  $c'$ -value leads to a shift of counterion at site II from  $A^-$  to  $B^-$ . Since  $B^-$  is much more strongly adsorbed than  $A^-$  (that is, it has a higher inductive constant  $I$ )  $B^-$  brings about a  $c$ -value rise of the next neighbor to the right, starting a chain of events (see Figures 5.3 and 5.4), and at the same time, causes a reciprocal  $c$ -value change at site I so that the  $c$ -value at the latter site changes to, say, 3. At  $c = 3$ , however, another cationic counterion  $G^+$  is preferred to either  $A^+$  or the virus  $B^+$ . Consequently,  $G^+$  is taken up by site I, displacing virus  $B^+$ . This exchange may, in turn, bring about a  $c'$ -value increase at site II. However, since partners other than  $A^-$  and  $B^-$  are not available, no further perturbation occurs. The net result is then characterized by changes of  $c$ - and  $c'$ -values at sites I, II, III, IV, . . . , and by the complete recovery of the virus  $B^+$ . Such  $c$ - and  $c'$ -value-ensemble changes alter the surface and zeta potentials (hence the electrophoretic mobility; see Chapter 10) as well as altering the antigenic specificity of the erythrocyte surface proteins in the manner discussed in the preceding paragraphs.\*

### 17.3. The Mechanism of Complement Fixation and Lysis

It has long been known (particularly since Ehrlich) that the presence of certain antibodies against bacteria or red blood cells does not bring about the destruction of these antigenic cells. To effect lysis, the antibodies must be aided by a component of the normal blood called *complement*; this is either a protein or a protein complex. We shall consider two aspects of this phenomenon.

The first is "complement fixation." Complement is bound to neither the antigenic cells nor the antibody, but only to the antigen-antibody adsorption complex (see Haurowitz, 1950, p. 297; Boyd, 1956, p. 361). As a mechanism for this action, let us first recall that the antibody and the surface of a bacterium are both semifixed-charge systems. As such, the tendency to form a large number of salt linkages and H-bonds must be great (see Section 7.3A). Through the *F*-effect, formation of these bonds will be associated with a change of the  $c$ -value ensembles of both the antigen and antibody. The altered  $c$ -value ensemble could thus make possible the preferential adsorption of the complement protein by sites which had little complement-binding ability before the antigen-antibody reaction.

\* In this model, the surface of the red blood cell is assumed to have both cationic and anionic fixed sites. This may appear to contradict our statement in Section 10.4 of the predominantly anionic nature of the surface sites of these cells. Actually there is no real conflict for two reasons: First, the adsorption-desorption cycle mentioned here does not have to involve more than a small fraction of the total number of fixed sites on the surface. Such a relatively small number of cationic sites would offer no contradiction to the conclusion reached in Section 10.4, since it is the total assemblage of fixed sites that determines the potential  $\psi$ . Second, the fixed cationic sites may, under normal conditions, exist as partners in salt linkages and thus may not function as fixed sites for free anion adsorption; it is the fixed sites available for free-ion adsorption which determines the potential  $\psi$ .

The second aspect of this problem is that the antigenic bacteria and red blood cells are lysed by neither the antibody nor complement alone, but readily succumb to the joint attack of the two. In presenting a model based on the association-induction hypothesis, we must first briefly describe what we consider to be the molecular mechanisms underlying lysis. The integrity of a cell depends intimately upon the three-dimensional structuring of its constituent proteins (see Section 9.4 on osmotic behavior of cells). Basically, however, proteins are linear polymers. That they can maintain the structure of the cell as the present hypothesis maintains, must depend to a large extent on certain intermolecular bonds that link linear polymers into a three-dimensional lattice. These bonds must consist of S—S bonds, H-bonds, and above all, salt linkages, all of which are subject to variation with changes in the *c*- and *c'*-value ensembles of the protein and protein complex (see Section 7.4C on dimensional changes of protein molecules). Lysis, therefore, must be basically a phenomenon connected with a critical change of the *c*-values of the partners in these S—S bonds, salt linkages, and H-bonds so that these cohesive linkages are energetically no longer favorable. This critical *c*-value-ensemble shift may be brought about not by complement alone nor by the antibody alone, but only by both of them acting together.

This action is reminiscent of the action of ATP and EDTA on contractile models, discussed in Chapter 15, in which ATP *alone* causes only contraction (formation of more salt linkages). EDTA *alone* causes no visible change. However, combined, they cause a marked relaxation, again a matter of dissociation of salt linkages. Thus, we can suggest that the cells depend critically upon certain salt linkages and H-bonds for their integrity. Using Figure 6.3 as a model, we can regard  $X_{\frac{1}{2}}^{f_0}$  as the mole fraction of such salt linkages;  $J'$ , the antibody alone, at the concentration present (say  $10^{-4}$  to  $10^{-3}$ ), may bring about no change or actually an intensification of such salt-linkage formation. When  $G$ , the complement, is added at a suitable concentration (say  $10^{-1}$ ), the critical salt linkages dissociate and lysis or hemolysis follows.

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# 18

## DEVELOPMENTAL PHYSIOLOGY AND CANCER

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In the course of our presentation of the association-induction hypothesis we have proceeded from the behavior of single isolated particles (ions in solution) to relatively simple semifixed-charge systems (protein in solution), thence to more organized nonliving fixed-charge systems (exchange resins, wool), and finally to living cells as true fixed-charge systems of protein, water, and salt. It was primarily the living state of single cells that was under consideration in our discussion of metabolism, accumulation of ions and nonelectrolytes, ionic permeability and diffusion, cellular potentials, and muscle contraction. In discussions of antigen-antibody reactions and excitation and inhibition, our interest began to move to interactions between cells. In this, the last chapter of the book, our attention will continue to be focused primarily at the cellular level; nevertheless, we shall begin to expand our concepts to include aspects of the living state of a multicellular organism.

### **18.1. The Activation of Eggs and the Physiology of Early Development**

#### **A. A THEORETICAL MODEL OF AN ACTIVATED OVUM; PREDICTIONS COMPARED WITH EXPERIMENTAL OBSERVATIONS**

The activation of an egg, a process which triggers the proliferation of the relatively quiescent ovum, is a nonspecific process. It may be brought about either by a variety of chemical agents, or by such physical factors as abrupt osmotic-pressure changes, temperature changes, and even simple pricking with a needle (see Delage, 1910; Loeb, 1909, for review). This diversity of eliciting stimuli is reminiscent of nerve excitation and, as early as 1886, Tichomirov remarked on the similarity between excitation of nerve tissue and parthenogenesis (Needham, 1942).

It is suggested that the activation of eggs in fertilization or in parthenogenesis

is a response of the labile  $c$ -,  $c'$ -, and  $\varepsilon^0$ -value ensembles of the egg protein system. This response may be interpreted as a cooperative change of the  $c$ -value ensemble from one metastable state to another through the mechanism of the indirect  $F$ -process.

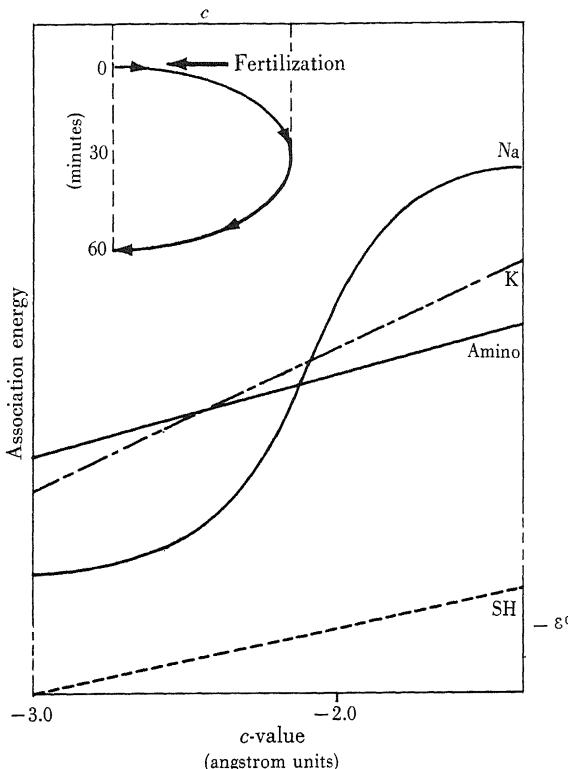
The present theory permits us to relate, through their underlying mechanisms, changes in such diverse biological variables as selective ionic accumulation, chemical reactivity, and viscosity. Few systems have been studied as intensively in these respects as the sea-urchin egg immediately following fertilization. Interesting changes in the variables of this system have a cyclical characteristic. From the theory presented thus far and with a few simple assumptions, it is possible to explain, in a unified way, these various changes discovered by different workers in the course of the last four decades.

The prototype for a time-dependent shift in  $c$ -value is found in Steinhardt and Zaiser's titration of hemoglobin (Figures 7.9, 7.10, and 7.14), in which, following the removal of the cardinal adsorbent ( $H^+$  ion) that initially brought about a change of preferred counterion, the action of the indirect  $F$ -effect slowly changes the  $c$ -value ensemble of the protein back to its original state and, therefore, back to its original ionic preference. Unfertilized sea-urchin eggs selectively accumulate  $K^+$  ions over  $Na^+$  ions in an environment containing a higher  $Na^+$ -ion than  $K^+$ -ion concentration (Bialaszewicz, 1929; Rothschild and Barnes, 1953). In accordance with this, let us suppose that the unfertilized sea-urchin egg possesses a modal  $c$ -value of about  $-2.7\text{\AA}$  (Figure 18.1). Following fertilization or parthenogenetic stimulation, let us assume a time-dependent shift of  $c$ -value as described above such that the modal  $c$ -value shifts in the course of 30 minutes to a value of  $-2.0\text{\AA}^*$  and that the system then returns to the region of the initial modal  $c$ -value in the course of another 30 minutes (see top curve, Figure 18.1). We have represented in Figure 18.1 the part of Figure 4.9 that shows the adsorption energies of  $Na^+$  and  $K^+$  ions in this  $c$ -value range. In addition, the model assumes that the cellular protein contains fixed amino groups having  $c'$ -values similar to those of the  $NH_4^+$  ion. Let us proceed to follow the time courses of change of various properties actually observed (Figure 18.2) and compare them with the predictions of the model.

### (1) Potassium-ion concentration

From our model one would expect the intracellular  $K^+$ -ion concentration to rise immediately after fertilization, reaching a maximum after about 10 minutes. This  $K^+$ -ion increase occurs at the expense of the salt linkages. From 10 minutes on, the  $K^+$ -ion concentration will steadily fall, reaching a minimum at about 30 minutes, which marks the point of reversal of  $c$ -value change; at this time, the  $c$ -value

\* These magnitudes for the  $c$ -values have been chosen only for illustration; in reality the shift may be much less than this.

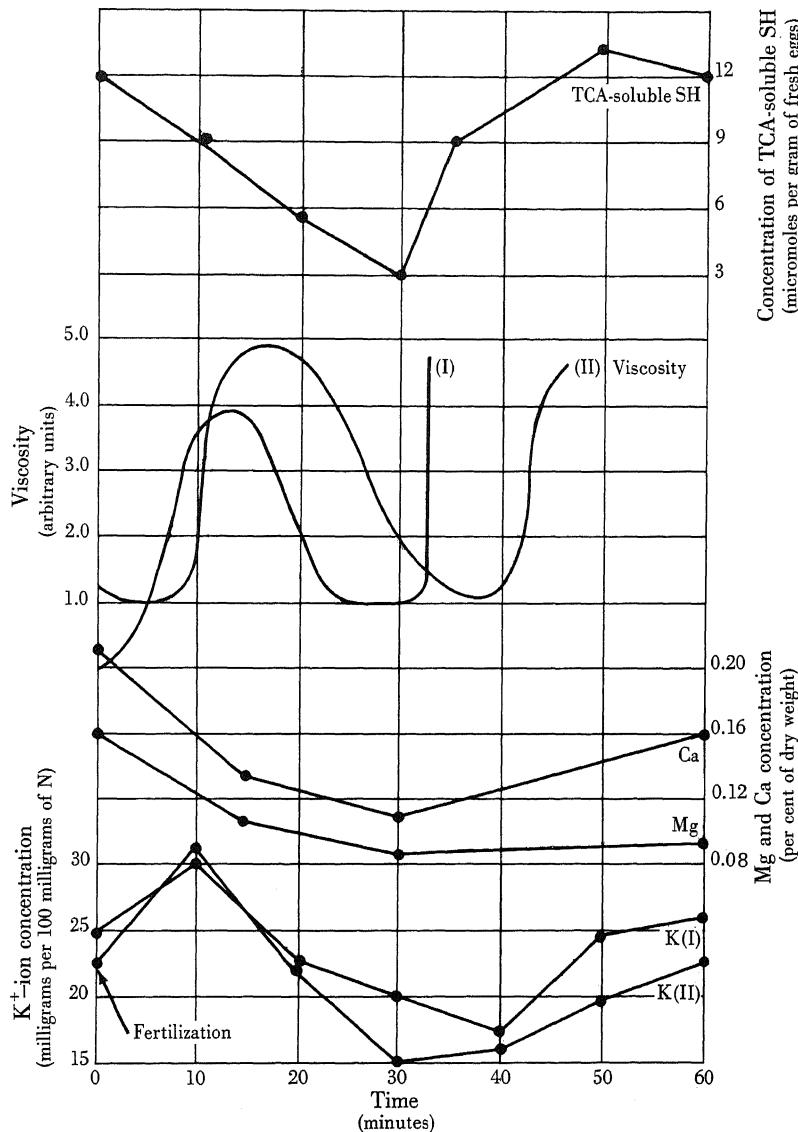


**Figure 18.1. THE THEORETICAL MECHANISM UNDERLYING THE SEQUENCE OF EVENTS IMMEDIATELY FOLLOWING THE FERTILIZATION OF SEA-URCHIN EGGS.** The top figure illustrates the proposed cyclic change of the modal *c*-value of the charged groups of the egg proteins. The middle one is a simplified version of Figure 4.9, where the adsorption energy  $\Delta E$  of the fixed cations on the fixed anionic groups is assumed to resemble that of the ammonium ion. The lowest curve is a diagram illustrating the direct relationship between the *c*-value and the oxidation-reduction potential  $\varepsilon^0$  of the protein sulphhydryl group. The *c*-value scales are the same for all three figures.

is such that the  $K^+$  ion is preferred to the  $Na^+$  ion to a smaller degree than before fertilization. From this time on, the *c*-value returns to the initial resting value, accompanied by a gradual increase of intracellular  $K^+$ -ion concentration. This is the sequence of events that has been observed (Monroy-Oddo and Esposito, 1951; Malm and Wachtmeister, 1950).

## (2) Sodium-ion concentration

Concomitant with the fall of  $K^+$ -ion concentration from 10 to 40 minutes, there should be a rise of intracellular sodium. Such a rise has been reported by Brooks



**Figure 18.2. EXPERIMENTALLY OBSERVED CHANGES IN SEA-URCHIN EGGS IMMEDIATELY FOLLOWING FERTILIZATION.** All curves are drawn to the same time scale. From top to bottom: Trichloroacetate (TCA) soluble sulphydryl groups in *Paracentrotus lividus*, from Rapkine (1931); viscosity (data shown in Curve I represent changes in the egg cortex), *Arbacia punctulata*, taken from D. E. S. Brown (1934); viscosity (Curve II represents changes of viscosity in the whole egg) taken from Heilbrunn (1920); Ca<sup>++</sup>- and Mg<sup>++</sup>-ion data, *Arbacia pustulosa*, from Monroy-Oddo (1946); K<sup>+</sup>-ion content changes shown in Curve I represent data from *Arbacia lixula*, those in Curve II from *Paracentrotus lividus*, from Monroy-Oddo and Esposito (1951).

(1938) and Malm and Wachtmeister (1950). Unfortunately, the complete time course of the change of  $\text{Na}^+$ -ion concentration is not available.

### (3) Alkaline-earth ions

In cationic exchange resins as well as in living cells, it has been found that divalent alkaline-earth ions may be replaced by alkali-metal ions (Gardner *et al.*, 1953; Cotlove *et al.*, 1951), and vice versa (Thiers *et al.*, 1960); calcium and magnesium ions can thus occupy anionic sites similar to those occupied by  $\text{K}^+$  ions (see Section 9.2B; also Ling, 1952). Such adsorption will, of course, vary with *c*-value shifts. Theoretical relationships between the *c*-value and the adsorption energies of alkaline-earth ions have not been derived.\* Empirically, however, Eisenman (unpublished) has observed a strong decrease of affinity for  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  ions accompanying a rise of *c*-value within the range discussed for numerous inorganic fixed-charge systems of high site density. If the modal *c*-value of the sea-urchin egg protein shifts following fertilization as postulated, changes of intracellular  $\text{Ca}^{++}$ - and  $\text{Mg}^{++}$ -ion concentrations are to be expected. A decline in intracellular  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  should coincide with the fall of intracellular  $\text{K}^+$ -ion concentration, being lowest when the  $\text{K}^+$ -ion concentration is lowest. This relationship has been observed by Mazia (1937) for the  $\text{Ca}^{++}$  ion, and by Monroy-Oddo (1946) for both the  $\text{Ca}^{++}$  and the  $\text{Mg}^{++}$  ions.† The curves shown in Figure 18.2 are taken from the latter author.

### (4) Changes in viscosity

Observed changes in viscosity are markedly dependent upon the method by which the viscosity is measured. Let us take the case of an actomyosin gel (at pH 7.0,  $[\text{KCl}] = 10\text{mM}$ ) into which  $10\text{mM}$  ATP is introduced. The viscosity, when measured on an Ostwald viscometer, will appear to *decrease* sharply with the formation of the dehydrated granular superprecipitate (Szent-Györgyi, 1951, p. 76). However, if the same change is followed by measuring the resistance to the movement of some particle embedded in the gel, it would appear that there is a marked *increase* rather than a decrease in viscosity. This latter type of measurement is the method most commonly used for studies of protoplasmic viscosity (see Heilbrunn, 1952). Such increases in viscosity may be attributed to an increase of intramolecular and intermolecular salt linkages (as between actin and myosin in actomyosin). This increase may occur at the expense of free cations. If we assume that the

\* These theoretical calculations would be further complicated by the introduction of another variable, very significant for divalent ions, that is, site-to-site distance.

† This  $\text{Ca}^{++}$ -ion loss has served as the basis of the well-known theory of calcium release as the initial step in cell excitation of the late Prof. L. V. Heilbrunn (1952).

amino curve in Figure 18.1 represents the cationic groups involved in the formation of intermolecular salt linkages, the changes shown in Figure 18.2 become explicable. Since the amino curve is similar to the  $K^+$ -ion curve, the rise in viscosity in the first 10 minutes should parallel the rise in  $K^+$ -ion concentration. With further increase of *c*-value, the anionic groups pass beyond the range of the preferred  $K^+$  or amino group and an abrupt fall of viscosity should follow. The reversal of the decline of *c*-value during the next 30 minutes would then be accompanied by a rise of viscosity; all these effects, as shown in Figure 18.2, were observed by Heilbrunn (1920), curve II for whole egg, and D. E. S. Brown (1934), curve I for egg cortex only, with some differences in the exact timing.

#### (5) Protein sulphhydryl groups

According to the present theory, *c*-value increases are accompanied by a decrease in the oxidation-reduction potential of the protein sulphhydryl groups with a concomitant increase in their reactivity with sulphhydryl agents. When such protein sulphhydryl groups are brought into contact with a mild oxidizing agent, the fall of  $\delta^0$  that accompanies the rise of *c*-value appears, when viewed from the point of view of classical theory, as an "increase" of titratable protein sulphhydryl groups. The later general *c*-value fall will reverse this trend and there will be a "decrease" in the titratable sulphhydryl groups. Hopkins (1925) established that reduced glutathione is a reversible reductant of protein sulphhydryl groups. We may assume that, immediately following activation of an egg, the sum of reduced glutathione and protein sulphhydryl groups must remain virtually constant. We may thus anticipate that in this virtually closed system a fall of reduced glutathione reflects a rise of protein sulphhydryl and vice versa. If this is correct and TCA-soluble sulphhydryl groups represent chiefly reduced glutathione (see Bolognari, 1952),\* then the cyclic change of TCA-soluble sulphhydryl observed by Rapkine (1931) and reproduced in Figure 18.2 reflects a similar but antiparallel time course of changes in protein sulphhydryl groups (see Brachet, 1950, p. 171). Such changes would be fully consistent with the *c*-value changes we have postulated.

We conclude that egg activation is interpretable in terms of a *c*-value-ensemble change through the indirect *F*-process. As a consequence of this, there is a sequential, semicyclical change in various physiological characteristics marking the earliest stages of zygotic ontogeny. The similarities of this model to that of denaturation (Section 7.4) are reminiscent of the observation of Mirsky on the similarity between certain egg protein fractions following fertilization and reversibly denatured myosin (Brachet, 1950; Mirsky, 1936b).

\* See the more recent findings of Sakai (1960). This footnote was added in proof, March 21, 1962.

## B. EMBRYOLOGY

### (1) Heterochromy in vital stains\*

The work of Spek (1930), Ries and Gersch (1935) and others, has shown that in a number of mosaic eggs, dyes that occur naturally or are artificially introduced into the cytoplasm exhibit different colors at the animal and vegetal poles.† Since the dyes are pH sensitive, this phenomenon is widely interpreted as indicating a maintained pH gradient in these cells.‡ An alternative interpretation exists, for, although proton association and dissociation is the most familiar mode of changing the absorption spectrum of a dye, the addition or removal of any electropositive or electronegative group will have a comparable effect. For example, dyes are known to change their color by adsorption onto proteins as illustrated by the bathochromic effect seen in the adsorption of erythrosin and phloxine on gelatin (Natanson, 1937). Another example is the adsorption on bovine serum albumin and other proteins of the azomercurial, 4-(*p*-dimethylaminobenzeneazo) phenylmercuric acetate (M. G. Horowitz and Klotz, 1956). Similarly, astacin, the lipochrome of lobster carapace and eggs is green in combinations with the carapace or egg proteins but pink in free form (Kühn and Lederer, 1933; Sørensen, 1935). Although Congo red, when it is bound to denatured albumin, remains red at pH 2, it turns into Congo blue below pH 3 to 4 (Haurowitz, 1950). Similarly, 2,6-dichlorophenol indophenol, when adsorbed onto denatured egg albumin, remains red in a medium of pH 6 to 7; in free solution, it would be blue at this pH (Todrick and Walker, 1937). In fact, these phenomena are so widespread that K. G. Stern has been led to comment that in "no instance has the absorption spectrum of the colored group as yet been found to tally with that of the whole chromo-protein" (1938).

On the other hand, a given dye may not adsorb onto a given protein in every circumstance. Astacin, just mentioned, combining as with native lobster carapace

\* These comments will be seen to have pertinence to the related fields of differential staining in histology and the genetics and physiology of plant pigments.

† The mosaic eggs offer further support to the present theory in that their cytoplasm clearly is not a dilute solution of salts and protein, but rather a complex, spatially fixed system. Thus Conklin (1905), the veteran American embryologist, long ago demonstrated that in the egg of the ascidian *Styela*, different regions of the cytoplasm, recognizable by their color, consistency, and other macroscopic characteristics, give rise separately to ectoderm, endoderm, and mesodermal organs. Strong centrifugation dislocates this cytoplasmic organization and leads to chaotic differentiation (see Needham, 1942, p. 131). The significance of these observations for readers of the present volume can be evaluated after consideration of two facts. First, all eggs possess some of the characteristics of the mosaic egg and some of the characteristics of the regulatory egg. Second, all living cells are descendants of these egg cells; the cytoplasm of most cell types resembles the cytoplasm of eggs—even though, in some specialized cells such as muscle cells, another element of regularity is added to the fundamental fixity of the cytoplasm (Figure 2.9).

‡ For beautiful illustrations, see colored plates shown in Figures 50 and 51 of Needham's monograph (1942).

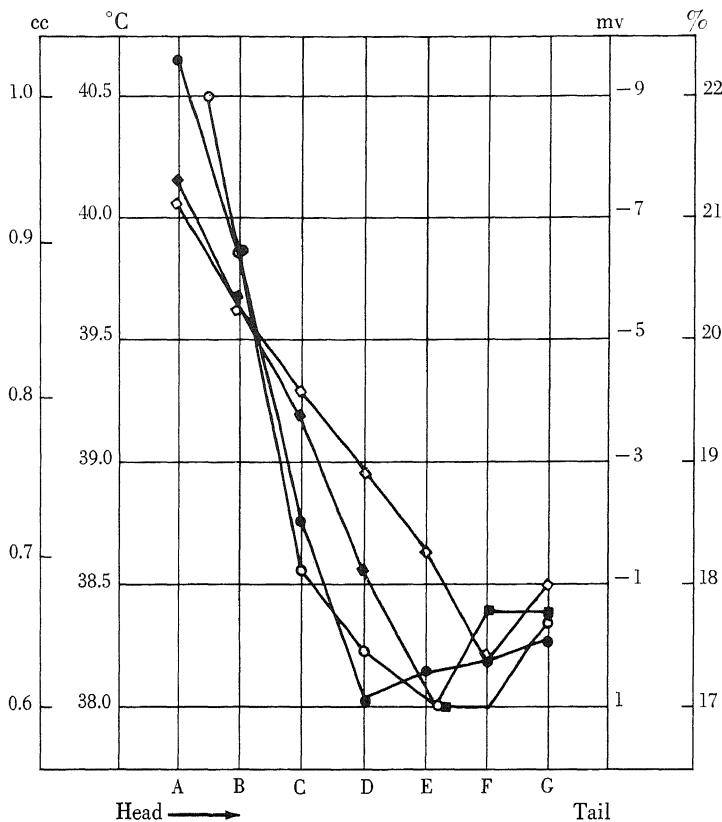
and egg protein, will not combine when these proteins are denatured (Kühn and Lederer, 1933). Congo red will combine with albumin only when it is denatured (Haurowitz, 1950). These facts again illustrate the principle of the variability of the preferred counterion discussed in Section 7.2A, and that an effective adsorption depends intimately on the  $c$ - and  $c'$ -values of the particular reacting groups which change with the change of state of proteins. We suggest that the mosaic egg possesses a  $c$ - and  $c'$ -value gradient directed from one pole to the other. The fact that the preferred counterion may vary permits the assumption that the degree of dye adsorption is different at the two poles. The one pole that does take up the dye will, in so doing, bring about a color change similar to that brought about by low pH. This, of course, will produce a differential coloring at two ends of a mosaic egg.

## (2) Physiological gradients

Since Boveri (1901) embryologists have observed gradients in the intensity of physiological manifestations along the axis joining the animal pole and the vegetal pole in a number of invertebrate animals and eggs. Thus in Figures 18.3 and 18.4 illustrative diagrams of the coincidence of some of these gradients in the flatworm *Planaria* and in the earthworm *Pheretima* are presented. Among the multitude of gradients observed, the variation in ability to reduce dyes, studied by Ranzi, is of special interest since it permits us to relate these gradients directly to the association-induction hypothesis. Ranzi and Falkenheim (1937) have measured oxidation-reduction potentials (expressed as  $rH$  value) with vital staining techniques and find them to be higher at the vegetal than at the animal pole of eggs. Their diagram is reproduced in Figure 18.5. They further showed that this measured oxidation-reduction potential may be altered by animalizing and vegetalizing agents. Based on our earlier discussion of the relation between the oxidation-reduction potential  $\varepsilon^0$  of SH groups, and the  $c$ -value ensemble (Section 7.2C), it is suggested that the *physiological gradients studied by Child (1940, 1942, 1943) and Ranzi and their schools are, in fact, direct or indirect expressions of  $\varepsilon^0$ -value and  $c$ - and  $c'$ -value gradients in the reactive groups of the cell proteins*. If this is the case, the animal pole has a high modal  $c$ -value and low  $\varepsilon^0$  value, while the vegetal pole has a low modal  $c$ -value and a high  $\varepsilon^0$  value.

Acceptance of the existence of a  $c$ - and  $\varepsilon^0$ -value gradient permits the prediction [from equation (10-12)] that an electrical potential gradient will be present. This arises from a difference in the relative preference for ions on the surface fixed ionic sites. For instance, the cells at the pole having a  $c$ -value greatly favoring  $K^+$ -ion adsorption will exhibit a high surface potential  $\psi$  in plasma or sea water, the condition seen in resting nerve and muscle; where the  $c$ -value is lower so that  $K^+$  ion is less preferred, the potential will also be lower. Such a potential gradient has been observed (paralleling gradients in other variables such as oxidizable substances and

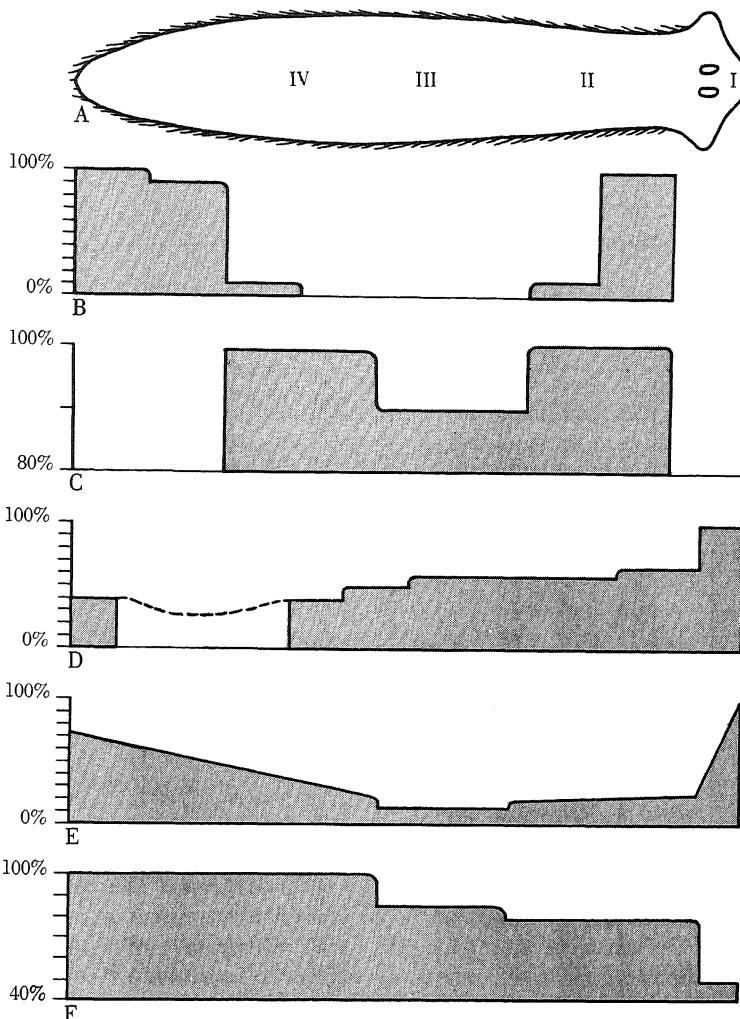
temperature sensitivity) in the earthworm *Pheretima* (J. S. Huxley and De Beer, 1934), shown by Figure 18.3, and in plants (Lund, 1947), shown in Figure 18.6.



**Figure 18.3. VARIOUS PHYSIOLOGICAL GRADIENTS IN THE EARTHWORM *PHERETIMA*.** Open circles and the outer left-hand scale indicate oxidizable substance as determined by the Manoilov reaction; ratio with respect to head region open squares and the outer right-hand scale indicate solid content in per cent. Solid squares and the inner left-hand scale indicate the temperature at which heat shortening occurs; solid circles and the inner right-hand scale indicate electrical potential in millivolts. (Watanabe, 1931: see J. S. Huxley and De Beer, 1934.)

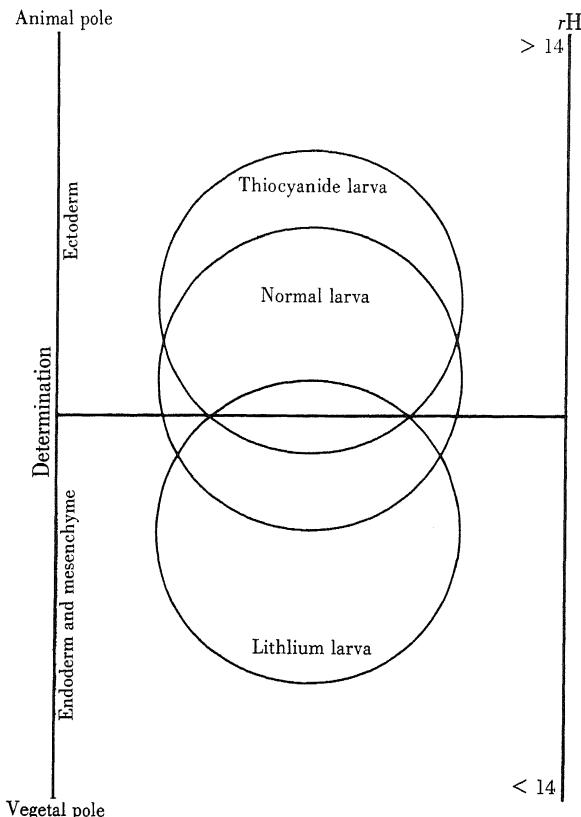
### (3) The action of animalizing and vegetalizing agents

If axial and embryological gradients are manifestations of a *c*- and  $\delta^0$ -value gradient, we may expect them to be varied by cardinal adsorbents. In embryology, these adsorbents are known as animalizing and vegetalizing agents. On the basis of the association-induction hypothesis, we expect that the  $\text{Li}^+$  ion, a strongly electronegative bioregulant, will, upon adsorption on an anionic site, draw elec-



**Figure 18.4. VARIOUS PHYSIOLOGICAL GRADIENTS IN THE FLATWORM *PLANARIA*.** A, different regions of the animal; B, gradient in the frequency of regeneration of heads in each piece after cutting into eight pieces; C, gradient in the rate of oxygen consumption; D, gradient in susceptibility to  $\text{H}_2\text{O}$ ; E, gradient in susceptibility to alcohol; F, growth gradient. (von Bertalanffy, 1941; see Brachet, 1950.)

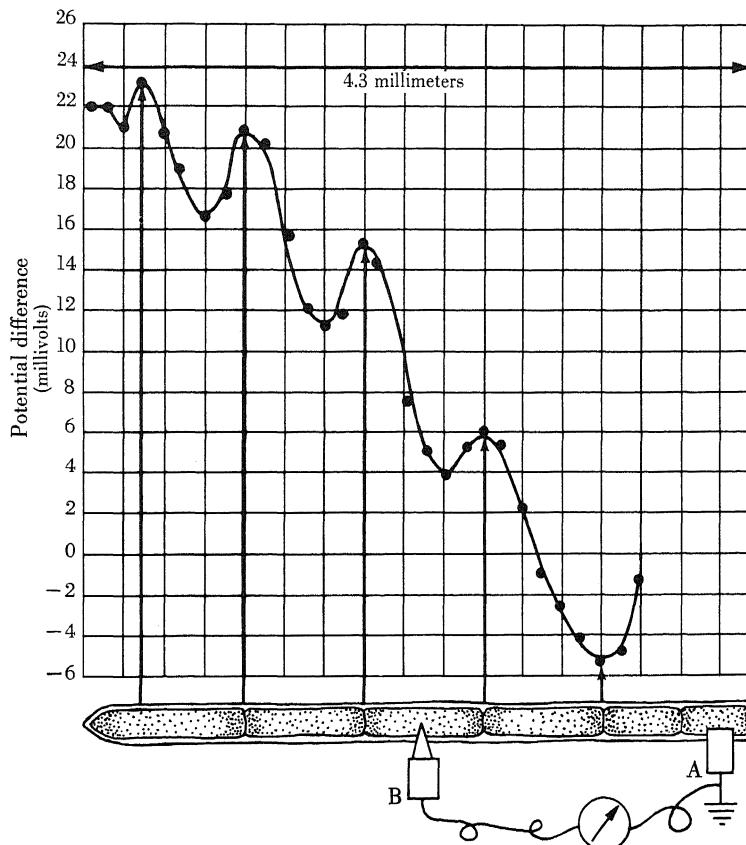
trons away from neighboring ionic sites or reducing groups such as SH. When a sufficient number has been adsorbed, an indirect  $F$ -effect will produce an increase in  $\varepsilon^0$  and  $c'$ -values and a decrease in  $c$ -value. Since the animal pole is characterized by high  $c$ - and low  $\varepsilon^0$ -values, lithium will have a vegetalizing effect (for other examples of  $\text{Li}^+$ -ion action on proteins, see Section 15.1). Strongly electropositive



**Figure 18.5. THE RELATION OF THE OXIDATION-REDUCTION POTENTIAL (EXPRESSED IN TERMS OF  $rH$  VALUE) TO INHERENT DEVELOPMENT POTENTIALITIES.** The potentialities for development of various regions into ectoderm, endoderm, and mesenchyme of normal sea-urchin larva and the effects of an animalizing agent, thiocyanide ( $SCN^-$ ), and a vegetalizing agent, lithium ion ( $Li^+$ ), on these potentialities are indicated. (Ranzi and Falkenheim, 1937: see Brachet, 1950.)

adsorbents such as thiocyanate  $SCN^-$  or iodide  $I^-$  (see Table 7.5), which repel electrons, will have a dramatically opposite effect and thus an animalizing action. These are the effects classically associated with these ions in embryology\* (Herbst, 1892; Runnström, 1928, 1929, 1931; Lindahl, 1939a,b; Hörstadius, 1939). For

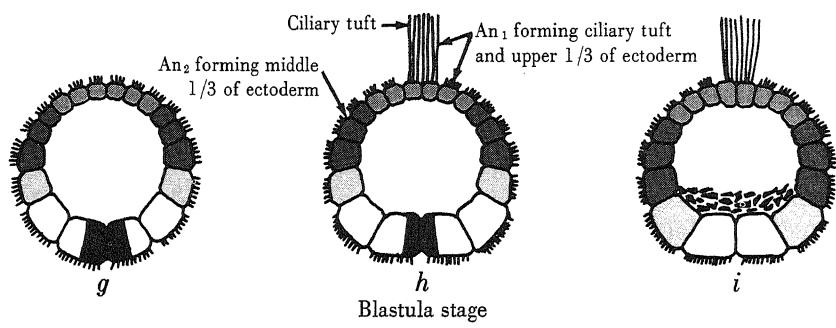
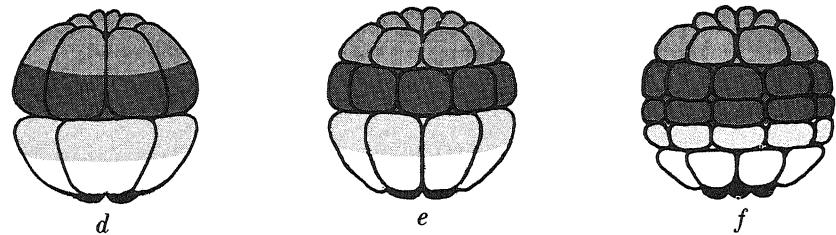
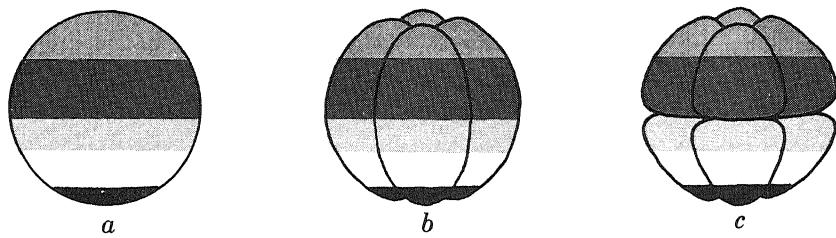
\* The observation by Herbst (1892) that the removal of  $SO_4^{2-}$  ion has an animalizing action on sea-urchin embryo is also explicable. The removal of the  $SO_4^{2-}$  ion, a weakly electropositive anion, is equivalent, because of mass action, to the addition of a stronger anion present in the medium, the  $Cl^-$  ion, for example (see Table 7.5).



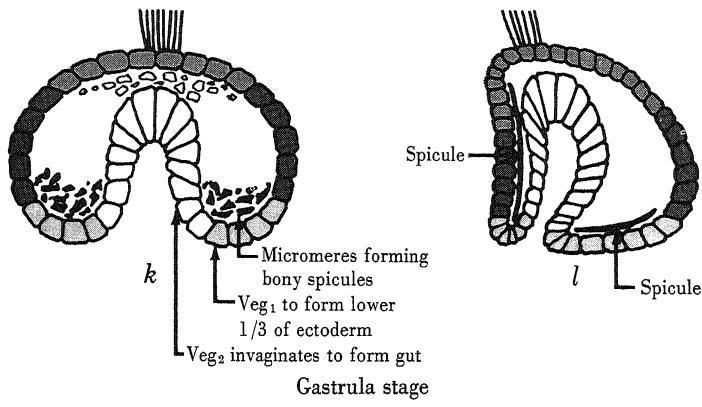
**Figure 18.6. THE ELECTRICAL POTENTIAL DIFFERENCE BETWEEN THE BASE AND MORE APICAL REGIONS OF A FILAMENT OF THE ALGA *PITHOPHORA*.** The potential difference between the basal portion in contact with the cuplike reference electrode A and the movable electrode B is plotted against the length of the algal filament, demonstrating a general electrical potential gradient oriented toward both the tip of the filament and the apical region of each cell. (Redrawn after Lund, 1947.)

those unfamiliar with the phenomena of animalization and vegetalization, we have provided Figure 18.7.

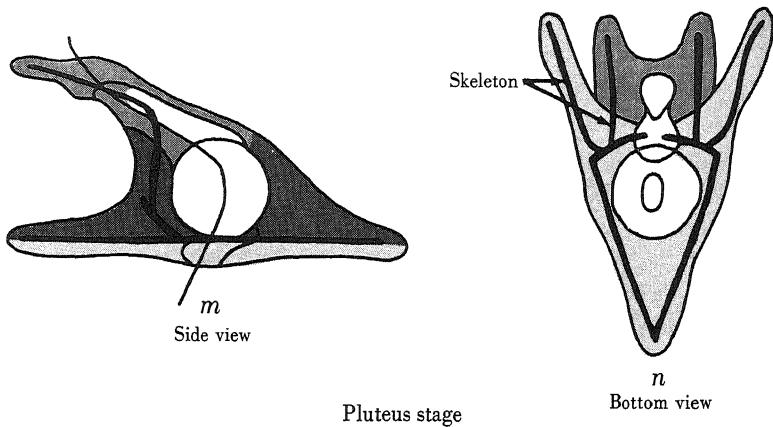
Ranzi (1957) and his co-workers have maintained that the animalizing action of the  $\text{Li}^+$  ion and the vegetalizing action of the  $\text{SCN}^-$  ion are due to their denaturing action on egg proteins. Their data show that vegetalizing agents delay the viscosity decrease brought about by urea, while animalizing agents enhance the urea effect. These observations are in accord with the principle of the additivity of the direct  $F$ -effect (Section 5.3A) and with the postulated electronegative nature of urea (Section 7.4C).



Blastula stage



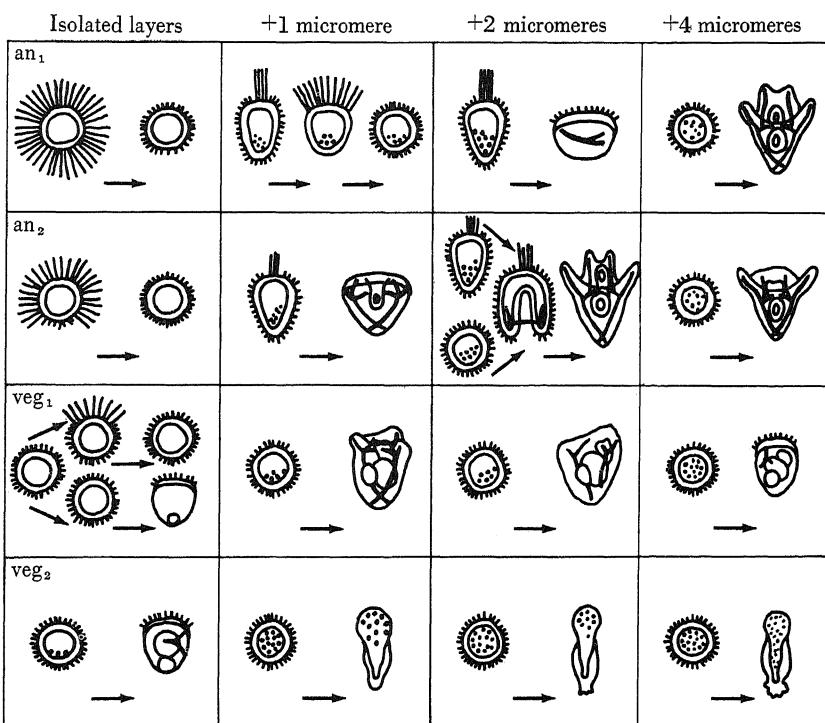
Gastrula stage



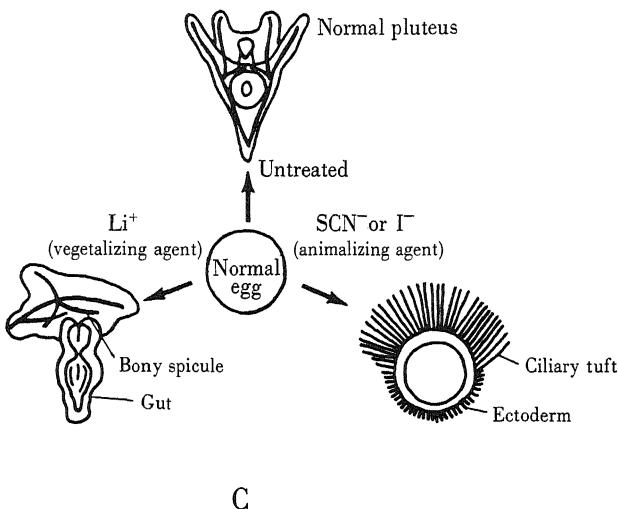
Pluteus stage

*n*  
Bottom view

A



B



C

**Figure 18.7A. NORMAL DEVELOPMENT OF A SEA-URCHIN EGG (*PARACENTROTUS LIVIDUS*).** Figures show the progress from an uncleaved egg to 4-, 8-, 16-, 32-, and 64-cell stages (*a* to *f*). At this stage the four types of cells, designated as  $\text{an}_1$ ,  $\text{an}_2$ ,  $\text{veg}_1$ ,  $\text{veg}_2$ , and the micromeres are differentiated. In the following (blastula) stages (*g* to *i*), the micromeres begin to migrate inside the hollow shell whereby they later form the spicule skeleton. Gastrula stages (*k* and *l*) then begin with the invagination of  $\text{veg}_2$  to form the endodermal gut. Final differentiation to the pluteus stage is represented by *m* and *n*. For a clear understanding of later figures we emphasize the following facts:  $\text{an}_1$  forms the ciliary tuft and upper third of the ectoderm;  $\text{an}_2$  forms the middle third of the ectoderm;  $\text{veg}_1$  forms the lower third of the ectoderm;  $\text{veg}_2$  forms the gut; the micromeres form the spicule skeleton. (Hörstadius, 1939: see Needham, 1942.)

**Figure 18.7B. ANIMALIZATION AND VEGETALIZATION BY SURGICAL MEANS: THE EFFECT OF IMPLANTING VARYING NUMBERS OF MICROMERES INTO DIFFERENT ISOLATED GROUPS OF CELLS IN THE SEA-URCHIN EMBRYO.** The figure shows that by combining surgically isolated  $\text{an}_1$ ,  $\text{an}_2$ ,  $\text{veg}_1$ , and  $\text{veg}_2$  with from 0 to 4 micromeres, normal plutei, animalized plutei, or vegetalized plutei may be formed. Animalized embryos show conspicuous and overwhelming development of the ciliary tufts and ectoderm ( $\text{an}_1$  plus no micromere), on the other hand, vegetalized embryos show overwhelming development of gut and spicule skeletons ( $\text{veg}_2$  plus 2 or 4 micromeres). (Hörstadius, 1939: see Needham, 1942.)

**Figure 18.7C. ANIMALIZATION AND VEGETALIZATION BY CHEMICAL MEANS.** Nucleophilic reagents (animalizing agents),  $\text{SCN}^-$  and  $\text{I}^-$ , produce, from normal eggs, animalized embryos similar to those produced by the surgical removal of vegetal cells. Similarly, electrophilic reagents (vegetalizing agents),  $\text{Li}^+$ , cause normal eggs to develop into vegetalized embryos similar to those produced by the surgical removal for animal cells.

#### (4) Evocation and organ induction

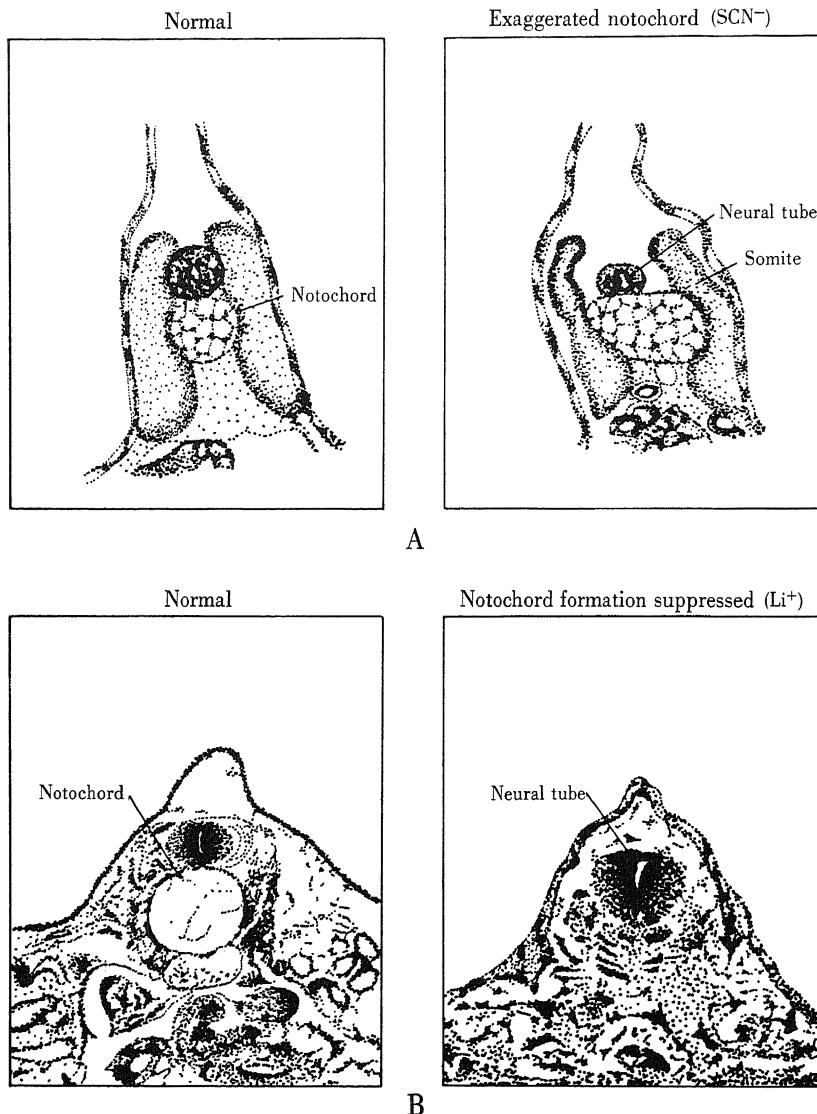
In various sections of his extensive treatise, Needham (1942, pp. 169, 170) has suggested that the mechanism for liberation of an evocator from, say, the dorsal lip of the blastopore of an amphibian embryo might involve a local protein denaturation which, in turn, sets free a protein-bound evocator, a substance of great potency. That denaturation can lead to the liberation of a substance bound to the native protein is well known; the molecular mechanism, based on the principle of the variability of the preferred counterion, is given in Section 7.2A. It is natural to extend this thesis to state that it is the variation of embryonic  $c$ - and  $c'$ -values that leads to the liberation of the evocator. Furthermore, on the basis of the same principle, one might expect that only an organ with a proper  $c$ - and  $c'$ -value ensemble, a "competent" organ, will accumulate the liberated evocator (Needham, 1942, p. 112). The consequent shift of the  $c$ - and  $c'$ -value ensembles of the "competent" organ following adsorption of the evocator will direct further development in a way similar to that of the animalizing action of the  $I^-$  and  $SCN^-$  ions and the vegetalizing action of the  $Li^+$  ion on development of an echinoderm egg.

That evocation is indeed the modification of the  $c$ -value ensemble of a "competent" organ is shown by the experiments of Lehmann (1933, 1937a,b) in which the  $Li^+$  ion completely inhibited notochord development in amphibia, and by the experiments of Ranzi and Tamini (1939) which showed that treatment with the  $SCN^-$  ion results in an abnormally large notochord (Figure 18.8). The neural tube evocator of the notochord may thus be expected to be an electropositive adsorbent like the  $SCN^-$  ion.

The principle of the additivity of  $F$ -effects states that  $c$ -value shifts in one direction produced by an electronegative substitution may be canceled by another electropositive substitution [equation (5-4)]. As examples, denaturation of protein by an electronegative denaturant, urea, is counteracted by the electropositive denaturant,  $HCl$  (Figure 7.16). Astacin liberated from protein by detergent is readsorbed by the protein in the presence of  $0.03M\ CaCl_2$ . If the above model is correct, then the  $c$ - and  $c'$ -value shifts produced by the evocator or organizer can conceivably be neutralized. The reversal of evocation has, in fact, been demonstrated. Thus, the anesthetic, chlorethane (chlorobutanol) (Lehmann, 1934), and urea (Jenkinson, 1906) have both been shown to abolish, in a quantitative and specific way, the lens evocation by the optic cup in an amphibian (Figure 18.9).

#### (5) The stability of the $c$ -value ensemble in different tissues

We have presented evidence that the part of a fertilized ovum which later gives rise to the ectoderm (that is, the animal pole) and the part that gives rise to the endoderm (that is, the vegetal pole) are basically the same material, and that one may be transformed into the other by the action of electropositive and electronegative adsorbents. According to our view, the animal pole has a low  $\varepsilon^0$ -value



**Figure 18.8. EXAGGERATED AND SUPPRESSED DEVELOPMENT OF THE NOTOCHORD SHOWN IN CROSS SECTION OF AN AMPHIBIAN EMBRYO.** A, the exaggerated notochord size in amphibian embryo treated with thiocyanide in comparison with a normal section. (Ranzi and Tamini, 1939; see Brachet, 1950.) B, the complete suppression of notochord development in amphibian embryo treated with  $\text{Li}^+$  ion in comparison with a normal section. (Figure after Lehmann, 1937b.)

and a high *c*-value ensemble. Prominent among tissues of ectodermal origin is the brain; prominent among tissue of endodermal origin are the liver and the gastrointestinal tract. One may recall from Table 9.5 and Figures 9.8 and 9.9, where the relative uptake of alkali-metal ion reflecting *c*-values of adult tissues and organs is given, that the high *c*-value ensemble of brain tissue persists in adult animals; similarly, the low modal *c*-value of the liver, stomach, and duodenum is preserved. This shows that the *c*-value ensemble, once established in the early embryonic state, not only persists in the sense that the original native protein maintains its unique *c*-value ensemble, but also in the equally fascinating way that similar modal *c*-values are found even in protein synthesized later for which the original cell protoplasm must serve as a template.

## 18.2. Growth, Differentiation, and Dedifferentiation

### A. DIFFERENTIATION AND DEDIFFERENTIATION

Embryologists have long known that at the cellular level there is a significant element of mutual exclusion between the state of active proliferation and the state of differentiation (Weiss, 1939; Needham, 1942). Thus, Flexner (1953) and his co-workers demonstrated in the guinea pig embryo a remarkable transformation in the embryonic nerve cells of the brain. Between the 41st and 45th day of pregnancy, the cerebral cortex showed an abrupt all-or-none onset of electrical activity (Figure 18.10), and the first fully positive peripheral muscular responses were invoked on cerebral cortical stimulation (Figure 18.11). From the present viewpoint, the two significant aspects of these observations are the abruptness of these transformations, and the lack of an increase of total protein nitrogen per kilogram of cells (Figure 18.12) indicating that there is no net synthesis of new protein nor, probably, of new cells.

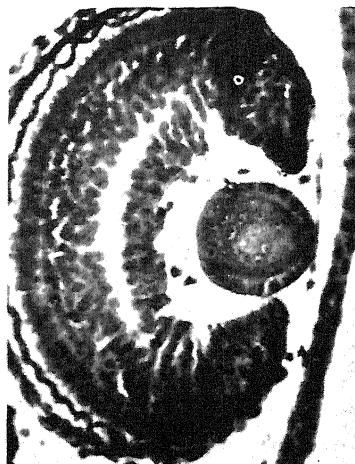
The abrupt onset of adult neural characteristics in guinea pig fetuses appears to indicate an all-or-none transformation from the rapidly proliferating phase to the adult differentiated phase. A similar transformation seems to occur also in the reverse direction. Thus it has been known for a long time that an isolated tissue in a culture medium may gradually dedifferentiate, assuming an indifferent character and, in so doing, give up many of its original morphological traits (Champy, 1912; Fischer, 1946). Fischer (1946, p. 214) and Strangeways (1924) observed that "the rate at which dedifferentiation proceeds is proportional to the rate of proliferation of the cells." A number of impressive instances were collected by Fischer to show that withdrawing embryonic extracts from the culture medium could reverse this dedifferentiation so that dedifferentiated iris epithelial cells could once more develop the characteristic black pigment (Doljanski, 1930a), liver cells could once more produce glycogen (Doljanski, 1930b), and certain brain

tissue cells could once more produce nerve fibers (Mihalik, see Fischer, 1946, p. 225). On the other hand, adding embryo extract to the medium brings about the opposite effect, an obliteration of the definite characteristics which the cells possessed before they entered into active proliferation.

It is a well-known histological fact that, in the adult animal, neurons undergo little proliferation. Yet, transplanted into tissue-culture media, they can divide



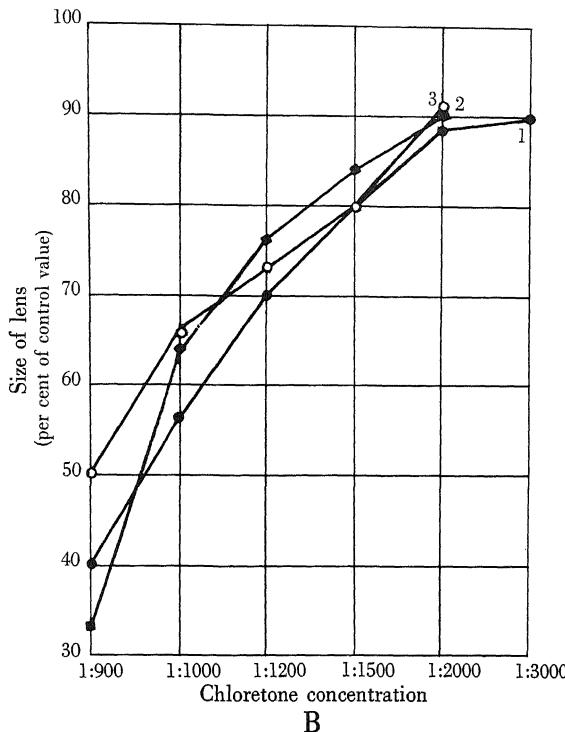
Normal lens



Abnormally small lens  
(chloretone concentration 1:1200)



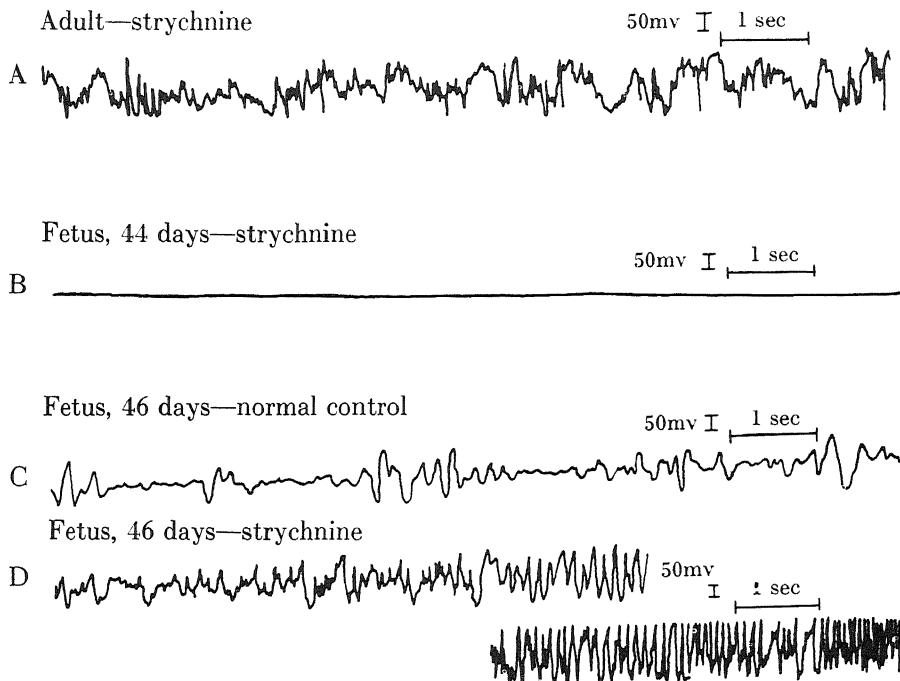
Lens formation suppressed completely  
(chloretone concentration 1:900)



**Figure 18.9. SUPPRESSION OF LENS FORMATION IN AMPHIBIAN EMBRYO BY CHLORETONE.** A, a cross-section of the optical cup in a normal embryo and those in embryos treated with different concentrations of chloretone. B, the relative lens size is plotted against the concentration of the chloretone employed. (A *from* and B *after* Lehmann, 1934.)

rapidly (Pomerat, 1951; Lumsden, 1951; Geiger, 1958). The transition from the inactive *in vivo* state to the rapidly reproducing state in culture media involves a period of inactivity. The length of this induction period varies with the age of the source animal (see Fischer, 1946). However, once growth has begun, there is no difference in the rate of growth, whatever the age of the tissue source. Thus, *in vitro*, we find that cells either assume the embryonic, rapidly proliferating phase or the adult differentiated phase, depending on the milieu in which they find themselves.

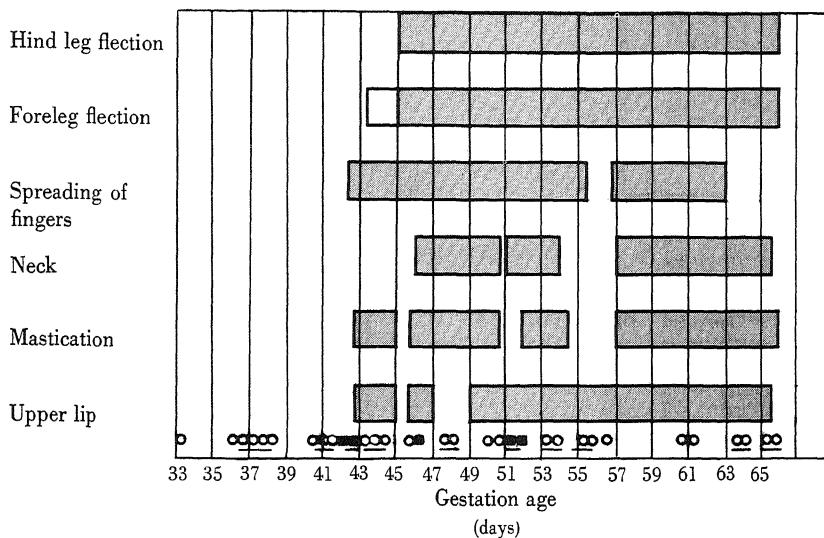
We suggest that the states of differentiation and of proliferation as observed *in vivo* in differentiating brain cells and *in vitro* in dedifferentiating cells in tissue cultures are essentially alike. In the developing brain, the cells go from the proliferating to the differentiated phase; in tissue culture, the differentiated cells revert back to the proliferative phase. As evidence, we cite the following: The



**Figure 18.10. BRAIN WAVES OF GUINEA PIG FETUSES BEFORE AND AFTER THE ABRUPT ALL-OR-NONE ONSET OF ELECTRICAL ACTIVITY.** The electrical activity of nerve cells in the brain may be recorded by placing a pair of electrodes over the exposed brain (or skull) surface. The recorded "brain waves" or electroencephalogram from electrodes placed over the surface of the brain (bipolar leads) record the algebraic sum of electrical potentials in the brain adjacent to the points at which the electrodes are placed. (For more description of electroencephalography in its important clinical use, see Gibbs, 1944.) Strychnine is a drug which powerfully stimulates the central nervous system (e.g., brain, spinal cord) eliciting powerful electrical activity in the adult brain, A. In 44-day-old fetus, this drug fails to produce any noticeable brain waves, B; in 46-day fetus, however, brain waves, similar to those from adult brains, were recorded in the normal state, C, or after strychnine stimulation, D. (Figures after Flexner *et al.*, 1950.)

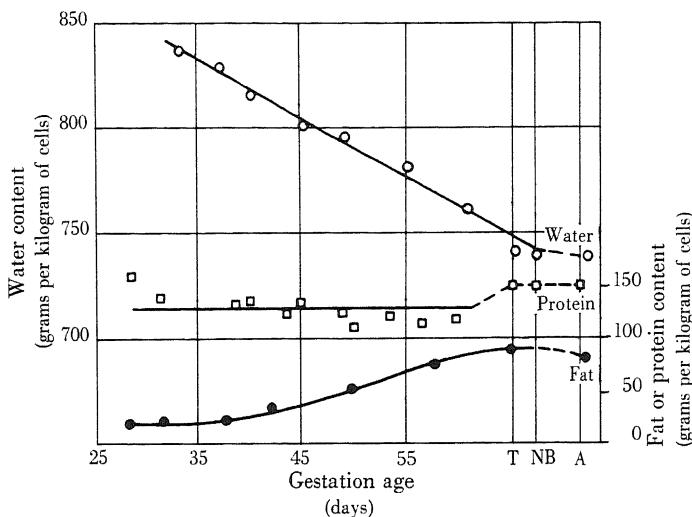
pre-41 day "neurones" of Peters and Flexner (1950) have few processes and no Nissl bodies; similarly, the actively growing brain cells in tissue culture (Mihalik, as quoted by Fischer, 1946) have no nerve fibers and look like epithelial cells. The post-41 day "neurones" of Peters and Flexner (1950) grow long nerve fibers; similarly, in the state of reduced multiplication, the cells of Mihalik grow a large number of nerve fibers. Generally speaking, cells of the pre-41 day guinea pig embryo have low enzyme activities which become activated abruptly on or

immediately after 41 days of gestation (Figure 18.13). Similarly, the enzyme activities of the tissue-culture cells have been found to be low when compared to normal adult cells (kidney, see Burlington, 1959; Figure 18.14). We suggest that the underlying mechanism is a shift of the modal *c*-value ensemble from one

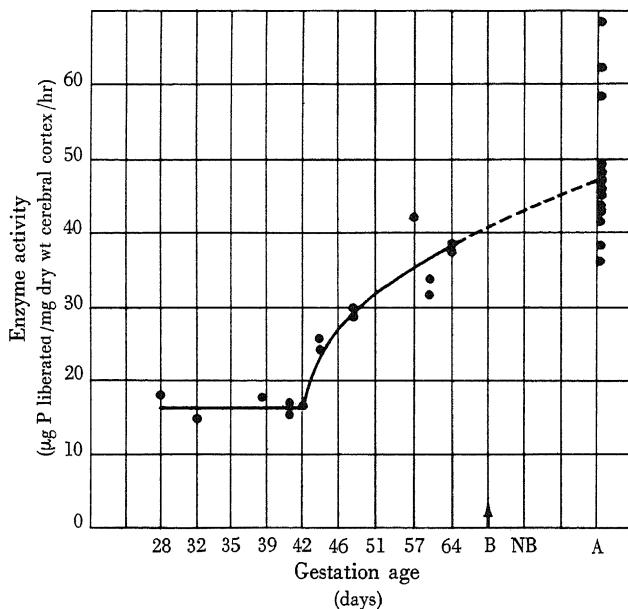


**Figure 18.11. THE ABRUPT ONSET OF FUNCTIONAL ACTIVITY OF THE GUINEA PIG NERVOUS SYSTEM.** Functional activity of the fetal brain was studied by electrically stimulating the motor area of the cerebral cortex and observing whether such stimulation elicits the typical responses in movements of appendages, neck, jaw, and lip, observed in adults. The shaded bar indicates the presence of the given response. Blank spaces indicate that the response was not obtained from the preparation studied at that particular stage of embryogenesis (gestation ages are shown at the bottom). The white square indicates a partial but typical response. Dots indicate fetuses whose ages were determined from crown-rump length; estimated error is plus or minus two days. Squares indicate dated pregnancies; error is plus or minus one day. Lines below dots and squares indicate littermates. (Figure after Kimel and Kavaler, 1951.)

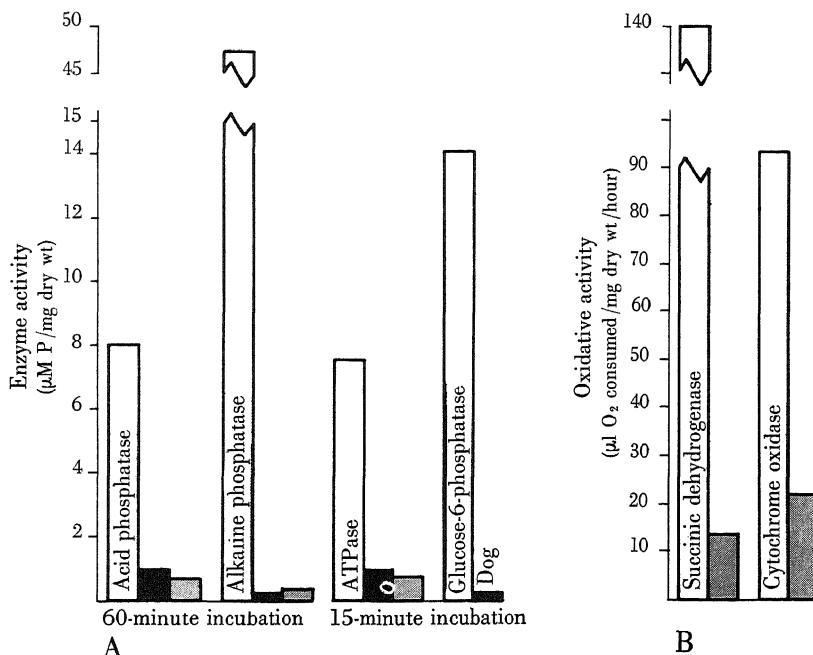
metastable state to another through an indirect *F*-process induced by agents produced within or outside the cell. Dedifferentiation follows as a consequence. That a *c*-value shift can produce an increase or a decrease of enzyme activity (Chapter 14) and changes of surface electrical potential (Chapter 10) has already been demonstrated and needs no repetition.



**Figure 18.12. PROTEIN, FAT, AND WATER CONTENT OF THE CORTICAL CELLS OF GUINEA PIG BRAIN AS A FUNCTION OF AGE.** Cells of frontal cerebral cortex. T indicates term embryos; NB, newborn; and A, adults. (Figure after Flexner and Flexner, 1950.)



**Figure 18.13. ENZYMATIC ACTIVITY OF APYRASE OF CEREBRAL CORTEX OF FETAL, NEWBORN, AND ADULT GUINEA PIGS.** (Figure after Flexner and Flexner, 1948.)



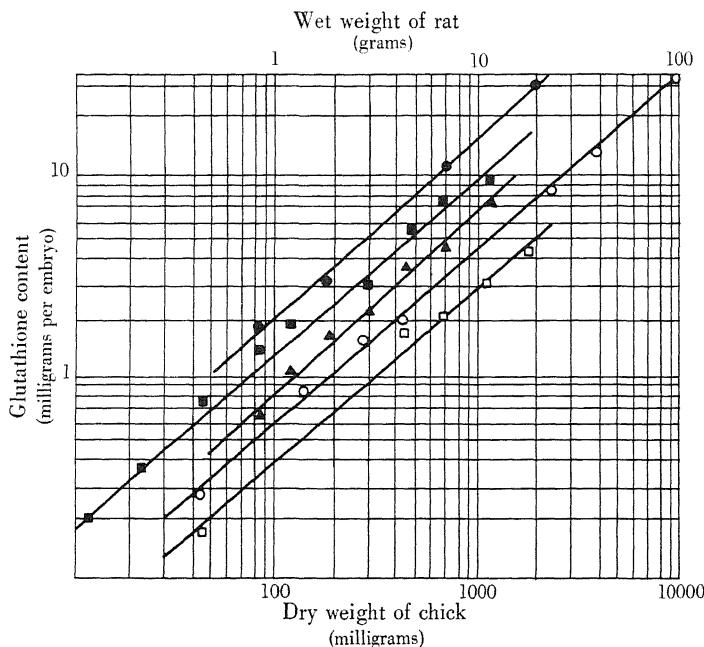
**Figure 18.14. THE DRASTIC REDUCTION IN ACTIVITIES OF A NUMBER OF ENZYMES OF KIDNEY CELLS IN TISSUE CULTURE.** A, white bars represent control in which kidney cells were isolated by treatment of tissue with trypsin; black bars represent dog kidney culture; and grey bars represent rabbit kidney culture. B, white bars represent control; grey bars represent dog kidney culture. (Figure after Burlington, 1959.)

#### B. DEDIFFERENTIATION AND INCREASED GROWTH OF CELLS IN TISSUE CULTURE IN RELATION TO THE OXIDATION-REDUCTION POTENTIAL

Although nothing is known about the factors immediately responsible for the suggested *c*-value shift in the brain of the 41- to 45-day fetal guinea pig, this is not the case in tissue culture. We know, at least, that the presence of embryo extract has a distinct influence on the transformation. The active factors in embryo extracts are complex. However, one feature, the high concentration of reduced glutathione in embryo extracts (which declines with age) is of particular interest to the present discussion (Needham, 1942, p. 420; Ephrussi, 1932; Rapkine, 1938; Borger and Peters, 1933) (Figure 18.15). There is evidence that active proliferation is promoted by low oxidation-reduction potentials in the system. First, the considerable amount of work by Hammett showing the growth-promoting action of a number of reduced thiols and the growth-inhibiting action of oxidized thiols (Hammett,

1929, 1934; Hammett and Hammett, 1933; Hammett and Lavine, 1940; Chalkley and Voegtlin, 1932) is in agreement with this point of view (see Figure 18.16).

Second, in microbiology it is well established that the growth of certain bacteria, the obligate anaerobes, depends upon a limited oxidation-reduction potential of the medium (Quastel and Stephenson, 1926; Knight and Fildes, 1930;

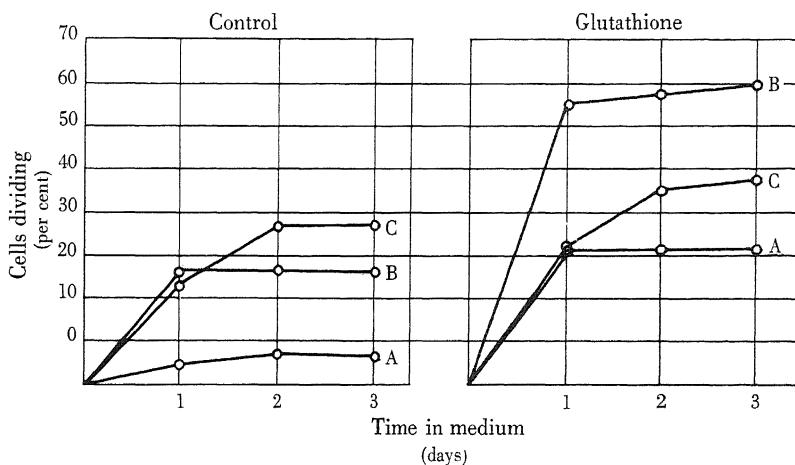


**Figure 18.15. THE DECLINE OF GLUTATHIONE CONCENTRATION IN EMBRYOS AS A FUNCTION OF AGE.** Glutathione in the embryo of chick and rat (various investigators) plotted on a double logarithmic grid. The decline in glutathione with age is shown by the fact that the sets of data are all inclined at angles less than  $45^\circ$  to the abscissa. Various symbols indicate sources of data. Chick: □, Murray (1926); ▲, Kamiya (1930); ■, Yaoi (1928); ●, Cahn and Bonot (1928). Rat: ○, Thompson and Voegtlin (1926). (Figure after Needham, 1942.)

Vennesland and Hanke, 1940). Growth ceases when the oxidation-reduction potential becomes higher than this limit. Some most convincing evidence for this view was given by Hanke and Katz (1943) who demonstrated the growth-regulating influence of the oxidation-reduction potential in experiments where this potential was varied by changing the voltage applied through a pair of platinum electrodes. Their results are shown in Table 18.1.

Third, in their synthetic media for tissue culture, Parker and his associates found that an increase of cysteine or reduced glutathione (these two agents being interchangeable) increased the rate of cell multiplication (see Parker, 1954).

The above evidence seems to indicate that a low oxidation-reduction potential is an important factor in the maintenance of the proliferative state. Our earlier



**Figure 18.16.** STIMULATION OF CELL DIVISION OF AMOEBAE BY REDUCED GLUTATHIONE. Increase of cell division in response to reduced glutathione ( $10^{-6}$  moles per liter). A, B, C, refer to amoebae of average cell volumes 0.001 to 0.0015, 0.0015 to 0.002, 0.002 to 0.0025 cubic millimeters, respectively. Control cells in standard saline. (Figure after Chalkley and Voegtlind, 1932.)

discussion has indicated that the oxidation or reduction of the SH and S—S groups alters the  $c$ -,  $c'$ -, and  $\varepsilon^0$ -value ensembles of the proteins (Section 7.4C). We suggest here that there is an optimal value of the ensemble for growth and that it is through the maintenance of this optimum that SH groups and appropriate oxidation-reduction potentials manifest their growth-stimulating effects. There are at least two possibilities whereby  $c$ -,  $c'$ -, and  $\varepsilon^0$ -value ensembles may regulate growth.

First, the entry of monomeric amino acids into cells must be the initial step

Time intervals, hr	Oxidation-reduction potential, mv			Bacterial growth	Electrolysis, coulombs
	Average	Average deviation			
1st interval: 0 to 4	128	15		—	1.5
2nd interval: 4 to 8	144	4		—	4.4
3rd interval: 8 to 10½	130	7		+	1.6
Average	135				
1st interval: 0 to 4	145	7		—	-0.65
2nd interval: 4 to 8	138	5		—	-1.30
3rd interval: 8 to 10½	130	6		+	3.5
Average	138				
1st interval: 0 to 3	141	10		—	1.9
2nd interval: 3 to 6	143	5		—	3.8
3rd interval: 6 to 9	146	2		—	4.5
4th interval: 9 to 12	144			+	4.5
5th interval: 12 to 15	146			+	6.5
Average	144				
1st interval: 0 to 4	161	4		—	4.7
2nd interval: 4 to 8	158	2		—	2.1
3rd interval: 8 to 12	157	2		—	2.6
4th interval: 12 to 16	155	12		—	3.8
5th interval: 16 to 20	158	3		—	3.3
Average	158				

**Table 18.1. THE EFFECT OF THE OXIDATION-REDUCTION POTENTIAL OF THE MEDIUM ON GROWTH OF THE ANAEROBIC BACTERIA, *BACTEROIDES VULGARIS*.** The oxidation-reduction potential of the bacteria culture was maintained electrolytically through a pair of platinum electrodes, thus obviating the need of the addition of extraneous agents. The growth of the bacteria was measured by the turbidity of the culture. The first three experiments show that at average potential of +135mv to +144mv, growth consistently occurred in from 10 to 15 hours; whereas at an average potential of +158mv, there was no growth in 20 hours. The last column (in Coulombs) indicates that an increase in reducing power of the cells accompanies and immediately precedes growth. (Table from Hanke and Katz, 1943.)

toward protein synthesis. Since synthesis involves the successful adsorption of the amino acid onto appropriate protein sites (as discussed under selective amino-acid accumulation and permeability in Chapter 9) the rate of entry of a rate-limiting amino acid may determine the rate of growth. If, on entering cells, a given amino acid is adsorbed onto cellular fixed ions through its  $\alpha$ -amino group and this  $\alpha$ -amino group has a  $c'$ -value equivalent to that of the  $\text{NH}_4^+$  ion, then, as Figures

4.9 to 4.11 show, maximum accumulation within cells could occur only at a definite *c*-value (see Section 4.4G). Such a *c*-value could conceivably be maintained as long as there is sufficient reduced glutathione to insure the proper reduced state of the protein SH groups.

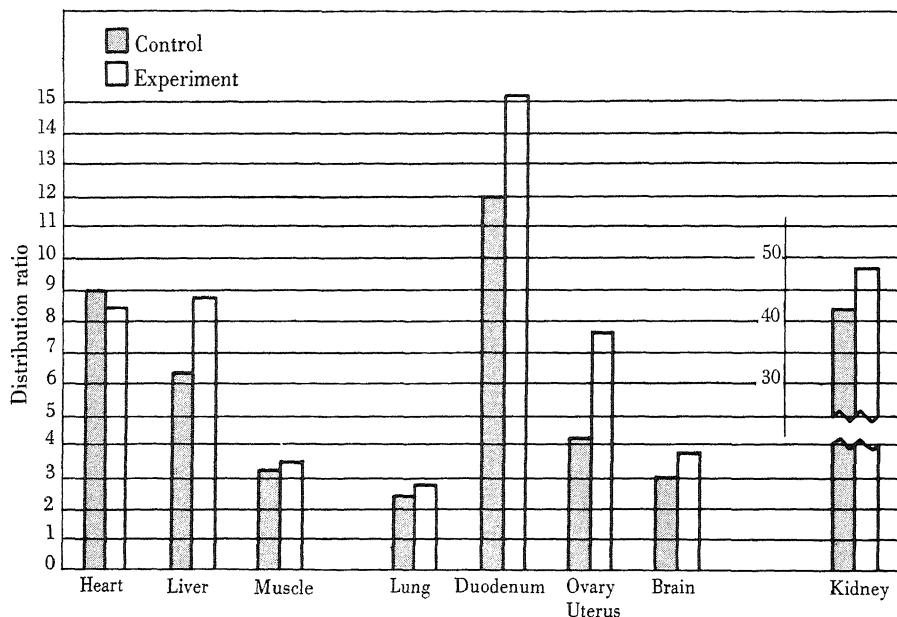
As a parallel to this concept, it is worth while to mention that estradiol, which promotes the rapid growth of virgin rat uterus, brings about a shift of the modal *c*-value of this tissue such that it goes from preferring the Cs<sup>+</sup> ion to a preference for the Rb<sup>+</sup> ion (Figure 16.1); note that the Rb<sup>+</sup> ion runs a course close to that of

Distribution ratio, uterus/serum	
Control	Experimental
5.55	16.6
4.23	13.6
5.01	14.7
4.83	9.62
Mean 4.90	Mean 13.6

**Table 18.2. THE EFFECT OF INJECTED ESTROGEN ON THE ACCUMULATION OF  $\alpha$ -AMINO ISOBUTYRIC ACID IN IMMATURE RAT UTERUS.** Each value represents results obtained from one animal. The concentration of  $\alpha$ -amino isobutyric acid in liver is not detectably altered by the injected estrogen. (Table after Noall *et al.*, 1957.)

the NH<sub>4</sub><sup>+</sup> ion in the  $\Delta E$ -versus-*c*-value plots (Figures 4.9 to 4.11). A counterpart of this experiment, more closely related to the present topic, is shown in Table 18.2. An analysis was made of the relative distribution ratio, not of Rb<sup>+</sup> ion, but of a nonmetabolized amino acid,  $\alpha$ -amino isobutyric acid (AIB), between the uterus and serum of virgin rats (Noall *et al.*, 1957). Estradiol sharply increased the uptake of this amino acid. Figure 18.17 illustrates the more general increase of AIB uptake in many tissues following treatment of rats with pituitary growth hormone as is also to be expected.

Second, let us assume that a microscopic portion of a certain protein molecule contains amino acids XYZ, in that order. This means that the synthesizing template must synthesize XYZ or nothing. Therefore, amino acid W, for example, must be able effectively to compete with X, Y, and Z for the third site on the template. If we apply the principle of the preferred counterion (Section 7.2A), we see that a unique *c*-value ensemble is necessary for protein synthesis. Our view as to how DNA may participate in the genetic control of such protein synthesis will be presented in a separate publication.



**Figure 18.17. THE EFFECT OF GROWTH HORMONE ON THE ACCUMULATION OF  $\alpha$ -AMINO ISOBUTYRIC ACID BY VARIOUS RAT TISSUES.** Administration of one unit per animal (body weight about 200 grams). The amount of  $\alpha$ -amino isobutyric acid accumulated is expressed as a ratio in comparison with plasma concentration. (Figure after Noall *et al.*, 1957.)

### 18.3. Cancer

So far, discussion of the ramifications of the association-induction hypothesis has centered about normal physiological processes. However, the conviction that life phenomena represent variations on a basic theme demands that there be, from the viewpoint of a single cell, only an artificial boundary between phenomena usually considered as representing normal function and those which are considered pathological processes. Thus, we shall end this monograph with a discussion of some studies on various aspects of carcinogenesis and of cancer, attempting to provide for them a unified interpretation.

We have discussed the major mechanisms which, in our opinion, are basic to life phenomena in detail. To avoid repetition, the following sections are deliberately synoptic; however, frequent references will be made to previous sections where parallel mechanisms have been discussed.

In Section 18.2 on growth and differentiation, it was shown that cells may modulate between two or more metastable states, one or more of these being

favorable to differentiation, the other to growth. We have also mentioned that rapidly proliferating cells in tissue culture may reverse the normal trend and gradually begin to show a loss of differentiation. It is well known that this is also the case with cancer cells; cells derived from tissues that are very different (thyroid, liver, breast, for example) are often indistinguishable following neoplastic change (Ewing, 1940). Such dedifferentiation is not merely morphological but involves changes in such functions as enzyme activity and the patterns of amino-acid and cation accumulation. In this section, we should like to show that the neoplastic change, similar to the alternation between proliferation and differentiation in growing tissue, represents a  $c$ -,  $c'$ -, and  $\delta^0$ -value shift from one metastable state to another.

More specifically, we propose that the carcinogenic process proceeds in the following manner. A carcinogenic agent\* is adsorbed onto cardinal sites of cell proteins or protein complexes. As this agent accumulates, it produces an inductive effect which leads to an unstable  $c$ -value ensemble. Finally, an abrupt shift of the  $c$ -value ensemble due to an indirect  $F$ -process leads to a new metastable state. This state, which represents the neoplastic state, is one of more rapid proliferation and is no longer under the normal regulating influence of bioregulants.

Most of the experimental findings on the action of carcinogens and the nature of neoplastic cells are not only consistent with the above scheme, but tend to verify its general applicability. To make this clear, we shall discuss these findings under three general headings: the specific binding of carcinogens to "target" cells, the action of carcinogens on the  $c$ -value ensemble of cell proteins, neoplastic transformation, a climactic indirect  $F$ -process, leading to a new metastable state.

#### A. SPECIFICITY IN THE BINDING OF CARCINOGENS

According to the principle of the variability of the preferred counterion, the adsorption of a given substance onto a protein (in competition with numerous other adsorbents) may occur only within a narrow range of  $c$ -values. Experiments have shown that the various organs of the rat have different modal  $c$ -values (Table 9.5); moreover, the  $c$ -value ensembles of specific organs, liver, for example, differ from one species to the next. Therefore, if the action of carcinogens does depend on their adsorption onto protein, one would expect a specific action on a particular organ, even though the entire animal is exposed to the carcinogenic agent. Species differences in sensitivity to various agents are also to be expected. Thus, 4-dimethylaminoazobenzene (DAB) causes cancer in no rat organ but the liver; paralleling this, bound DAB is found only in the liver (Table 18.3). Furthermore, DAB causes cancer in the liver only of rats and, to a lesser extent, mice; again, as a

\* Although organic carcinogens are dealt with throughout, it is not our intention to suggest that these are uniquely responsible for the etiology of cancer. Any of the known carcinogenic agents (viruses, radiation, mechanical stimulation, and so on) may act, directly or indirectly, in a similar manner.

parallel, DAB is found in the liver of rats, to a smaller extent in the liver of mice, and not at all in the other rodents and fowl studied (Table 18.4). Other experimental findings indicating the specificity of binding of carcinogens are, with some qualifications, N-2-fluorenylacetamide binding in rat liver (Dyer and Morris,

Tissue	A Animals fed DAB and basal diet	B Animals fed basal diet	A - B
Liver	0.330	0.050	0.280
Lung	0.044	0.030	0.014
Heart	0.088	0.058	0.030
Kidney	0.074	0.054	0.020
Skeletal muscle	0.038	0.038	0.000
Small intestine	0.034	0.046	-0.012
Red blood cells	0.356	0.332	0.024
Spleen <sup>b</sup>	0.304	0.190	0.114
Blood plasma <sup>a</sup>	0.078	0.018	0.060

**Table 18.3. CONCENTRATION OF BOUND 4-DIMETHYLAMINOAZOBENZENE IN RAT TISSUES.** The concentration of bound dye (DAB) was determined by colorimetric measurement at 520m $\mu$  of alkaline hydrolytic product of tissue powder. (Data from Miller and Miller, 1947.)

<sup>a</sup> The blood was removed by perfusing the whole animals.

<sup>b</sup> Spleens of dye-fed rats were enlarged and their high color intensity must be due to their high contents of blood and breakdown products as indicated by a different absorption spectrum of the color from that of the dye.

1956; Weisburger and Weisburger, 1958, p. 383), and 3,4-benzpyrene binding in mouse epidermis (Miller, 1951; C. Heidelberger, 1959). In fact, this observation has such general significance, it led Heidelberger to conclude: "it may be stated emphatically at the outset that *the binding of chemical carcinogens to the proteins of the susceptible tissue has been found in every case thus far investigated . . .*" (Heidelberger, 1959, p. 179; present author's italics.)

#### B. ALTERATIONS PRODUCED BY CARCINOGENS IN THE *c*-VALUE ENSEMBLE OF CELL PROTEINS

##### (1) The principle of additivity

Miller and Miller (1947, 1953) have shown that different amino azo dyes are taken up by liver in different amounts; those dyes which are most strongly adsorbed and are, therefore, taken up in greatest amount are the ones which cause cancer. From equation (5-2) we see that these two factors are most important in the production

Animal	Dyes in liver						Susceptibility to DAB	
	Concentration of free dyes in blood, $\mu\text{M/l}$		"Free dyes," $\mu\text{M/kg}$ fresh tissue			Bound dyes, $A/100\text{g}$ liver powder		
	DAB	AB	DAB	MAB	AB			
Albino rat (Sprague-Dawley)	0	68.0	1.41	0.89	9.29	0.236	high	
Albino mouse (abc)	2.02	421	0.75	0.51	11.2	0.070	low	
Guinea pig	0	0	0	0	0	0	none	
Rabbit	0	0	0	0	1.02	0	very low	
Cotton rat	0	7.87	0.40	0.05	7.81	0	very low	
Chicken (White leghorn)	0	60.4	0.18	0.19	3.96	0	none	

**Table 18.4.** 4-DIMETHYLAMINOAZOBENZENE ACCUMULATION IN LIVERS OF ANIMALS SUSCEPTIBLE TO THIS DYE. Concentration of bound dye determined by colorimetric measurement at  $520\text{m}\mu$  of alkaline hydrolytic product of tissue powder and expressed as optical absorption  $A$ . The dye cannot be released by boiling organic solvents or by hot TCA which removes all of the nucleic acid. This and the fact that it is released after prolonged tryptic digestion suggests that it is bound to proteins. Concentration of "free" dyes were determined in extracts of livers that had previously been perfused with Ringer's solution. The last column represents the susceptibility of the various animals to induction of liver tumors by DAB. High levels of bound DAB were found in rats, which develop a high incidence of liver tumor in response to DAB. In mice, which develop liver tumors slowly, only low levels of bound dye accumulate in the liver. Other species, known to be DAB resistant, accumulate negligible amounts of dye. Note also that a similar relation holds for the "free" DAB. The "free" dye is extracted with strongly alkaline alcohol solution from fresh minced tissue. For this reason, the present author added the quotation marks, as such a "free" dye does not necessarily exist in the state in which it exists as a free aqueous solution. In fact, we believe that this is also adsorbed, but onto different types of sites than the bound dye. Support for this can be found in a comparison of the "free" DAB and AB in liver with the levels of these in blood. It is clear that there is also a high degree of selective accumulation of "free" DAB particularly in rat liver. The data represent averages from two animals fed the carcinogen-containing diet for two months. The carcinogens were DAB, *p*-dimethylaminoazobenzene; MAB, *p*-monomethylaminoazobenzene; AB, *p*-aminoazobenzene. Conversion of dye concentrations to molar values were made on the basis of the following molecular weights: AB, 197.23; MAB, 212.26; DAB 225.28. (Data of Miller and Miller, 1947.)

Group No.	Carcinogens fed for the period	Survival <sup>a</sup>	No. rats with gross cirrhosis		No. rats bearing tumors in the				Small intestine	Negative survivors
			Mild	Moderate	Liver	Ear duct				
Series A	(0-90 days)	(90-180 days)	(180 days)	(180 days)	(180 days)	(270 days)	(270 days)	(270 days)	(270 days)	
1	0.018 per cent AAF + 0.020 per cent 3'-Me-DAB	same	16/16	9 7	10	16	3	0	0	
2	0.018 per cent AAF	same	16/16	10 2	1	12	3	1	1 <sup>b</sup>	
3	0.020 per cent 3'-Me-DAB	same	16/16	0 0	0	0	0	0	0	16
4	0.036 per cent AAF	0.040 per cent 3'-Me-DAB	16/16	9 7	10	14	3	4	0	
5	0.040 per cent 3'-Me-DAB	0.036 per cent AAF	15/16	10 5	4	12	0	0	0	1 <sup>b</sup>
6	0.036 per cent AAF	none	16/16	7 4	9	13	3	7	0	
7	0.040 per cent 3'-Me-DAB	none	16/16	0 0	1	2	0	0	0	14
Series B	(0-130 days)	(130 days)	(130 days)	(130 days)	(220 days)	(220 days)	(220 days)	(220 days)	(220 days)	
8	0.018 per cent AAF + 0.020 per cent 3'-Me-DAB	20/20	6 10	9	19	2	2	0	0	
9	0.018 per cent AAF	20/20	9 0	0	2	0	0	0	16	
10	0.020 per cent 3'-Me-DAB	19/20	0 0	0	0	0	0	0	0	17
11	0.025 per cent ABT + 0.020 per cent 3'-Me-DAB	15/15	0 0	0	1	8	1	6		
12	0.018 per cent AAF + 0.064 per cent 4'-Me-DAB	14/15	4 1	0	3	0	0	0	8 <sup>b</sup>	
13	0.025 per cent ABT	14/15	0 0	0	0	3	3	3	8	
14	0.064 per cent 4'-Me-DAB	15/15	0 0	0	0	0	0	0	7	

**Table 18.5. THE SYNERGISTIC ACTION OF TWO HEPATIC CARCINOGENS.** Note particularly that, in series B, continued feeding of a diet containing 0.018 per cent 2-acetylaminofluorene (AAF) and 0.020 per cent 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB) created many more instances (Experiment 8) of liver tumor than the sum of tumor incidents in rats treated with the same concentrations of dyes, each separately (Experiments 9, 10). ABT, 3-acetylaminodibenzothiophene; 4'-Me-DAB, 4'-methyl-dimethylaminoazobenzene. (Data from MacDonald *et al.*, 1952.)

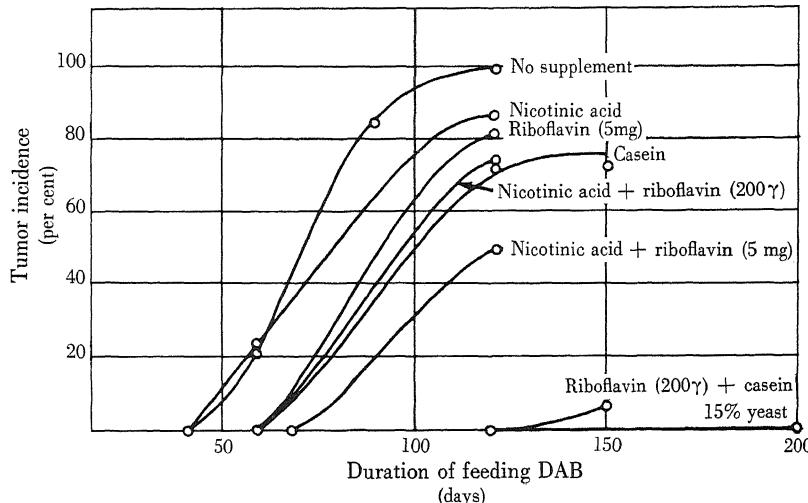
<sup>a</sup> Survival is expressed as the number of rats alive at the end of the feeding period over the number started.

<sup>b</sup> Other tumors found in this series included a myxofibroma (Group 5), benign cystadenoma (Group 9), a fibrosarcoma (Group 13), and a mammary adenocarcinoma (Group 2).

of the indirect *F*-effect; that is, a large quantity of adsorbent must be present and this adsorbent must have a high binding energy. Both these criteria are, apparently, fulfilled by the cancer-producing amino azo compounds.

## (2) The pattern of action of carcinogens

Carcinogens show synergistic\* and antagonistic† relationships with other carcinogens (see Table 18.5). They also show similar interactions with vitamins, drugs,



**Figure 18.18. THE ANTAGONISTIC EFFECT OF VITAMINS AND OTHER DIETARY FACTORS ON THE CARCINOGENIC ACTION OF *p*-DIMETHYLAMINOAZOBENZENE (DAB).** (Figure after Kensler *et al.*, 1941.)

hormones, and SH reagents‡ (Figure 18.18). For example, carcinogenesis may be brought about, not only by manifestly noxious agents but by purely physiological agents such as hormones (Lacassagne, 1932; Gardner, 1953; Mühlbock, 1956) (Table 18.6). Conversely, small amounts of carcinogens may actually promote normal growth, much as vitamins or hormones do (Morigami, 1940; Cook *et al.*, 1939; Hollaender *et al.*, 1939) (Figure 18.19). Such evidence indicates that

\* See MacDonald *et al.*, 1952; Greenstein, 1954; Simpson and Cramer, 1945.

† See Berenblum, 1935; Lacassagne *et al.*, 1945; Crabtree, 1946; Steiner and Edgcomb, 1952; Richardson and Cunningham, 1951.

‡ See W. U. Gardner *et al.*, 1944; Schaefer *et al.*, 1950; Copeland and Salmon, 1946; Haddow and Sexton, 1946; György *et al.*, 1941; du Vigneaud *et al.*, 1942; Crabtree, 1945.

carcinogens must act on the same apparatus as other bioregulants; within the framework of the present theory, this means that they act on the  $c$ -,  $c'$ -, and  $\varepsilon^0$ -value ensembles of the cell proteins.

Strain of mice		No. of animals	Total number of hypophyseal glands implanted per animal, average	Animals developing mammary tumors, %
$C_3H_f/He/A$	Control	8	0	0
	Experimental	8	114	100
$F_i(\text{♀ } O_{20} \times \text{♂ } DBA_f)$	Control	8	0	0
	Experimental	8	148	87
$F_i(\text{♀ } C_{57} \text{ black} \times \text{♂ } DBA_f)$	Control	7	0	0
	Experimental	7	206	86

**Table 18.6. THE EFFECT OF SUCCESSIVE TRANSPLANTS OF HYPOPHYSIS ON THE INCIDENCE OF MAMMARY-GLAND TUMOR IN MICE.** Females of certain strains of mice, receiving subcutaneous transplantation of five hypophyses, once weekly for a year or longer, developed high incidences of mammary tumors. The data show that hormonal imbalance may lead to tumor development. (Table from Mühlbock, 1956.)

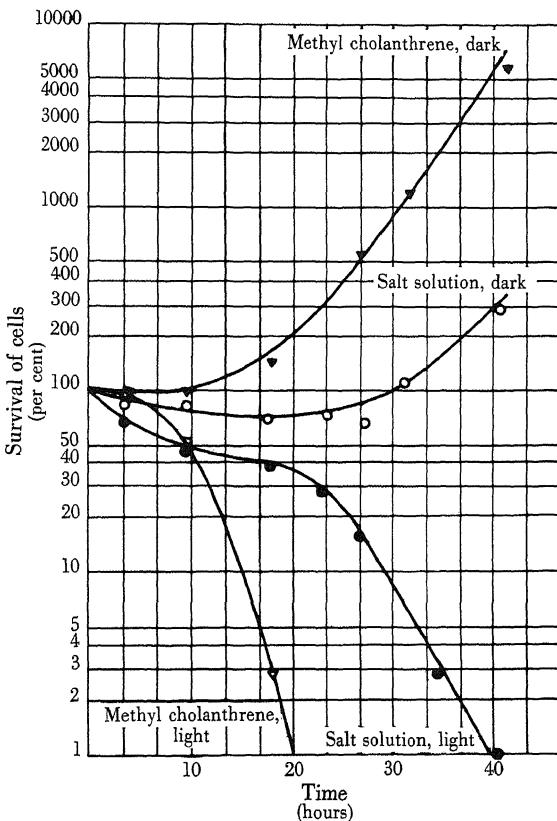
### (3) Electronic distribution and the effectiveness of carcinogens

One of the most exciting developments in oncology is the discovery of correlations between the electronic configuration of a compound and its effect as a carcinogen.\* Although later theories are more exact, the work of Mme. Pullman (1947) illustrates simply the principle involved; she showed, using a long series of polycyclic hydrocarbons, that carcinogenicity bears a direct relation only to the total charge (or electron density) of a part of the molecule called the K-region (Table 18.7). Threshold activity was found with a total charge of 1.291; above this, carcinogenicity increased roughly in proportion to the charge as shown by Figure 18.20.

Lacassagne *et al.* (1956) showed a similar relation between  $pK$  and carcinogenic activity in the benzacridine series (Table 18.8). In addition, they pointed out a correlation (Figure 18.21) between the electron density and  $pK$ ; this shows the existence of a direct relationship between electron density and  $c$ -value if we recall the relation between the proton dissociation energy and the  $c$ -value as plotted in Figure 4.9. The fact that carcinogenicity among polycyclic hydrocarbons is related to the  $pK$  and other manifestations of the electronic distribution, rather than to the steric attributes of these agents, is evidence indicating the importance of the principle of the variability of the preferred counterion in biological specificity.

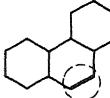
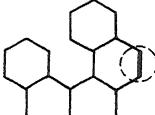
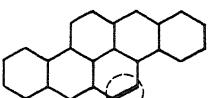
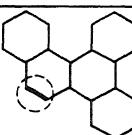
\* See O. Schmidt, 1938; 1939a,b,c; Svartholm, 1942; Pullman and Pullman, 1955b; Daudel and Daudel, 1950; Coulson, 1953; Badger, 1954; B. Pullman, 1959; Lacassagne *et al.*, 1956.

A high electron density at a particular locus on a molecule signifies a high *c*-value analogue for that group. This group would, in general, tend to adsorb onto cardinal sites of high *c'*-value (Section 6.2). Such cardinal adsorption would then bring about cooperative changes of the *c*-value ensemble (see Bergmann, 1942;



**Figure 18.19. THE STIMULATORY EFFECT OF METHYLCHOLANTHRENE ON YEAST GROWTH IN THE DARK AND THE TOXIC EFFECT OF METHYLCHOLANTHRENE IN NEAR ULTRAVIOLET LIGHT.** Methylcholanthrene, a known carcinogen, at a concentration of  $10^{-8}$  stimulates the rate of growth of yeast in the dark; this beneficial effect changes to toxicity when the treated yeasts are exposed to light with wave lengths near the ultraviolet range. (Figure after Hollaender *et al.*, 1939.)

Badger, 1954). On the other hand, since electron density is related to chemical reactivity (see Section 7.2C), the actual reaction may well be the formation of some additional complex (Miller and Miller, 1952; Wiest and Heidelberger, 1953), or even a manifest chemical reaction such as the reduction of the recipient molecule (Pullman and Pullman, 1954). The point of crucial importance is that the carcinogen must react with the cell proteins and, in so doing, must bring about a

Compound	Structural formula showing K-region	Carcinogenic activity
Phenanthrene		—
3,4,5,6-Dibenzphenanthrene		—
Anthanthrene		—
3,4,8,9-Dibenzpyrene		++++
1,2,3,4-Dibenzphenanthrene		+

**Table 18.7. THE K-REGION OF A FEW EXAMPLES OF HETEROCYCLIC HYDROCARBON COMPOUNDS AND THEIR CARCINOGENIC ACTIVITY.** The K-region is shown as an encircled heavy line. (Data selected from Pullman and Pullman, 1955b.)

*c*-value shift strong enough to produce the particular indirect *F*-effect corresponding to carcinogenesis.

#### C. CARCINOGENESIS AS AN INDIRECT *F*-PROCESS LEADING TO A NEW METASTABLE STATE

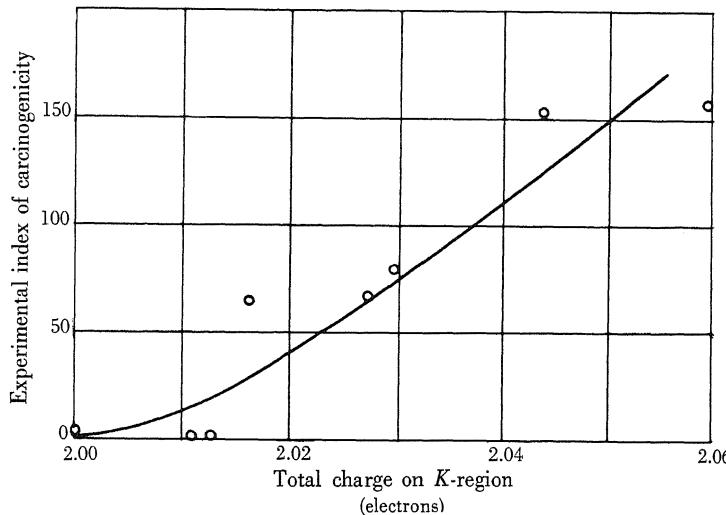
As mentioned, neoplasms tend to be strikingly uniform despite differences in their tissues of origin. Thus, we may say that the uniformity of the response to neoplastic transformation stands in sharp contrast to the variety of eliciting stimuli. In this respect the response to carcinogenesis bears a marked resemblance to the phenomena of nerve excitation, muscle contraction, egg activation, and protein denaturation.\* We shall present a collection of experimental observations

\* Rondoni (1955) has discussed the resemblance between carcinogenesis and denaturation, noting that an "increase of entropy takes place in the process of protein denaturation, just as it most probably does in carcinogenesis." The present theory is concerned with attributing both protein denaturation and carcinogenesis to a similar underlying event, but differs from Rondoni in that both processes are viewed as being due basically to a change in free energy through the indirect *F*-process.

demonstrating the singleness of the neoplastic state. This strongly indicates an abrupt all-or-none transformation; otherwise intermediate stages are bound to be observed.

### (1) The adsorption of 4-dimethylaminoazobenzene (DAB)

Miller and Miller (1947) have shown that when normal rats are fed the carcinogenic dye, 4-dimethylaminoazobenzene, their livers concentrate the dye much more

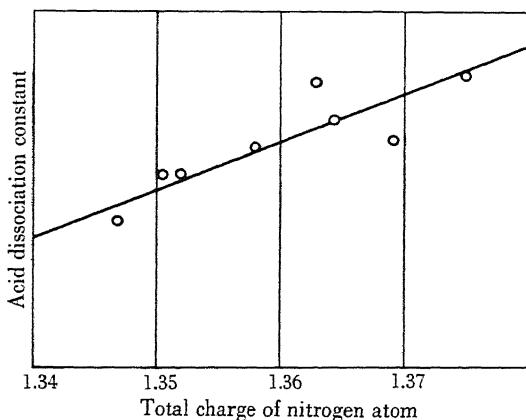


**Figure 18.20. CORRELATION BETWEEN EXPERIMENTALLY OBSERVED INDEX OF CARCINOGENICITY AND THE TOTAL ELECTRONIC CHARGE ON THE K-REGION.** The index of carcinogenicity, according to Iball (1939), depends on two factors: (1) the per cent of cancer induced in the mice that survived beyond the shortest time of the latent period; (2) the average length of this latent period in the animals developing cancer. The index is  $[(1)/(2)] \times 100$ . Total electronic charge was calculated theoretically. (Daudel, 1948: see Coulson, 1953.)

effectively than any other tissue. However, once the cancer arises among the liver cells, the dye disappears, even though the rats are fed the dye continually (Table 18.9). This observation is the main support for the Millers' theory of carcinogenesis through protein depletion (Miller and Miller, 1947; Miller *et al.*, 1949; see also C. Heidelberger, 1959). Although we disagree with the idea that there is an actual physical loss of protein in the carcinogenic process, we feel that depletion could perhaps also be interpreted as a loss of certain normal traits and functions of some particular proteins. Invoking the principle of the variability of the preferred counterion, it is suggested that, following the neoplastic change, the cellular *c*-value ensemble switches to preferring ions or molecules other than DAB.

Benz[c]acridine series	<i>pKa</i>	P	I	Benz[a]acridine series	<i>pKa</i>	P	I
7,9-dimethyl	4.26	81	41	9,12-dimethyl	5.13	11	0
7,10-dimethyl	3.99	56	64	12-methyl	4.60		
7,8,9,11-tetramethyl	3.98	50	20	8,9,12-trimethyl	4.59	0	0
10,11-dimethyl	3.74	0	0	9-methyl	4.52		
10-methyl	3.68		0	10-methyl	4.22		
8-methyl	3.67		0	unsubstituted	3.95		
5,7-dimethyl	3.63	0	0				
unsubstituted	3.24						

**Table 18.8.** THE RELATION BETWEEN THE ACID DISSOCIATION CONSTANT AND THE CARCINOGENIC ACTIVITY OF BENZACRIDINE DERIVATIVES. Acid dissociation constants were experimentally determined (Pages-Flon *et al.*, 1953). Definition of carcinogenic index is given in Figure 18.20. P represents the carcinogenic index by painting; I represents the carcinogenic index by injection. (Table from Lacassagne *et al.*, 1956.)



**Figure 18.21.** INCREASE OF ACID DISSOCIATION CONSTANT OF BENZACRIDINES WITH THE TOTAL CHARGE OF THE NITROGEN ATOM. (Figure after Lacassagne *et al.*, 1956.)

That is, in carcinogenesis there is a loss of the characteristic by which we recognize these proteins, the *c*-value ensemble. (See Section 7.1 on the characterization of protein.)

(2) The *c*-value change as shown by the change of the  $K^+/Ca^{++}$  ratio

The present theory predicts that each tissue, as a function of its modal *c*-value, will selectively adsorb a characteristic molar quantity of  $K^+$  ion when bathed in

plasma containing appropriate amounts of ions such as  $K^+$  and  $Na^+$ . This is merely a corollary to the prediction of the theoretical curves (Figures 4.9 to 4.11) that, as the  $c$ -value ensemble changes, there will be a gain or loss of intracellular  $K^+$  ion at the expense of salt linkages,  $Na^+$  ion, and so forth. Thus, a prominent

"Free" aminoazo dyes, $\mu M/kg$ of fresh tissue						
	Liver			Tumor		
Sample	DAB <sup>b</sup>	MAB <sup>b</sup>	AB <sup>b</sup>	DAB	MAB	AB
1	0.88	0.42	8.12	0.44	0.28	7.61
2	0.57	1.46	5.58	0.22	0.85	9.65
3	1.06	1.13	9.65	1.01	0.61	1.52
Average <sup>a</sup>	0.84	1.04	7.62	0.71	0.33	6.60
Range <sup>a</sup>	(0.31-1.72)	(0.42-1.84)	(2.54-15.2)	(0.18-1.45)	(0.00-0.85)	(1.01-13.2)
Bound aminoazo dyes, $A^c/100\text{mg}$ powder						
4		0.176			0.066	
5		0.208			0.048	
6		0.184			0.050	
7		0.195			0.042	
Average (4-7)		0.191			0.052 <sup>d</sup>	

**Table 18.9. THE CONCENTRATIONS OF FREE AND BOUND DYES IN LIVER TUMOR AND IN ADJACENT LIVER TISSUE.** Rats were fed *p*-dimethylaminoazobenzene from four to six months. After perfusion of the excised liver, the tumorous and nontumorous portions were separated and analyzed for free and bound aminoazo dye. Data show the presence of "free" dye in tumor as well as in nontumor tissue, but consistent absence of bound dye in tumor tissue. (Data of Miller and Miller, 1947.)

<sup>a</sup> Average and range of eight samples including samples one to three.

<sup>b</sup> DAB, *p*-dimethylaminoazobenzene; MAB, *p*-monomethylaminoazobenzene; AB, *p*-aminoazobenzene.

<sup>c</sup> Uncorrected colorimetric reading, see legend of Table 18.4.

<sup>d</sup> Normal liver powder, 0.050.

change of accumulated  $K^+$  ion would be the most direct evidence for an abrupt  $c$ -value-ensemble shift during carcinogenesis. In fact, a large body of evidence showing a rise of  $K^+$  ion in malignancies has been accumulated over a number of years, usually in conjunction with observations on the fall of  $Ca^{++}$ -ion content (a rise of  $K^+/Ca^{++}$  ratio) (see K. Stern and Willheim, 1943, for review; Brunschwig *et al.*, 1946; Dunham *et al.*, 1946; Fujiwara *et al.*, 1937). This marked  $K^+$ -ion-con-

centration change suggests that, in the process of carcinogenesis, there is indeed an abrupt *c*-value shift (Tables 18.10 and 18.11; see also Section 18.1 on fertilization).

(3) The change of enzymatic activities from a specific pattern to a common one

In Chapter 15, we offered evidence for the view that enzyme activity is an expression of the *c*-, *c'*-, and  $\delta^0$ -values of the sites on proteins. It follows that the specific

Patient	Water, % of fresh tissue <sup>a</sup>		Calcium, mM/kg of fresh tissue <sup>a</sup>		Potassium, mM/kg of fresh tissue <sup>a</sup>		Total nitrogen, g/kg of fresh tissue <sup>a</sup>	
	Mucosa	Neoplasm	Mucosa	Neoplasm	Mucosa	Neoplasm	Mucosa	Neoplasm
A	85.1	83.8	3.69	2.52	48.6	92.6	22.8	23.0
B	87.9	86.8	3.67	1.57	35.3	65.5	18.1	20.6
C	83.1	82.9	3.87	1.87	61.9	96.9	24.4	23.7
D	85.5	83.0	2.77	1.87	51.1	88.5	19.6	22.3
E	86.5	85.5	2.15	1.95	36.1	66.7	19.4	21.9
F	83.2	80.0			52.9	66.0	20.4	24.9
G (2 cases)	82.7	83.6	3.26	2.69	57.8	88.2	22.7	23.8
		83.6		2.40		97.7		23.0
H	84.2	86.0	2.52	2.15	41.9	56.0	19.6	23.8
I	81.0	83.2	2.69	0.66	55.5	89.0	21.2	21.9
J	85.0	84.1	2.37	1.58	44.2	76.2	19.6	22.6
K	83.5	82.0	2.69	2.50	54.7	88.7	23.2	24.3
Average	84.3	83.7	3.02	1.97	49.1	81.1	21.0	23.0

**Table 18.10. ANALYSES OF GASTRIC CARCINOMA AND ADJACENT NORMAL MUCOSA.** Specimens of strips of normal mucosa were separated from neoplastic portions of the stomach. The potassium and calcium contents of the controls are similar to those of mucosa from noncancerous control stomachs. (Brunschwig *et al.*, 1946.)

<sup>a</sup> Weight of fresh tissue after removal of fat was used in calculations.

pattern of enzyme activity observed in a particular tissue follows from the specific *c*-value ensemble characteristic of that tissue.

Greenstein (1954), in his treatise on the chemistry of cancer cells, reviewed the extensive literature on the enzymatic activities of normal and neoplastic tissues. He reached the conclusion that while "each normal tissue is characterized by the possession of an individual pattern of enzymatic activity . . . the tumor ends by possessing a chemical pattern very largely the same as that of other tumors of quite different etiology or histogenesis" (Tables 18.12 and 18.13). This general observation is again consistent with the concept of the change from the specific *c*-value ensemble to a common pattern shared by all neoplastic tissues.

- (4) The *c*-value change as shown by changes from specific patterns of amino-acid accumulation in normal differentiated tissue to a common pattern in neoplastic tissues

Each normal tissue has a typical pattern of accumulation of the free amino acids. Tumors, on the other hand, regardless of their origin, show a similar pattern

Patient	% H <sub>2</sub> O <sup>a</sup>		Calcium, <sup>a</sup> mg/100g of fresh tissue		Potassium, <sup>a</sup> mg/100g of fresh tissue		Nitrogen, g/kg of fresh tissue	
	Mucosa	Neoplasm	Mucosa	Neoplasm	Mucosa	Neoplasm	Mucosa	Neoplasm
A	85.6	85.6	9.9	6.2			20.9	20.3
B	85.6	83.4	8.2	5.3	187	413	19.3	22.0
C	85.6	84.5	7.6	6.2		189	19.3	19.4
D	82.7	82.9	6.7	5.7	234	190	22.0	24.8
E	86.5	85.5	7.2	6.2	244	320	20.1	22.3
F	86.1	84.2	8.6	7.2	214	324	20.9	22.6
G	88.5	84.7	12.7	12.2	289	322	16.9	24.5
H	85.0	83.0	8.5	5.5	209	295	21.4	22.9
I	86.3	83.6			164	184	18.0	21.5
J	85.0	83.0	7.8	4.9	206	285	20.5	24.7
K	87.2	83.2	9.7	9.4	170	260	17.2	22.7
L	84.0	90.0	9.7	8.1	180	325	22.7	22.2
M	81.0	83.0	11.8	7.7	211	304	20.1	23.1
N	80.0	86.0	12.4	9.0	205	390	22.2	24.6
Aver-								
age	84.9	84.5	9.3	7.2	209.4	292.4	20.1	22.7

Table 18.11. ANALYSES OF CARCINOMA OF THE COLON AND ADJACENT UNINVOLVED MUCOSA. Details of the experiment were similar to those for Table 18.10. (Dunham *et al.*, 1946.)

<sup>a</sup> Weight of fresh tissue after removal of fat was used in calculations.

(E. Roberts and Tishkoff, 1949; Roberts and Frankel, 1949, Figure 18.22). In Section 9.3C, we pointed out that this preferential adsorption of an  $\alpha$ -amino acid depends upon the *c*- and *c'*-values of the fixed ionic group of the tissue proteins. That each normal tissue possesses a typical pattern is to be expected once we have established that each tissue and organ has a specific *c*-value ensemble (Table 9.5, Figures 9.8 and 9.9). That tumor cells lose the specific pattern of free amino-acid accumulation characteristic of their source tissue and instead take on a pattern common to all cancer cells suggests that, whatever the origin of the cells, once they have become malignant, they tend to assume similar *c*-value ensembles.

The experimental findings collected above point out that the carcinogenic

Tissue	Arginase	Catalase	Xanthine dehydrogenase	Acid phosphatase	Alkaline phosphatase	Deoxyribonucleopolymerase	Ribonuclease-polymerase	Esterase	Cytochrome oxidase	Cystine desulfurase	Deoxyribonucleo-deaminase	Ribonuclease	Arginase for BA	Dihydropeptidase II	Dihydropeptidase I
<b>Normal tissue</b>															
Liver	246	8.00	10	12	4	14	0.12	411	8	6	7	16	34	15	20
Hyperplastic breast <sup>a</sup>	67	0.02	45	18	9	8	0.04	—	—	—	—	—	—	—	—
Lymph nodes	20	0.02	240	49	8	25 <sup>b</sup>	0.46	25	3	0	25	60	—	0	—
Bone marrow	4	0.01	45	22	23	7	0.60	—	—	—	—	—	—	—	21
Spleen	6	0.12	30	73	17	16	0.28	106	2	—	88	96	35	—	—
Kidney	42	3.20	15	15	1072	10	0.08	108	11	3	64	82	7	23	22
Skeletal muscle	4	0.01	92	19	2	12	0.07	13	6	0	0	0	3	0	18
Cardiac muscle	7	0.01	50	18	12	9	0.04	13	19	0	—	—	—	0	—
Skin	27	0.01	45	30	5	10	0.17	3	—	0	—	—	—	0	—
Lung	50	0.22	>300	33	36	8	0.06	68	4	0	0	34	—	0	—
Intestinal mucosa	80	0	6	34	2789	15	0.68	973	1	0	5	10	—	0	20
Gastric mucosa	4	0	>300	27	17	6	0.27	48	1	0	—	—	—	0	—
Thymus <sup>c</sup>	2	0	>300	5	3	3	0.12	3	—	0	—	—	—	0	—
Pancreas	8	0.01	30	10	1	5	0.78	1820	2	2	—	—	7	4	19
Brain	3	0	15	15	12	4	0.18	7	10	0	0	30	11	0	20
Adult bone	—	—	—	50	420	—	—	1	—	0	—	—	—	—	—
<b>Tumor<sup>d</sup> and strain of mice</b>															
Hepatoma 1 <sup>e</sup> ( <i>I</i> × DBA)	40	0.60	10	10	1	11	—	—	—	0	—	—	—	—	0
Hepatoma A <sup>e</sup> (C3H)	34	0.05	38	21	0	21	0.26	172	3	0	40	42	—	—	0
Hepatoma 98/15 <sup>f</sup> (C3H)	30	0.08	35	22	1	21	0.30	—	3	0	44	50	34	20	0
Hepatoma 7A/77 <sup>g</sup> (A)	37	0.80	18	22	4	15	0.29	207	4	0	—	—	—	—	0
Hepatoma 587 <sup>f</sup> (A)	33	0.24	20	21	5	15	0.27	103	4	0	42	50	32	20	0
Mammary carcinoma (C3H)	114	0.01	30	21	22	20	0.27	26	6	0	—	—	—	20	0
Lymphoma 72942 <sup>f</sup> (A)	26	0.01	25	10	10	4	0.17	—	—	0	—	—	—	—	0
Lymphoma Y-103 <sup>f</sup> (DBA)	31	0.01	40	12	4	4	0.11	—	—	0	25	60	—	—	0
Lymphoma 163 <sup>f</sup> (C3H)	28	0	22	12	6	4	0.12	8	1	0	—	—	—	—	0
Pigmented melanoma <sup>h</sup> (DBA, C)	49	0.01	—	19	1	3	0.09	27	2	0	—	—	28	20	0
Sarcoma 37 (DBA, C, Y)	49	0	15	23	2	3	0.16	6	2	0	—	—	25	20	0

CR-180 (DBA)	49	0	15	21	2	3	0.15	6	2	0	0	43	—	18	0
Intestinal adenocarcinoma (A)	26	0	30	19	3	14	0.20	11	3	0	2	10	—	22	0
Gastric adenocarcinoma (C3H)	—	—	—	16	0	6	—	—	—	0	—	—	—	—	0
Squamous cell carcinoma of stomach (A)	52	0	17	6	3	3	0.16	19	3	0	—	—	32	21	0
Lung tumor F (A)	29	0	25	11	1	3	0.11	6	2	0	0	34	30	19	0
Sarcoma <sup>i</sup> (C3H)	25	0	22	19	2	4	0.12	6	2	0	—	—	—	—	0
Spindle-cell sarcoma (C3H)	28	0	40	12	1	4	0.10	6	2	0	—	—	—	—	0
Rhabdomyosarcoma (C)	28	0	42	8	24	15	0.18	—	—	0	—	—	—	—	0
Osteogenic sarcoma <sup>j</sup> (C3H)	27	0	35	135	1100	3	0.10	—	—	0	—	—	—	—	0

**Table 18.12. A COMPARISON OF ENZYMATIC ACTIVITIES OF NORMAL AND NEOPLASTIC MOUSE TISSUES.** The extensive and meticulous data of Greenstein are tabulated in his monograph (1954) where the exact experimental details are given. It need only be mentioned here that enzymatic activities for the normal and neoplastic tissues were assayed in exactly the same way and the results given in numbers. Normal tissues from four strains of mice, C, C3H, A, and DBA, were studied while neoplastic tissues studied were from one or another of these strains, as indicated. (Table from Greenstein, 1954.)

<sup>a</sup> Induced by subcutaneous implantation of stilbesterol pellets.

<sup>b</sup> Probably somewhat higher than the value given.

<sup>c</sup> Animals two months old.

<sup>d</sup> With exception of the spontaneous mammary carcinoma and primary sarcoma induced by methylcholanthrene, all the tumors studied were transplants in late generation.

<sup>e</sup> Originally induced by injection of 2-amino-5-azotoluene.

<sup>f</sup> Originally spontaneous.

<sup>g</sup> Originally induced by injection of carbon tetrachloride.

<sup>h</sup> The cytochrome oxidase activity of the melanotic and amelanotic melanomas are nearly identical. The melanotic tumor contains an active tyrosinase and dopa oxidase, together with a cyanide-insensitive system or systems which oxidize *p*-phenylenediamine; the amelanotic melanoma is completely lacking in all these enzymes.

<sup>i</sup> Primary tumor induced by administration of methylcholanthrene.

<sup>j</sup> Representing an early (third) transplant of this tumor; the later (17th, etc.) transplants lost the osseous elements together with the phosphatase activity.

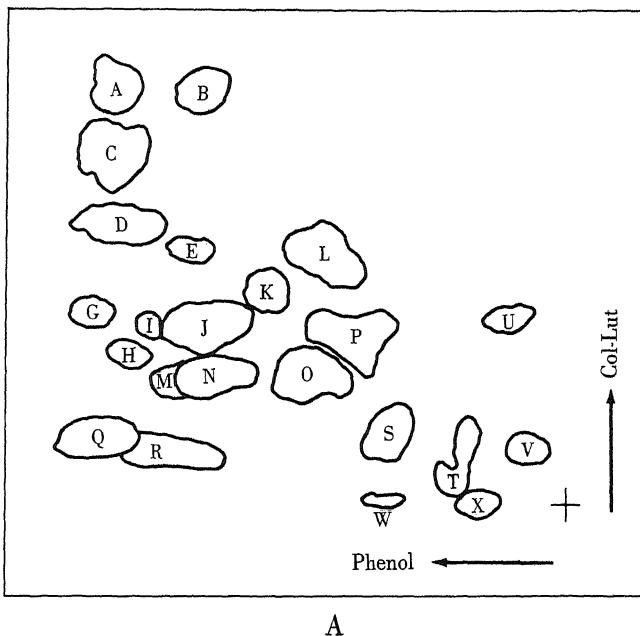
Tissues	Arginase	Catalase	Acid phosphatase	Alkaline phosphatase	Ribonucleodeaminase	Deoxyribonucleodeaminase	Dehydropteridine I	Dehydropteridine II	Exocystine desulfurase	BAA amidase (benzoylarginineamidase)	Cytochrome oxidase	Esterase
Normal												
Liver	213	2.0	25	4	20	4	20	20	45	32	11.8	312
Kidney	60	1.0	95	1500	80	60	22	20	16	12	14.0	70
Pancreas	4	0.1	18	3	10	0	17	10	8	8	—	1600
Spleen	10	0.4	42	21	96	84	20	0	0	50	4.3	82
Brain	3	0	16	14	32	0	18	0	0	0	13.0	4
Muscle	8	0.1	16	2	0	0	18	0	0	0	6.3	4
Neoplastic												
Transplanted hepatoma 31	21	0	52	542	50	36	20	1	0	56	3.1 <sup>a</sup>	104
Transplanted Jensen sarcoma	46	0	22	44	52	0	19	0	0	43	1.6 <sup>a</sup>	83
Transplanted tumor 2226 <sup>b</sup>	48	0	32	18	50	5	20	0	0	40	2.0	72

**Table 18.13.** A COMPARISON OF ENZYMATIC ACTIVITIES OF NORMAL AND NEOPLASTIC RAT TISSUES. Enzyme activities are studied the same way as for Table 18.12 (Table from Greenstein, 1954.)

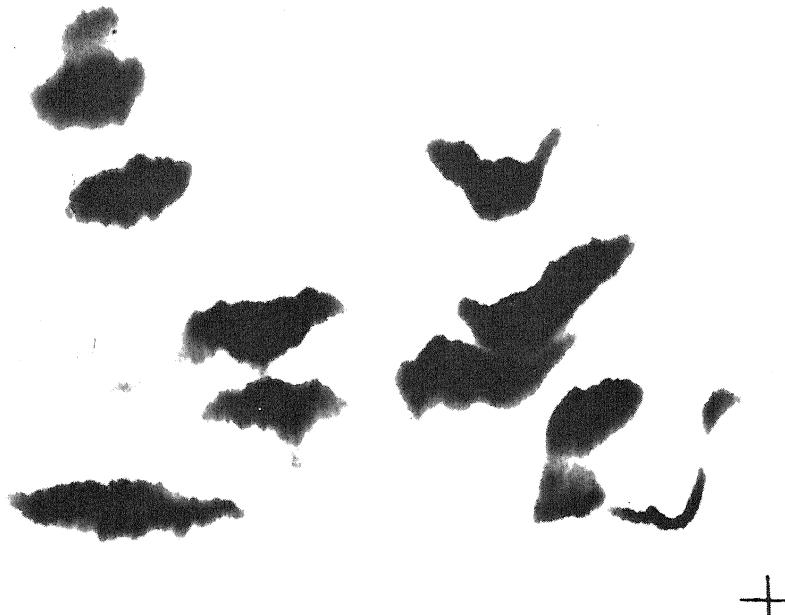
<sup>a</sup> According to Schneider and Potter (1943) the cytochrome oxidase activity of hepatoma 31 and the Jensen sarcoma is very nearly the same.

<sup>b</sup> Very anaplastic epithelial type of tumor.

process involves a *c*-value-ensemble change from one metastable state specific to each normal tissue, to another shared by all neoplastic tissues. While this uniformity among neoplastic tissues of diverse origin is, in itself, most fascinating, it does not present a process totally unknown in physiology, for it bears a close resemblance, though the direction is reversed, to the normal process of differ-



**Figure 18.22. FREE AMINO ACIDS IN NORMAL AND NEOPLASTIC TISSUES OF MICE.** Amino acids in ethyl alcohol extracts of fresh tissues were analyzed by two-dimensional paper chromatography. A, a map of the positions on the paper square of known amino acids. This diagram serves as a key to all subsequent figures. A, phenylalanine; B, tyrosine; C, leucine; D, valine; E, methionine sulfone; G, proline; H, histidine; I, hydroxyproline; J, alanine; K, threonine; L, taurine; M,  $\beta$ -alanine or citrulline; N, glutamine; O, glycine; P, serine; Q, arginine; R, lysine; S, glutamic acid; T, aspartic acid; U, cysteic acid; V, "oxidized" glutathione; W, "underglutamic acid"; X, glutathione. The cross at lower right corner in this and other figures indicates the spot where the sample was applied. Note the great diversity in patterns of free amino-acid accumulation in normal tissues and the uniform pattern in all neoplastic tissues irrespective of their original source tissue. (Figures after E. Roberts and Frankel, 1949.)



B I

Normal epidermis



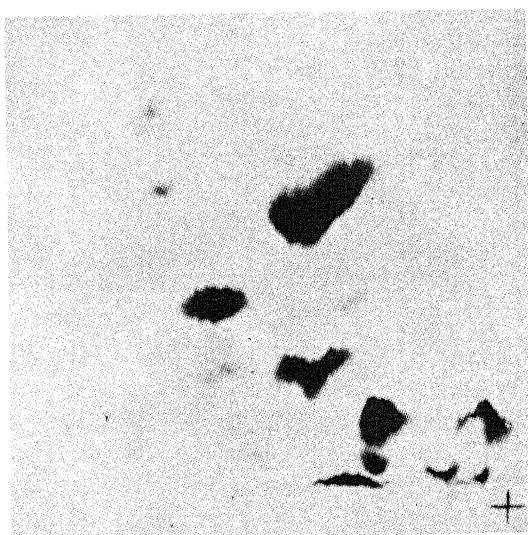
B II

Squamous cell carcinoma



C I

Normal muscle



C II

Rhabdomyosarcoma



D I                    Normally lymph nodes



D II                    Lymphosarcoma



E I

### Normal liver



E II

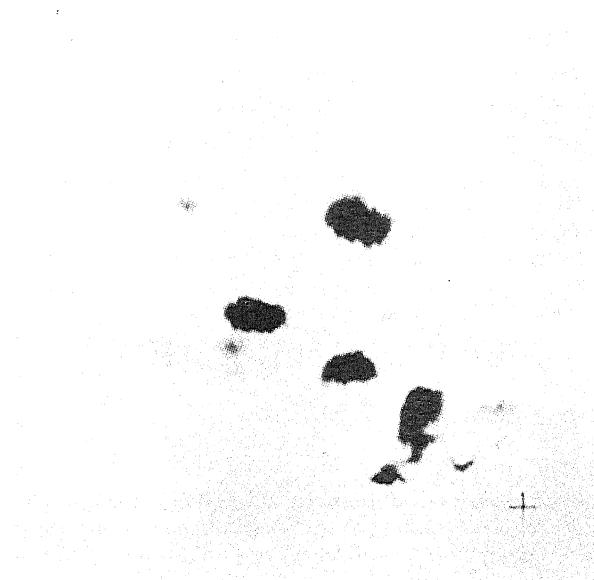
### Hepatoma



F I                   Normal testis

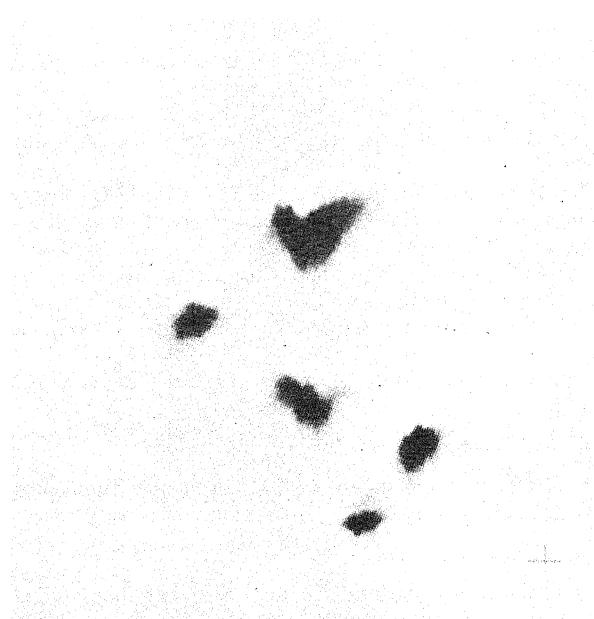


F II                   Interstitial cell tumor



G

Sarcoma 37



H

Tumor MA 387

entiation which transforms a single fertilized ovum into a great variety of cells that together form the advanced physiology and histology of higher living organisms. In such processes of specialization, the development of an undifferentiated cell into one type of tissue or into another may be controlled by a variety of agents (the  $\text{Li}^+$  ion, for example). It is, therefore, no surprise that carcinogenesis, the reverse of the normal process, may be brought about by another great variety of physiological or noxious agents, known as carcinogens.

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## B. CARCINOGENESIS

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- Nowinski, W. W., "Fundamental Aspects of Normal and Malignant Growth." Elsevier, Amsterdam and New York, 1960.
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### REVIEWS AND ORIGINAL ARTICLES

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# APPENDIX A

## Equilibrium Distribution of One Species of Counterion Between a Fixed-Charge System and Free Solution

In the fixed-charge system, we shall assume that there is a total number  $N$  of fixed anionic sites which are rigidly held in space and more or less evenly distributed in the bulk phase. Filling the bulk phase of the fixed-charge system are water molecules, each of which moves in a free volume determined by the position of its neighbors.

There are also  $N$  free counterions, either adsorbed or dissociated, and they distribute themselves one to each microcell (Figure 3.1). Such dissociated counterions will be called interstitial ions. In each microcell there are, on the average,  $\rho$  equivalent sites corresponding to the adsorbed state and  $\sigma$  equivalent sites corresponding to the dissociated interstitial state. The notational symbols may then be tabulated in the following way:

<u>Species</u>	<u>Allowed energies</u>	<u>Degeneracies</u>	<u>Distribution numbers</u>
Solvent			
(water)	$\eta_1, \eta_2, \dots, \eta_i, \dots$	$(\omega_i)$	$m_1, m_2, \dots, m_i, \dots$
Adsorbed			
counterion	$\eta'_1, \eta'_2, \dots, \eta'_i, \dots$	$(\omega'_i)$	$m'_1, m'_2, \dots, m'_i, \dots$
Interstitial			
counterion	$\eta''_1, \eta''_2, \dots, \eta''_i, \dots$	$(\omega''_i)$	$m''_1, m''_2, \dots, m''_i, \dots$

To this we may add the limiting conditions,

$$\sum_i m_i = M_s \quad (\text{A-1})$$

$$\sum_i m'_i = M_{\text{ads}} \quad (\text{A-2})$$

$$\sum_i m''_i = M_{\text{ins}} \quad (\text{A-3})$$

$$M_{\text{ads}} + M_{\text{ins}} = N. \quad (\text{A-4})$$

Here,  $M_s$  is the total number of solvent molecules,  $M_{ads}$ , the total number of adsorbed counterions, and  $M_{ins}$ , the total number of interstitial ions.

In a total of  $N$  microcells, there are  $(M_{ins} + M_{ads})! / M_{ins}! M_{ads}!$  distinguishable ways in which the counterions may be distributed. Since, for each adsorbed ion, there are  $\rho$  equivalent but spatially different sites and for each interstitial ion there are  $\sigma$  sites, the total number of possible complexions for the whole assembly is

$$\Omega = \sum_i \frac{(M_{ins} + M_{ads})!}{M_{ins}! M_{ads}!} (\rho)^{M_{ads}} (\sigma)^{M_{ins}} \frac{M_{ins}!}{\prod_i m_i''!} \frac{M_{ads}!}{\prod_i m_i'!} \frac{M_s!}{\prod_i m_i!}. \quad (\text{A-5})$$

From this, we proceed directly to the Helmholtz free energy  $F$  of the fixed-charge phase, eliminating the intermediate steps of the full statistical treatment (see Rushbrooke, 1949),

$$F = -kT \ln \left[ \frac{(M_{ins} + M_{ads})!}{M_{ins}! M_{ads}!} (\rho)^{M_{ads}} (\sigma)^{M_{ins}} (\text{p.f.})_1^{M_{ads}} (\text{p.f.})_2^{M_{ins}} (\text{p.f.})_3^{M_s} \right] \quad (\text{A-6})$$

where

$$(\text{p.f.})_1 = \sum_i \omega_i' e^{-\eta_i'/kT} \quad (\text{A-7})$$

$$(\text{p.f.})_2 = \sum_i \omega_i'' e^{-\eta_i''/kT} \quad (\text{A-8})$$

$$(\text{p.f.})_3 = \sum_i \omega_i e^{-\eta_i/kT}. \quad (\text{A-9})$$

By using Stirling's theorem, one can next derive the chemical potentials,

$$\begin{aligned} \mu_{ads} &= \left( \frac{\partial F}{\partial M_{ads}} \right)_{M_{ins}, M_s} \\ &= -kT[\ln(M_{ins} + M_{ads}) - \ln M_{ads} + \ln \rho + \ln (\text{p.f.})_1], \end{aligned} \quad (\text{A-10})$$

$$\begin{aligned} \mu_{ins} &= \left( \frac{\partial F}{\partial M_{ins}} \right)_{M_{ads}, M_s} \\ &= -kT[\ln(M_{ins} + M_{ads}) - \ln M_{ins} + \ln \sigma + \ln (\text{p.f.})_2]. \end{aligned} \quad (\text{A-11})$$

At thermodynamic equilibrium,

$$\mu_{ads} = \mu_{ins} \quad (\text{A-12})$$

and consequently,

$$\frac{M_{ads}}{\rho(M_{ads} + M_{ins})(\text{p.f.})_1} = \frac{M_{ins}}{\sigma(M_{ins} + M_{ads})(\text{p.f.})_2} \quad (\text{A-13})$$

$$\frac{M_{ins}}{M_{ads}} = \frac{\sigma(\text{p.f.})_2}{\rho(\text{p.f.})_1}. \quad (\text{A-14})$$

This equation is valid if the two sets of energy levels are measured from the same energy zero. More explicitly, if we measure the energy levels of the adsorbed and interstitial ion from their own ground levels, we obtain the equation,

$$\frac{M_{\text{ins}}}{M_{\text{ads}}} = \frac{\sigma(\text{p.f.})_2}{\rho(\text{p.f.})_1} \exp\left(\frac{\Delta E}{RT}\right), \quad (\text{A-15})$$

where  $\Delta E$  is the difference between the levels. Since  $M_{\text{ins}}$  and  $M_{\text{ads}}$  refer to the number of interstitial and adsorbed ions in a common volume  $V$ , one can divide both values by  $V$  and Avogadro's number  $L$ , and multiply by the number of cubic centimeters in a liter, that is, 1000. This yields

$$\frac{\frac{M_{\text{ins}}}{V \times L} \cdot 1000}{\frac{M_{\text{ads}}}{V \times L} \cdot 1000} = \frac{C_{\text{ins}}}{C_{\text{ads}}} = \frac{\sigma(\text{p.f.})_2}{\rho(\text{p.f.})_1} \exp\left(\frac{\Delta E}{RT}\right). \quad (\text{A-16})$$

It should be noticed that  $\Delta E$ , in terms of kilogram calories per mole, is the adsorption energy of the counterion, and  $C_{\text{ins}}$  and  $C_{\text{ads}}$  are the molar concentrations of the interstitial and adsorbed ions, respectively, per liter.

# APPENDIX B

## Equilibrium Distribution of Two Species of Counterions Between a Fixed-Charge System and Free Solution

Next, we shall consider the more complex problem of a fixed-charge system containing two species of counterions, A and B, in equilibrium with an external free solution, also containing these two species. This time, we use the notational symbols,

<u>Species</u>	<u>Allowed Energies</u>	<u>Degen-</u> <u>eracies</u>	<u>Distribution Numbers</u>
Solvent in fixed-charge system	$\eta_1, \eta_2, \dots, \eta_i, \dots$	$(\omega_i)$	$m_1, m_2, \dots, m_i, \dots$
Adsorbed counterion A	$\eta'_1, \eta'_2, \dots, \eta'_i, \dots$	$(\omega'_i)$	$m'_1, m'_2, \dots, m'_i, \dots$
Interstitial counterion A	$\eta''_1, \eta''_2, \dots, \eta''_i, \dots$	$(\omega''_i)$	$m''_1, m''_2, \dots, m''_i, \dots$
Adsorbed counterion B	$\eta'''_1, \eta'''_2, \dots, \eta'''_i, \dots$	$(\omega'''_i)$	$m'''_1, m'''_2, \dots, m'''_i, \dots$
Interstitial counterion B	$\eta''''_1, \eta''''_2, \dots, \eta''''_i, \dots$	$(\omega''''_i)$	$m''''_1, m''''_2, \dots, m''''_i, \dots$
Solvent in ex- ternal solu- tion	$\varepsilon_1, \varepsilon_2, \dots, \varepsilon_i, \dots$	$(\omega_i)$	$n_1, n_2, \dots, n_i, \dots$
A in external solution	$\varepsilon'_1, \varepsilon'_2, \dots, \varepsilon'_i, \dots$	$(\omega'_i)$	$n'_1, n'_2, \dots, n'_i, \dots$
B in external solution	$\varepsilon''_1, \varepsilon''_2, \dots, \varepsilon''_i, \dots$	$(\omega''_i)$	$n''_1, n''_2, \dots, n''_i, \dots$

In addition, we write the limiting conditions,

$$\sum_i m_i = M_s \quad (B-1)$$

$$\sum_i m'_i = M_{ads}^A \quad (B-2)$$

$$\sum_i m''_i = M_{ins}^A \quad (B-3)$$

$$\sum_i m'''_i = M_{ads}^B \quad (B-4)$$

$$\sum_i m''''_i = M_{ins}^B \quad (B-5)$$

$$M_{ads}^A + M_{ins}^A = M_A \quad (B-6)$$

$$M_{ads}^B + M_{ins}^B = M_B \quad (B-7)$$

$$M_{ads}^A + M_{ins}^A + M_{ads}^B + M_{ins}^B = M. \quad (B-8)$$

Here,  $M_s$  is the total number of solvent molecules in the fixed-charge phase;  $M_{ads}^A$ ,  $M_{ins}^A$  and  $M_A$  are the numbers of adsorbed, interstitial, and total A;  $M_{ads}^B$ ,  $M_{ins}^B$ , and  $M_B$  are the numbers of adsorbed, interstitial, and total B;  $M$  is the total number of counterions and is also equal to the total number of fixed charges. All the above terms refer to the fixed-charge phase. On the other hand, we have the conditions,

$$\sum_i n_i = N_s \quad (B-9)$$

$$\sum_i n'_i = N_A \quad (B-10)$$

$$\sum_i n''_i = N_B \quad (B-11)$$

where  $N_s$ ,  $N_A$ , and  $N_B$  are, respectively, the numbers of solvent molecules, A ions, and B ions in the external free solutions. The total number of complexions for the whole assembly (the fixed-charge phase and the external free-solution phase) is

$$\Omega = \sum \frac{(M_{ads}^A + M_{ins}^A + M_{ads}^B + M_{ins}^B)!}{M_{ads}^A! M_{ins}^A! M_{ads}^B! M_{ins}^B!} (\rho)^{M_{ads}^A + M_{ads}^B} (\sigma)^{M_{ins}^A + M_{ins}^B} \times \\ \times \prod_i \frac{M_{ads}^A!}{m'_i!} \frac{M_{ins}^A!}{m''_i!} \frac{M_{ads}^B!}{m'''_i!} \frac{M_{ins}^B!}{m''''_i!} \frac{M_s!}{m_i!} \frac{(N_A + N_B + N_s)!}{N_A! N_B! N_s!} \frac{N_A!}{n'_i!} \frac{N_B!}{n''_i!} \frac{N_s!}{n_i!} \quad (B-12)$$

The Helmholtz free energy of the whole assembly  $F$  is the sum of the free energy of the fixed-charge phase  $F_f$  and that of the free-solution phase  $F_s$ , that is,  $F = F_f + F_s$ . Here,  $F_f$  and  $F_s$  are given by the equations,

$$F_f = -kT \ln \left[ \frac{(M_{\text{ads}}^A + M_{\text{ins}}^A + M_{\text{ads}}^B + M_{\text{ins}}^B)!}{M_{\text{ads}}^A! M_{\text{ins}}^A! M_{\text{ads}}^B! M_{\text{ins}}^B!} (\rho)^{M_{\text{ads}}^A + M_{\text{ads}}^B} (\sigma)^{M_{\text{ins}}^A + M_{\text{ins}}^B} \times \right. \\ \left. \times (\text{p.f.})_2^{M_{\text{ads}}^A} (\text{p.f.})_3^{M_{\text{ins}}^A} (\text{p.f.})_4^{M_{\text{ads}}^B} (\text{p.f.})_5^{M_{\text{ins}}^B} (\text{p.f.})_1^{M_s} \right] \quad (\text{B-13})$$

$$F_s = -kT \ln \left[ \frac{(N_A + N_B + N_s)!}{N_A! N_B! N_s!} (\text{p.f.})_7^{N_A} (\text{p.f.})_8^{N_B} (\text{p.f.})_6^{N_s} \right]. \quad (\text{B-14})$$

Here, the symbols used are defined by the equations,

$$\begin{aligned} (\text{p.f.})_1 &= \sum_i \omega_i \exp(-\eta_i/kT) & (\text{p.f.})_5 &= \sum_i \omega_i'''' \exp(-\eta_i''''/kT) \\ (\text{p.f.})_2 &= \sum_i \omega_i' \exp(-\eta_i'/kT) & (\text{p.f.})_6 &= \sum_i \omega_i \exp(-\varepsilon_i/kT) \\ (\text{p.f.})_3 &= \sum_i \omega_i'' \exp(-\eta_i''/kT) & (\text{p.f.})_7 &= \sum_i \omega_i' \exp(-\varepsilon_i'/kT) \\ (\text{p.f.})_4 &= \sum_i \omega_i''' \exp(-\eta_i'''/kT) & (\text{p.f.})_8 &= \sum_i \omega_i'' \exp(-\varepsilon_i''/kT). \end{aligned}$$

By Stirling's approximation, the chemical potentials,  $\mu_{\text{ads}}^A$ ,  $\mu_{\text{ins}}^A$ ,  $\mu_{\text{ads}}^B$ , and  $\mu_{\text{ins}}^B$  can be derived for the adsorbed and interstitial A and B in the fixed-charge phase. The derivations yield the values,

$$\begin{aligned} \mu_{\text{ads}}^A &= \left( \frac{\partial F_f}{\partial M_{\text{ads}}^A} \right)_{(M_{\text{ins}}^A, M_{\text{ads}}^B, M_{\text{ins}}^B, M_s)} \\ &= -kT[\ln(M_{\text{ads}}^A + M_{\text{ins}}^A + M_{\text{ads}}^B + M_{\text{ins}}^B) - \ln M_{\text{ads}}^A + \ln \rho + \ln(\text{p.f.})_2] \end{aligned} \quad (\text{B-15})$$

$$\begin{aligned} \mu_{\text{ins}}^A &= \left( \frac{\partial F_f}{\partial M_{\text{ins}}^A} \right)_{(M_{\text{ads}}^A, M_{\text{ads}}^B, M_{\text{ins}}^B, M_s)} = -kT[\ln(M_{\text{ads}}^A + M_{\text{ins}}^A + M_{\text{ads}}^B + M_{\text{ins}}^B) \\ &\quad - \ln M_{\text{ins}}^A + \ln \sigma + \ln(\text{p.f.})_3] \end{aligned} \quad (\text{B-16})$$

$$\begin{aligned} \mu_{\text{ads}}^B &= \left( \frac{\partial F_f}{\partial M_{\text{ads}}^B} \right)_{(M_{\text{ads}}^A, M_{\text{ins}}^A, M_{\text{ins}}^B, M_s)} = -kT[\ln(M_{\text{ads}}^A + M_{\text{ins}}^A + M_{\text{ads}}^B + M_{\text{ins}}^B) \\ &\quad - \ln M_{\text{ads}}^B + \ln \rho + \ln(\text{p.f.})_4] \end{aligned} \quad (\text{B-17})$$

$$\begin{aligned} \mu_{\text{ins}}^B &= \left( \frac{\partial F_f}{\partial M_{\text{ins}}^B} \right)_{(M_{\text{ads}}^A, M_{\text{ins}}^A, M_{\text{ads}}^B, M_s)} = -kT[\ln(M_{\text{ads}}^A + M_{\text{ins}}^A + M_{\text{ads}}^B + M_{\text{ins}}^B) \\ &\quad - \ln M_{\text{ins}}^B + \ln \sigma + \ln(\text{p.f.})_5]. \end{aligned} \quad (\text{B-18})$$

Similarly, for the free solution, we find the chemical potential for A,  $\mu_{fr}^A$ , and that for B,  $\mu_{fr}^B$ ,

$$\mu_{fr}^A = \left( \frac{\partial F_s}{\partial N_A} \right)_{(N_B, N_s)} = -kT[\ln(N_A + N_B + N_s) - \ln N_A + \ln(\text{p.f.})_7] \quad (\text{B-19})$$

$$\mu_{fr}^B = \left( \frac{\partial F_s}{\partial N_B} \right)_{(N_A, N_s)} = -kT[\ln(N_A + N_B + N_s) - \ln N_B + \ln(\text{p.f.})_8]. \quad (\text{B-20})$$

At equilibrium, the conditions

$$\mu_{\text{ads}}^{\text{A}} = \mu_{\text{ins}}^{\text{A}} \quad (\text{B-21})$$

$$\mu_{\text{ads}}^{\text{B}} = \mu_{\text{ins}}^{\text{B}} \quad (\text{B-22})$$

$$\mu_{\text{ins}}^{\text{A}} = \mu_{\text{fr}}^{\text{A}} \quad (\text{B-23})$$

$$\mu_{\text{ins}}^{\text{B}} = \mu_{\text{fr}}^{\text{B}} \quad (\text{B-24})$$

hold. Therefore, we have the equations,

$$\frac{M_{\text{ads}}^{\text{A}}}{\rho(M_{\text{ads}}^{\text{A}} + M_{\text{ins}}^{\text{A}} + M_{\text{ads}}^{\text{B}} + M_{\text{ins}}^{\text{B}})(\text{p.f.})_2} = \frac{M_{\text{ins}}^{\text{A}}}{\sigma(M_{\text{ads}}^{\text{A}} + M_{\text{ins}}^{\text{A}} + M_{\text{ads}}^{\text{B}} + M_{\text{ins}}^{\text{B}})(\text{p.f.})_3} \quad (\text{B-25})$$

$$\frac{M_{\text{ads}}^{\text{B}}}{\rho(M_{\text{ads}}^{\text{A}} + M_{\text{ins}}^{\text{A}} + M_{\text{ads}}^{\text{B}} + M_{\text{ins}}^{\text{B}})(\text{p.f.})_4} = \frac{M_{\text{ins}}^{\text{B}}}{\sigma(M_{\text{ads}}^{\text{A}} + M_{\text{ins}}^{\text{A}} + M_{\text{ads}}^{\text{B}} + M_{\text{ins}}^{\text{B}})(\text{p.f.})_5} \quad (\text{B-26})$$

$$\frac{M_{\text{ins}}^{\text{A}}}{\sigma(M_{\text{ads}}^{\text{A}} + M_{\text{ins}}^{\text{A}} + M_{\text{ads}}^{\text{B}} + M_{\text{ins}}^{\text{B}})(\text{p.f.})_3} = \frac{N_{\text{A}}}{(N_{\text{A}} + N_{\text{B}} + N_{\text{S}})(\text{p.f.})_7} \quad (\text{B-27})$$

$$\frac{M_{\text{ins}}^{\text{B}}}{\sigma(M_{\text{ads}}^{\text{A}} + M_{\text{ins}}^{\text{A}} + M_{\text{ads}}^{\text{B}} + M_{\text{ins}}^{\text{B}})(\text{p.f.})_5} = \frac{N_{\text{B}}}{(N_{\text{A}} + N_{\text{B}} + N_{\text{S}})(\text{p.f.})_8}. \quad (\text{B-28})$$

Written somewhat differently, equations (B-25) and (B-26) become

$$\frac{M_{\text{ins}}^{\text{A}}}{M_{\text{ads}}^{\text{A}}} = \frac{\sigma(\text{p.f.})_3}{\rho(\text{p.f.})_2} \exp\left(\frac{\Delta E_{\text{A}}}{RT}\right) \quad (\text{B-29})$$

and

$$\frac{M_{\text{ins}}^{\text{B}}}{M_{\text{ads}}^{\text{B}}} = \frac{\sigma(\text{p.f.})_5}{\rho(\text{p.f.})_4} \exp\left(\frac{\Delta E_{\text{B}}}{RT}\right). \quad (\text{B-30})$$

We can convert the number of particles to molar concentration and write out the partition functions,

$$\frac{M_{\text{ins}}^{\text{A}}}{M_{\text{ads}}^{\text{A}}} = \frac{M_{\text{ins}}^{\text{A}} \cdot 1000/VL}{M_{\text{ads}}^{\text{A}} \cdot 1000/VL} = \frac{C_{\text{ins}}^{\text{A}}}{C_{\text{ads}}^{\text{A}}} = \frac{\sigma(\text{p.f.})_{\text{A}}^{\text{ins}}}{\rho(\text{p.f.})_{\text{A}}^{\text{ads}}} \exp\left(\frac{\Delta E_{\text{A}}}{RT}\right) \quad (\text{B-31})$$

$$\frac{M_{\text{ins}}^{\text{B}}}{M_{\text{ads}}^{\text{B}}} = \frac{M_{\text{ins}}^{\text{B}} \cdot 1000/VL}{M_{\text{ads}}^{\text{B}} \cdot 1000/VL} = \frac{C_{\text{ins}}^{\text{B}}}{C_{\text{ads}}^{\text{B}}} = \frac{\sigma(\text{p.f.})_{\text{B}}^{\text{ins}}}{\rho(\text{p.f.})_{\text{B}}^{\text{ads}}} \exp\left(\frac{\Delta E_{\text{B}}}{RT}\right). \quad (\text{B-32})$$

From this, follows the mass action relation,

$$\frac{C_{\text{ins}}^{\text{A}}}{C_{\text{ads}}^{\text{A}}} \frac{C_{\text{ads}}^{\text{B}}}{C_{\text{ins}}^{\text{B}}} = \frac{(\text{p.f.})_{\text{A}}^{\text{ins}}}{(\text{p.f.})_{\text{A}}^{\text{ads}}} \frac{(\text{p.f.})_{\text{B}}^{\text{ads}}}{(\text{p.f.})_{\text{B}}^{\text{ins}}} \exp\left(\frac{\Delta E_{\text{A}} - \Delta E_{\text{B}}}{RT}\right). \quad (\text{B-33})$$

We also know that

$$\frac{M_{\text{ins}}^{\text{A}}}{N_{\text{A}}} = \frac{\sigma(M_{\text{ads}}^{\text{A}} + M_{\text{ins}}^{\text{A}} + M_{\text{ads}}^{\text{B}} + M_{\text{ins}}^{\text{B}})}{(N_{\text{A}} + N_{\text{B}} + N_{\text{S}})} \frac{(\text{p.f.})_3}{(\text{p.f.})_7} \exp\left(\frac{\psi\mathcal{F}}{RT}\right) \quad (\text{B-34})$$

$$\frac{M_{\text{ins}}^{\text{B}}}{N_{\text{B}}} = \frac{\sigma(M_{\text{ads}}^{\text{A}} + M_{\text{ins}}^{\text{A}} + M_{\text{ads}}^{\text{B}} + M_{\text{ins}}^{\text{B}})}{(N_{\text{A}} + N_{\text{B}} + N_{\text{S}})} \frac{(\text{p.f.})_5}{(\text{p.f.})_8} \exp\left(\frac{\psi\mathcal{F}}{RT}\right) \quad (\text{B-35})$$

where  $\psi$  is the macroscopic surface potential (for example, the resting potential

of muscle cell) between the fixed-charge phase and the free solution, and  $\mathfrak{F}$  is the Faraday constant.

Using equation (B-8) and the fact  $N_A + N_B + N_s$  is the total number of water molecules per liter of pure water, it is possible to write equations (B-34) and (B-35) differently. Let  $V_f$  and  $V_s$  be the volumes of the fixed-charge and free-solution phases, respectively, and multiply both sides by equation (B-34) and equation (B-35), by the quantity

$$\frac{1000}{V_f L} / \frac{1000}{V_s L}.$$

This yields the equations,

$$\frac{C_{\text{ins}}^A}{C_A} = \frac{\sigma C_f}{C_{A+B+S}} \frac{(\text{p.f.})_3}{(\text{p.f.})_7} \exp\left(\frac{\psi\mathfrak{F}}{RT}\right) \quad (\text{B-36})$$

$$\frac{C_{\text{ins}}^B}{C_B} = \frac{\sigma C_f}{C_{A+B+S}} \frac{(\text{p.f.})_5}{(\text{p.f.})_8} \exp\left(\frac{\psi\mathfrak{F}}{RT}\right) \quad (\text{B-37})$$

where  $C_{A+B+S}$  is equal to the molar concentration of pure water and equal to 55.5. Writing the partition function more explicitly yields the equations,

$$\frac{C_{\text{ins}}^A}{C_A} = \frac{\sigma C_f}{55.5} \frac{(\text{p.f.})_A^{\text{ins}}}{(\text{p.f.})_A^{\text{fr}}} \exp\left(\frac{\psi\mathfrak{F}}{RT}\right) \quad (\text{B-38})$$

$$\frac{C_{\text{ins}}^B}{C_B} = \frac{\sigma C_f}{55.5} \frac{(\text{p.f.})_B^{\text{ins}}}{(\text{p.f.})_B^{\text{fr}}} \exp\left(\frac{\psi\mathfrak{F}}{RT}\right). \quad (\text{B-39})$$

For the case in which  $\sigma \gg \rho$ ,  $\sigma C_f$  approaches 55.5 M/l; then, equations (B-38) and (B-39) simplify to the equations,

$$\frac{C_{\text{ins}}^A}{C_A} = \frac{(\text{p.f.})_A^{\text{ins}}}{(\text{p.f.})_A^{\text{fr}}} \exp\left(\frac{\psi\mathfrak{F}}{RT}\right) \quad (\text{B-40})$$

$$\frac{C_{\text{ins}}^B}{C_B} = \frac{(\text{p.f.})_B^{\text{ins}}}{(\text{p.f.})_B^{\text{fr}}} \exp\left(\frac{\psi\mathfrak{F}}{RT}\right). \quad (\text{B-41})$$

From equations (B-31), (B-32), (B-38), and (B-39), it is also possible to derive the relations,

$$\frac{C_{\text{ads}}^A}{C_A} = \frac{\rho C_f (\text{p.f.})_A^{\text{ads}}}{55.5 (\text{p.f.})_A^{\text{fr}}} \exp\left(\frac{\Delta E_A + \psi\mathfrak{F}}{RT}\right) \quad (\text{B-42})$$

$$\frac{C_{\text{ads}}^B}{C_B} = \frac{\rho C_f (\text{p.f.})_B^{\text{ads}}}{55.5 (\text{p.f.})_B^{\text{fr}}} \exp\left(\frac{\Delta E_B + \psi\mathfrak{F}}{RT}\right). \quad (\text{B-43})$$

# APPENDIX C

## The 1952 Model of the Fixed-Charge System for Selective Ionic Accumulation

Equation (C-1) gives the probability of finding a counterion within a spherical shell of thickness  $dr$  and a distance  $r$  from the center of a fixed ion

$$p = 4\pi r^2 \exp(Z_1 Z_2 \epsilon^2 / D' k T r) dr. \quad (C-1)$$

The values of  $D'$  were chosen from Grahame (1950), corresponding to  $m = \frac{1}{2}$ , where  $m$  is an arbitrary constant; for  $m$ , a value of  $\frac{1}{2}$  has been suggested by the author as most probable. By means of graphic integration, a plot of the probability of the counterion being distributed at certain distances from the center of a fixed anionic charge could be obtained as shown in Figure 4.1.\* Again, by following reasoning similar to that of Bjerrum (1926), it could be shown that the probability that a microcell is unoccupied by a counterion, doubly occupied, or triply occupied is low; most of the microcells are, therefore, singly occupied. More important, Figure 4.1 shows that, for most of the microcells, the counterion would tend to approach the fixed ion as closely as possible. Now let us suppose that the fixed-charge system is in equilibrium with a second phase of free solution, containing a number of free ions at equimolar concentrations. The respective distances of closest approach of these free ions to the fixed ion are  $a_i$ ,  $a_j$ , and so on. If  $a_i \neq a_j$ , then the relative ratio of accumulation of the  $i$ th ion to the  $j$ th ion in the fixed-charge phase could be represented by the equation

$$\frac{C_i}{C_j} = \frac{\int_{a_i}^{r_{\max}} r^2 \exp(\pm Z_i \epsilon^2 / D' r k T) dr}{\int_{a_j}^{r_{\max}} r^2 \exp(\pm Z_j \epsilon^2 / D' r k T) dr}. \quad (C-2)$$

The exact ratio for any pair of counterions would, to some extent, depend on the fixed-ion density (that is, the radius of the microcells  $r_{\max}$ ), and on the values of the distances of closest approach for different ions.

\* A slight difference exists between this figure and the figure published in 1952 (Ling, 1952, Figure 6). No account was taken of the volume factor in the earlier version as its significance is greatly reduced because of the small size of the microcells.

	$\text{Li}^+$		$\text{Na}^+$		$\text{K}^+$		$\text{Rb}^+$		$\text{Cs}^+$		Primary, I, or secondary, II	Source
Method	Radius, Å	Hydra- tion No.	Radius, Å	Hydra- tion No.	Radius, Å	Hydra- tion No.	Radius, Å	Hydra- tion No.	Radius, Å	Hydra- tion No.		
Mobility	2.26	—	1.74	—	1.17	—	1.12	—	1.11	—	I	Lorenz, 1910
Mobility	2.30	—	1.79	—	1.22	—	1.17	—	1.17	—	—	Hartley and Raikes, 1927
Mobility	2.35	3.5-7	1.90	2-4	—	—	—	—	—	—	I	Ulich, 1930
Entropy deficiency	2.65	5	2.10	3.5	1.81	2	1.83	2	—	—	I	Ulich, 1930
Compressibility	1.97	3	2.19	4	2.03	3	—	—	—	—	—	Moesveld and Hardon, 1931
Mobility	2.99	7.5	2.14	3	1.45	1	—	—	—	—	—	Brüll, 1934
Crystal radii and deformability	3.2	—	2.1	—	1.4	—	1.23	—	1.17	—	—	Kielland, 1937
Mobility	2.35	—	1.8	—	1.25	—	1.2	—	1.18	—	—	Gorin, 1939
Mobility	2.8	—	2.15	—	1.45	—	1.4	—	1.4	—	—	Gorin, 1939
Conductivity and transference	3.09	—	2.562	—	1.978	—	—	—	—	—	—	Gorin, 1939
Activity change	2.45	5.5	2.95	9	1.86	6	—	—	—	—	—	Eucken, 1948
Viscosity	2.51	6.0	2.30	4.6	1.88	2.3	1.75	1.7	—	—	—	Bockris, 1949
Mobility	2.51	6.0	2.02	3.1	1.68	1.5	1.77	1.75	—	—	—	Bockris, 1949
Mobility	3.07	—	2.25	—	1.63	—	1.57	—	1.56	—	—	Bockris, 1949
Density	1.74	2	1.49	1	1.30	0	1.50	0	—	—	—	Bockris, 1949
Transference	8.25	158	6.32	171	4.27	22	—	—	—	—	—	Riesenfeld and Reinhold, 1909
Transference	10.03	—	7.90	—	5.32	—	5.09	—	5.05	—	—	Remy, 1915
Transference	3.46	12.6	2.86	8.4	2.22	4.0	—	—	—	—	—	Baborovsky, 1927
Transference	3.66	13.9	2.88	8.4	2.45	5.4	—	—	—	—	—	Washburn, 1919
Molar volume and molar heat	3.14	10.5	3.06	9.7	3.18	11.0	3.37	12.0	—	—	I and II	Eucken, 1948
Dialysis coefficient	—	—	—	—	3.10	9.5	—	—	—	—	I and II	Spandan and Spandan, 1943
Salting out	—	—	3.75	14.5	3.34	11.5	—	—	—	—	I and II	Freundlich and Schnell, 1928
Crystal radii	0.68	—	—	—	—	—	—	—	—	—	—	Kortum and Bockris, 1951
	—	—	1.01	—	1.30	—	0.50	—	1.75	—	—	Pauling, 1948

Table C.1. VALUES OF THE RADII OF HYDRATED ALKALI-METAL IONS, COLLECTED FROM THE LITERATURE IN 1956.

For a semiquantitative estimate of ionic selectivity, the relative ratio of accumulation of  $K^+$  ion and  $Na^+$  ion within the system under study could be investigated. Two assumptions were then made. First, the negatively charged group on the fixed-charge system is regarded as a monovalent oxyacid which is not hydrated. Second, the distance of closest approach of a free counterion and the fixed anion is determined by the sum of the radius of a naked oxygen atom and the radius of a hydrated cation. A reasonable estimate of the radii of the hydrated  $Na^+$  ion and of the hydrated  $K^+$  ion at 0.1M ionic strength could be obtained by fitting the transference-number data of Allgood *et al.* (1940) and the conductivity values that Gorin calculated from the data of Longsworth (1932) and Shedlovsky (1932) into Gorin's equation (Gorin, 1939). The values obtained are 2.0 $\text{\AA}$  for the radius of a hydrated  $K^+$  ion and 2.8 $\text{\AA}$  for an  $Na^+$  ion, both at 18°C. Based on these hydrated ionic radii, a  $K^+/Na^+$  selectivity ratio of 11.2 was calculated with the assumption of a microcell diameter of 14 $\text{\AA}$ , and a ratio of 7.3 was obtained for a microcell 20 $\text{\AA}$  in diameter.

In Table C.1, we have tabulated most data available prior to 1953 on measured hydrated ionic diameters. If the average of these values were used, a better selectivity ratio might be achieved. However, it was thought more correct to use the values calculated on the basis of Gorin's equation, as this alone takes into account the ionic strength of the solution, while all values given in Table C.1 refer to hydrated ionic diameter at infinite dilution.

## APPENDIX D

### A Rapid and Convenient Procedure for the Determination of ATP, ADP, and AMP

The following procedure, which has been modified from Kalckar (1947), has been used successfully in the author's laboratory for the past seven years. It is presented for two reasons. First, several important arguments (see especially Chapters 8 and 9) depend upon the accurate determination of the ATP and ADP contents of tissues. Second, there is a real need for an easy, time-saving, and accurate procedure for the assay of the AMP, ADP, and ATP contents of a large number of tissue samples. The following procedure requires neither special apparatus nor the experience of an enzymologist.

The basis of this procedure is the fact that adenosinemonophosphate (AMP, or 5'-adenylic acid) has an absorption peak at  $265m\mu$ , whereas deaminated AMP (inosinic acid) has no absorption peak at this wavelength. The transformation from adenosinemonophosphate (AMP) to inosinic acid is brought about by the action of the enzyme, 5'-adenylic acid deaminase, which is specific for this reaction. The determination of ATP and ADP utilizes enzymes specific for the transformation of these compounds into AMP. In the determination of ATP, the enzyme used is potato apyrase which also converts ATP into AMP. In the determination of ADP, the enzyme, myokinase, which is specific for the reaction  $ADP \rightarrow AMP$ , is used; one-half of the ADP present in the sample is converted to AMP.

#### (1) Purification of adenylic acid deaminase (method modified after Nikiforuk and Colowick,\* 1955)

A rabbit is killed by a blow on the head and skinned. The muscles are removed as quickly as possible and placed in a beaker which is sitting in an ice bath. The muscles are weighed and ground in a meat grinder in a cold room ( $4^{\circ}C$ ). An equal part of 0.9 per cent NaCl is added to the ground muscle and the mixture is shaken or

\* The author is indebted to Dr. S. Colowick for making available to him in 1952 the then unpublished manuscript of Nikiforuk and Colowick (1956). The present modified procedure is based on this, as well as on the original work of G. Schmidt (1928).

stirred vigorously in the cold for 15 minutes. It is then filtered through two layers of cheesecloth. The filtrate is put aside in the cold. The ground muscle is again shaken for 15 minutes in the cold with an equal volume of 0.9 per cent NaCl and again filtered through cheesecloth. The second filtrate is combined with the first and saved for the later preparation of myokinase [see Section D(2)]. The ground muscle is washed twice more using the same procedure; the third and fourth filtrates are discarded.

After the fourth washing, the sediment is shaken for 90 minutes at room temperature with two volumes of 2 per cent NaHCO<sub>3</sub>. The resultant sticky mixture is filtered through three layers of cheesecloth; the filtrate volume is made as large as possible by squeezing. The sediment is again shaken for 15 minutes with two volumes of 2 per cent NaHCO<sub>3</sub> (at room temperature). After filtering through cheesecloth, the second filtrate is added to the first and both are measured in a large graduate. Celite, a diatomaceous filtering agent (Johns Manville), is added to a concentration of 4 per cent (weight/volume) and the mixture is filtered through large Buchner funnels employing Whatman No. 1 paper. Since this procedure may be very slow, it is advisable to add only a very thin layer of the extract to the funnel at a time. If it is necessary to complete the filtering overnight, the funnel and flask must be placed in the cold; a frequent change of filter paper may hasten the procedure somewhat. A translucent filtrate is obtained.

The filtrate is adjusted to pH 7.0 with a 0.3M acetic-acid-sodium-acetate buffer at pH 5.0. It can now be frozen in a deep freeze; in this condition it will remain active for several months. When purified adenylic acid deaminase is needed, part of the filtrate may be thawed and further purified to isolate the enzyme as follows: The thawed Celite filtrate is adjusted to pH 6.0 by drop-by-drop addition of the 0.3M acetate buffer at pH 5.0. To one volume of this adjusted Celite filtrate, 0.3 volumes of saturated ammonium sulfate, also adjusted to pH 6.0, are added. This should be added drop by drop in the cold, using extreme care to mix well during the addition. The mixture is then allowed to stand for 15 minutes in the cold and spun for 10 minutes at high speed (approximately 12,000  $\times g$ ). The precipitate is discarded and twice the original volume of ammonium sulfate is added to the clear filtrate in the manner described above. The resultant mixture is again allowed to stand in the cold for 10 minutes and again spun at high speed. Following this, the supernatant is discarded and the precipitate treated in the following way.

Into each centrifuge tube, one or two drops of 2 per cent NaHCO<sub>3</sub> are pipetted and the precipitate is then taken up in twice the minimum amount of a mixture of one part 0.3M sodium-acetate-acetic-acid buffer at pH 6.0 and two parts of a 0.75M NaCl solution. The resultant solution should be clear and the pH should be 6.0. Otherwise, the pH should be adjusted with the sodium-acetate-acetic-acid buffer and spun once more to get rid of particulate matter. The completely soluble enzyme is now ready for use and is free from ATPase or myokinase activity.

## (2) Myokinase and potato apyrase preparation

A satisfactory myokinase preparation may be obtained from the combined NaCl washings of the rabbit muscle discussed in Section D.1, using the method of Colowick-Kalckar (1943):

The muscle extract is acidified with 0.05 volume of 1*N* HCl and heated to 90°C for three minutes. It is then cooled rapidly in an ice bath, neutralized with 2*N* NaOH to pH 6.5, filtered, and the precipitate is discarded. Ammonium sulfate is added to the supernatant to about 80 per cent of saturation at room temperature and the precipitate spun down at the same temperature. The resultant precipitate is dissolved in 15 to 20 milliliters of H<sub>2</sub>O. The preparation is then frozen in the deep freeze; a small amount may be thawed and used as needed.

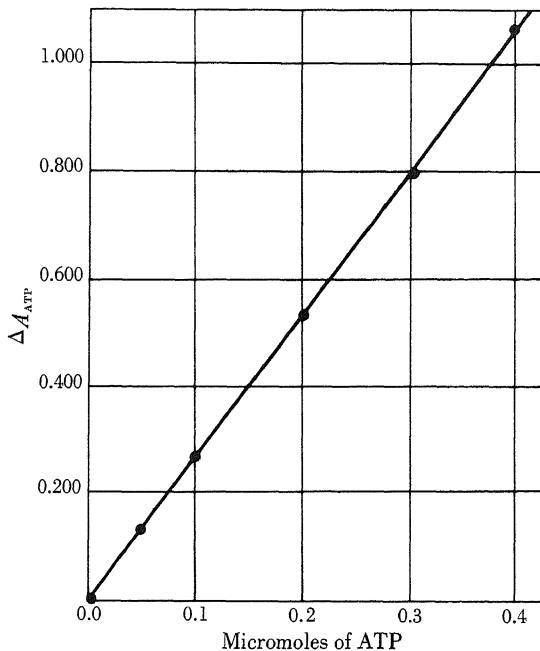
Potato apyrase may be satisfactorily prepared by the method of Krishnan (1949). One kilogram of peeled potatoes is ground in a Waring blender with an equal volume of 0.01*M* NaCN solution and the supernatant centrifuged in the cold to remove particulate matter. After returning to room temperature solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> is added to the supernatant with stirring to a concentration of 45 grams per 100 milliliters of extract. The mixture is filtered through Whatman No. 1 filter paper at 4°C. The "precipitate" is then taken up in water, dialyzed against distilled water (changed frequently) in the cold for 24 hours. The dialysate is then lyophilized. The powdery preparation, if kept in the deep freeze, will remain active at least for several months. For use, the powder is dissolved in a small amount of distilled water.

## (3) Determination of ATP, ADP, and AMP

The tissue, in cold 2 per cent perchloric acid, is homogenized at low temperature and centrifuged. To the clear supernatant is added one drop of 0.1 per cent phenolphthalein in alcohol. The pH is then adjusted to near neutrality with NaOH. A final pH of 7.0 is achieved by further adjustment with the aid of brom-thymol-blue on a porcelain spot test plate. The neutralized samples can be frozen and preserved overnight or even somewhat longer. Due to the ease of ATP hydrolysis in basic or acidic medium, extreme caution must be exercised that a neutralized sample does not become contaminated by droplets of acid or base on the side of the centrifuge tubes. For the determination of AMP, ADP, and ATP, three aliquots, each 0.5 milliliters in volume, are needed for each piece of tissue.

(a) *Reagents.* The reagents used are (1) succinic acid buffer containing 15 parts 0.1*M* succinic acid adjusted to pH 5.9 with NaOH, 1 part 0.3*M* MgCl<sub>2</sub>, and 9 parts 1.5*M* NaCl; (2) 2 per cent perchloric acid neutralized to pH 7.0 with NaOH; (3) samples prepared by the cold perchloric-acid extraction method given above; and (4) solutions of adenylic acid deaminase, myokinase, and potato apyrase as prepared above, kept in small test tubes immersed in an ice-water mixture.

(b) *Determinations.* The succinic acid buffer (2.5ml) and 0.5ml of sample (well shaken before pipetting) are pipetted into a silica cuvette. The blank contains 0.5ml of neutralized 2 per cent perchloric acid instead of a sample. The solutions are stirred with small polyethylene stirring rods and the absorption is read



**Figure D.1. THE RELATIONSHIP BETWEEN  $\Delta A_{ATP}$  AND THE AMOUNT OF ATP IN SAMPLE.** A fresh preparation of crystalline  $\text{Na}_2\text{ATP}\cdot 3\text{H}_2\text{O}$  (Sigma Chemical Co., St. Louis) was employed.

on a Beckman DU spectrophotometer at  $265\text{m}\mu$ . This initial reading is only tentative and aids in the rapid reading of the following samples.

(i) *AMP determination.* Add 0.05ml of 5-adenylic acid deaminase to the samples and to the blank prepared as above. The contents of the cuvettes are rapidly stirred and the absorption at  $265\text{m}\mu$  read three times in rapid succession. The points so obtained are plotted on semilogarithmic graph paper; the initial absorption is obtained by extrapolation to zero time. The samples and blank are then poured into small stoppered tubes and kept in the refrigerator overnight or longer. After that the absorption at  $265\text{m}\mu$  is determined to give the final reading. The difference between the initial and final readings will be referred to as  $\Delta A_1$ .

(ii) *ADP determination.* The procedure is exactly the same as that for AMP, except that 0.05ml of myokinase is added to the samples and to the blank when the 5-adenylic deaminase is added. The difference between the initial and final readings at 265m $\mu$  will be called  $\Delta A_2$ .

(iii) *ATP determination.* The procedure is exactly the same as that for AMP, except that 0.05ml of potato apyrase is added to the samples and to the blank when the 5-adenylic deaminase is added. The difference between the initial and final readings at 265m $\mu$  will be referred to as  $\Delta A_3$ .

(c) *Calculations.* The relationships between the  $\Delta A$  values obtained above and the concentrations of ATP, ADP, and AMP in the sample expressed in terms of the changes of absorption at 265m $\mu$  are given by the equations,

$$\begin{aligned}\Delta A_{\text{AMP}} &= \Delta A_1 \\ \Delta A_{\text{ADP}} &= 2(\Delta A_2 - \Delta A_1) \\ \Delta A_{\text{ATP}} &= \Delta A_3 - 2\Delta A_2 + \Delta A_1.\end{aligned}$$

If the procedure is followed *exactly* as outlined a  $\Delta A$  value of 0.260 corresponds to 0.1 micromole of adenosine compound in the sample. The relationship between  $\Delta A_{\text{ATP}}$  and the micromoles of ATP in the sample as determined for the purest preparation of ATP available as a standard is shown in Figure D.1. Note that the relationship is a straight line to at least 0.4 micromoles of ATP. The same relationship holds true for both  $\Delta A_{\text{AMP}}$  and  $\Delta A_{\text{ADP}}$ .

# APPENDIX E

## The Nature of the Cell Surface

We have presented the view that the activity of nerve cells reflects an indirect *F*-process, a process made possible by the high polarizability of the resonating polypeptide chain. Although it is conceivable that the cell surface is made up of a protein complex containing lipid components, from a functional standpoint, this surface must be proteinaceous. Nevertheless, it has long been held by many that the surface layers of cells, usually referred to as the "cell membrane" are lipid in nature. Let us here examine the evidence in support of this view.

### (1) High specific resistance

Since lipoid material is highly insulating, a high specific resistance across the surface of a cell is generally considered to be evidence supporting a lipid "membrane" concept. An examination of the orders of magnitude of the resistances of several cell "membranes" as well as of various lipoid and nonlipoid substances may perhaps clarify this matter.

The *specific transverse resistance*\* of giant squid axon "membranes" is usually found to be only 400 to 1100 ohm cm<sup>2</sup>; that of frog muscle fibers measures only 1500 to 4000 ohm cm<sup>2</sup> (see Davson, 1951, Table 30, p. 385); however values as low as 23 ohm cm<sup>2</sup> have been measured for functioning squid axons (Cole and Curtis, 1936). Assuming a cell membrane about 100Å in thickness (Hodgkin, 1947), the specific resistance of this "membrane" would be  $2 \times 10^7$  to  $4 \times 10^9$  ohm cm. This is not an extraordinarily high specific resistance. Ordinary dielectric materials have values many times higher (white beeswax,  $6 \times 10^{14}$  ohm cm; ordinary glass,  $9 \times 10^{13}$  ohm cm). In fact, a collodion membrane less than one micron thick (showing red and green interference colors) (Blinks, 1931), which is a *nonlipoid*

\* The electrical resistance of a homogeneous material is directly proportional to the thickness *l* of the substance and its specific resistance *R*, and inversely proportional to the surface area *A*; this is expressed by the relation,

$$\text{Resistance} = Rl/A.$$

The specific transverse resistance is the specific resistance multiplied by the thickness and is thus equal to *Rl*.

fixed-charge system, has a *transverse specific resistance* of several million ohm cm<sup>2</sup>. This gives a specific resistance of the order of 10<sup>11</sup> ohm cm. From these considerations, we conclude that specific resistances of the order of those of the cell surface offer no evidence that the cell surface is lipoid in nature.

#### (2) Osmium tetroxide staining

With the recent rapid development of electron microscopy, the surfaces of many types of cells which have been fixed with osmic acid have been shown to contain a microscopic layer which appears dark colored in electron micrographs. Since osmium tetroxide does stain lipoid material, giving similar dark lines and has been considered a fat stain (Wigglesworth, 1957), can we then regard this as evidence in support of a lipoid "membrane"?

Bahr (1957) has studied this problem and concluded that osmium-tetroxide fixation favors neither protein nor lipid. Robertson, in his recent review, reached a similar conclusion after discussing at some length reactions of OsO<sub>4</sub> with proteins (Robertson, 1960, pp. 396-399).

#### (3) The correspondence of the oil-water distribution coefficients of nonelectrolytes and the rate of penetration of these substances into plant cells

Collander and Bärlund (1930) have shown that the rates of penetration of non-electrolytes into certain plant cells follow the same order as their oil-water distribution coefficients. Such findings have often been cited as evidence in favor of a lipoid membrane. However there are really two different processes involved here. The oil-water distribution coefficient describes an equilibrium distribution which is a function of the difference in free energy between the substance in oil and the substance in water; the penetration of nonelectrolytes is a rate process which depends on at least two steps. The first step involves the entry into or association with the supposedly lipid membrane; the second step involves dissociation from the "lipid membranes." These two processes, being independent, do not necessarily behave in parallel fashion. Thus, for example, a substance may have an infinite oil-water partition coefficient. This substance will tend to enter and stay in the oily phase; its rate emerging from the other side of the oily phase into another aqueous phase certainly does not have to be faster than that of another substance preferred by the oil phase to a much smaller degree. For this reason, a correspondence between the equilibrium distribution and the order of the rate of penetration cannot be used to establish that the "membrane" is lipoid in nature.

#### (4) The action of phospholipases on the surfaces of cells

This subject has already been dealt with in detail in Chapter 8 and need not be further elaborated. Suffice it to say that the action of phospholipases on the sur-

faces of muscle and nerve cells appears to be a function of the charge of certain molecules in the preparation and not of its specific enzymatic activity.

#### (5) The lack of action of proteolytic enzymes on the surfaces of cells

As we mentioned in Chapter 10, Tobias (1955, 1958) has found that proteolytic enzymes have no effect on the surfaces of nerve and muscle cells; this fact has often been regarded as evidence for the nonprotein nature of these surfaces. However, if we remember that trypsin, a protein with powerful proteolytic activity, does not act on itself, it is clear that a lack of action proves neither the nonproteinaceous nature of trypsin nor the nonproteinaceous nature of the cell surface. In fact, it has long been known that proteolytic enzymes attack many proteins only in the denatured state (see Green and Neurath, 1954, p. 1172). From this fact, as well as from the specificity of the *c*-value ensembles of the various proteins, a concept which forms an integral part of the present thesis, we could well expect cell surfaces of a protein nature not to react with such enzymes.

#### (6) Immunological properties of cell surfaces

It has long been known that intact cells are antigenic; the best-known example, of course, is the red blood cell. After an early period of controversy, it is now generally accepted that lipids, as such, are not antigens (see Boyd, 1956, p. 162; Haurowitz, 1950, p. 280). This offers a strong argument against the concept that cell "membranes" are made of lipid material, and suggests that the cell surfaces are basically proteinaceous since a great majority of antigens are protein in nature.

#### (7) The action of tannic acid on cell surfaces

Tannic acid is a polymer of various hydroxybenzoic acids. Commercially it is used to tan leather and to manufacture an artificial material used for horn-rimmed glasses (by reacting with albumin and gelatin). Both of these uses indicate the high degree of reactivity of tannic acid with proteins. On the other hand, a search of the extensive literature on tannic acid failed to reveal any known reaction of tannic acid with lipid. (It has been found to be insoluble in lipid solvents such as benzene, ether, and petroleum ether). On the other hand this compound has a pronounced effect on the surface viscosity of collagen at concentrations of 1 to 6 mg/liter. Since tannic acid (not uncommonly, but incorrectly, called tannins) has a molecular weight of 1701.18, 1 to 6 mg/liter corresponds to about  $10^{-6}$  moles/liter\* (see Ellis and Parkhurst, 1954).

It has been known for a long time that tannic acid at low concentration pro-

\* Since gallic acid, resorcinol, phenol, and so on, produce no viscosity increase, the action of tannic acid may be added to the class of reactions in which polymers react while monomers do not (recall the action of poly-L-lysine on cells, Section 10.4). An entropic interpretation of these phenomena has been offered in terms of the present association-induction hypothesis.

duces a profound effect on permeability of red blood cells (Handovsky and Heubner, 1923; Jacobs *et al.*, 1943; Bohlmann, 1944). This action is instantaneous in spite of the higher molecular weight and is readily reversible by addition of protein solutions; these facts are compatible only with the assumption that tannic acid acts on the outer surface of the cell (Edelberg, 1952), and again suggest that the surfaces of these cells are made of protein.

In concluding this section we say that there is no conclusive evidence to support the conventional lipid "membrane" concept. On the other hand, there is considerable evidence supporting the view that the cell surface is proteinaceous in nature.

# APPENDIX F

## Electrical Properties of Cell Surfaces

The following brief discourse is presented primarily for the reader whose principle interest does not lie in cellular electrical phenomena to show how the present theory offers natural mechanisms for much of the electrical behavior of cell surfaces.

### (1) Resistance

The high electrical resistance of cell surfaces bearing predominantly anionic sites which strongly prefer the  $K^+$  ion over the  $Na^+$  ion, as in the case of nerve and muscle cells, follows from the mechanism of selective ionic permeability discussed in Chapter 11 and does not depend upon the presence of lipid components (see Appendix E).

### (2) Capacity

Surfaces of nerve, muscle and other cells have an electrical capacity ranging from  $0.8 \mu\text{F}/\text{cm}^2$  for erythrocytes to  $4.5 \mu\text{F}/\text{cm}^2$  for frog muscle; the usual value is near  $1 \mu\text{F}/\text{cm}^2$  (see for example, Davson, 1951, Table 30). The existence of this capacity is a consequence of the predominantly anionic surface of the fixed-charge system with its cationic counterions more or less oriented in one direction by the surface potential; a model for this system may be found in the phenomenon of electrocapillarity. The capacitance of a liquid-solid interface as presented by this system has been a major subject of study since Helmholtz (1879). His original simple model of a double layer gives the capacity per unit area  $C$  as

$$C = \frac{de}{dV} = \frac{D}{4\pi\delta}$$

where  $e$  is the charge per unit surface on each side of the double layer,  $D$  is the dielectric constant of the medium,  $V$  is the electrical potential difference between the two layers, and  $\delta$  is the distance between the layers.

### (3) Rectification properties of cell surfaces

An asymmetrical resistance to the passage of electrical current across the cell surface is commonly called the *rectifying* property. This also follows naturally from the present thesis. The anionic nature of the surface fixed ions bars the entry of and thus reduces the over-all permeability to  $\text{Cl}^-$  and to intracellular anions (many of which are also strongly adsorbed onto the fixed-charge system); this makes the external  $\text{Cl}^-$  and intracellular anions relatively ineffective current carriers. The remaining ions are the external  $\text{Na}^+$  and  $\text{K}^+$  ions, which could carry positive current inward, and the intracellular  $\text{Na}^+$  and  $\text{K}^+$  ions, which could carry current outward.

In Chapter 11, we provided a rather detailed presentation of the theory for the low  $\text{Na}^+$ -ion permeability  $P_{\text{Na}}$  and the high  $\text{K}^+$ -ion permeability  $P_{\text{K}}$  through the surface fixed-charge system of the cell. Since

$$[\text{Na}]_{\text{ex}} \gg [\text{Na}]_{\text{in}}$$

and

$$[\text{K}]_{\text{in}} \gg [\text{K}]_{\text{ex}}$$

the quantity,  $(P_{\text{K}}[\text{K}]_{\text{in}} + P_{\text{Na}}[\text{Na}]_{\text{in}})$ , which is proportional to the conductance of positive current outward must be greater than the quantity  $(P_{\text{K}}[\text{K}]_{\text{ex}} + P_{\text{Na}}[\text{Na}]_{\text{ex}})$ , which is proportional to the conductance of positive current inward. This is, of course, revealed as a rectifying property. The above is the most direct explanation of the rectifying property. Other asymmetry factors arising from the fact that the cytoplasm is not a dilute salt solution but a fixed-charge system can provide answers to other rectifying properties not directly explicable in terms of the above.

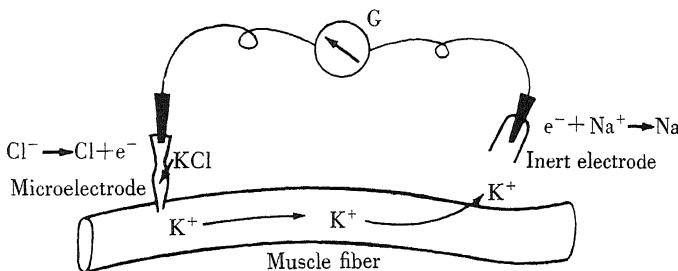
### (4) The demarcation current

It has been repeatedly demonstrated that a current, the demarcation current, can be shown to flow from the intracellular phase for long periods of time when the intracellular and extracellular phases are connected by a suitable device. How is it possible to reconcile this with the contention that cellular ionic distribution and electric potential represent equilibrium conditions? The key to understanding this seeming paradox lies in the phrase "suitable device." Thus, the equilibrium state referred to exists only in the intact cell; to measure a current, the cytoplasm must be connected directly with a liquid phase ( $3M$  KCl, for example, in the micro-electrode). After the introduction of such a microelectrode into the cell the earlier equilibrium is no longer maintained.

If we could somehow ideally synthesize a muscle or nerve cell at absolute zero, the system will contain exactly the same number of negative charges and positive charges; there would be no cellular potential  $\psi$ . As we warmed the system to room temperature, a number of counterions, mostly in the form of  $\text{K}^+$  ions, would begin to diffuse out to seek the greater entropic freedom in the external free solution.

This process would continue until  $[K]_{in} = [K]_{ex}$ , were it not for the development of a macroscopic potential  $\psi$ , as cations diffused out unaccompanied by anions. This potential makes further outward migration more difficult. When the final equilibrium is reached, the outward migration of the  $K^+$  ion, favored by a gain of entropy, exactly equals the inward  $K^+$ -ion migration which is favored by  $\psi$ . This, then, is the condition of normal cellular equilibrium.

When we connect the positive and negative terminals of a dry cell with a piece of perfectly conducting wire, current flows through the wire as long as the potential difference between the poles is maintained; once the potential difference

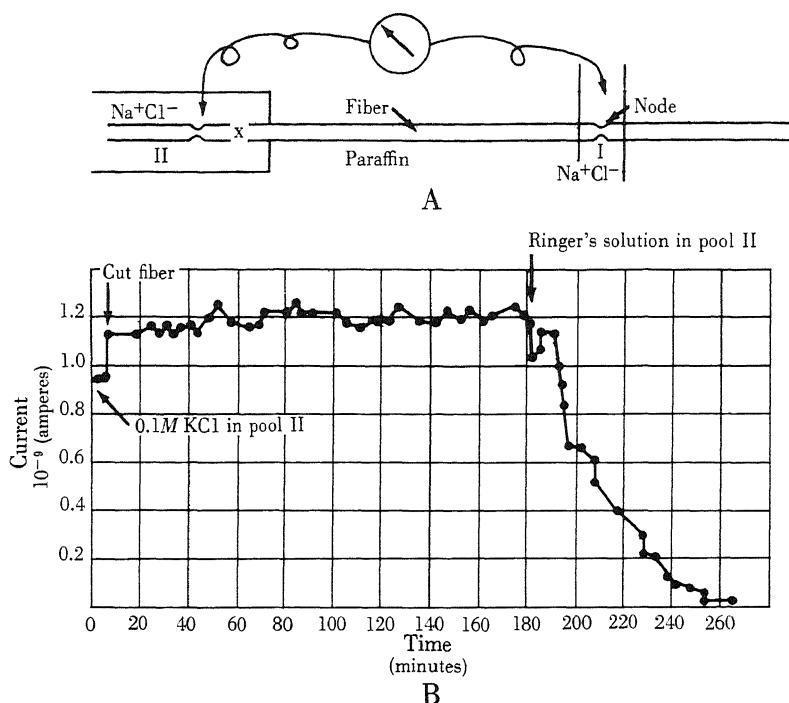


**Figure F.1. THE MECHANISM OF THE MAINTAINED DEMARCACTION CURRENT.**

reaches zero, the current stops. To measure such an electric current, an ammeter can be interposed in the wire. If the battery is not a dry cell, but contains an aqueous phase, say, KCl solution, it is usually necessary to interpose nonpolarizable electrodes between the battery and the wires. Such electrodes serve to stabilize the transfer of ions in the solution into a current of electrons flowing through the wire. While the exact chemical reaction depends on the nature of the electrode, in essence, at one electrode the positively charged  $K^+$  ion maintains the current flow by picking up an electron and becoming a K atom which then secondarily reacts with water, releasing  $H_2$  gas. At the other electrode, the negatively charged  $Cl^-$  ion gives off an electron to the electrode to become atomic Cl which is usually evolved as  $Cl_2$  gas. The loss of electrons at one end of the wire and gain at the other leads to the flow of a current through the wire and through the ammeter.

Now if such electrodes are connected, not to a battery, but on the inside and outside of a cell through a microelectrode, the surface potential  $\psi$  across the cell surface will act as the potential of a battery and lead to the flow of electrons (Figure F.1). At the calomel junction of the microelectrode, the reaction  $Cl^- \rightarrow Cl_2 + e^-$  continually removes anions from the cytoplasm-microelectrode system. The extra

cation  $K^+$  can then enter the cytoplasm and serve to maintain a current outward across the cell surface into the Ringer's solution without affecting the surface



**Figure F.2. DEMARCATIION CURRENT IN A SINGLE MYELINATED FROG NERVE.** A, the experimental setup. An isolated nerve fiber is placed between pools I and II; the main portion of the fiber rests in a narrow trough filled with liquid paraffin. The end on the right (not shown) also rests in a pool of aqueous solution and is treated in the same way as the end in pool I. B, at the first arrow,  $0.1M$  KCl is introduced into pool II and shortly after this the fiber is cut (at X in A). The figure shows that as long as  $0.1M$  KCl is in contact with the cut end of the nerve, the current is maintained; it rapidly degenerates when Ringer's solution is substituted for KCl in pool II. (after Müller, 1958.)

potential. As long as there is a large store of KCl in the muscle-microelectrode system, the potential and current will be maintained. The energy source of the continued current is the free energy of dilution and mixing of the  $K^+$  ion as it goes from the cytoplasm-electrode system into the Ringer's solution. A somewhat similar conclusion has been drawn by Müller in a paper published in 1958 from an experiment on the single myelinated nerve fiber of a frog (Figure F.2). Here instead of a microelectrode, a "pool" of solution was used to make contact with

the intracellular phase. If this pool contains 0.1*M* KCl the current is maintained indefinitely. If NaCl is substituted, the current soon dies out because, once the Na<sup>+</sup> ion has replaced all the K<sup>+</sup> ion, there will no longer be an outward concentration gradient. Accompanying the replacement of the K<sup>+</sup> ion within the cell by the Na<sup>+</sup> ion, there will be a gradual loss of K<sup>+</sup> ion from the potential-determining surface sites; this will lead to a gradual fall of  $\psi$  as predicted by equation (10-12). When  $\psi$  reaches zero, the current ceases.

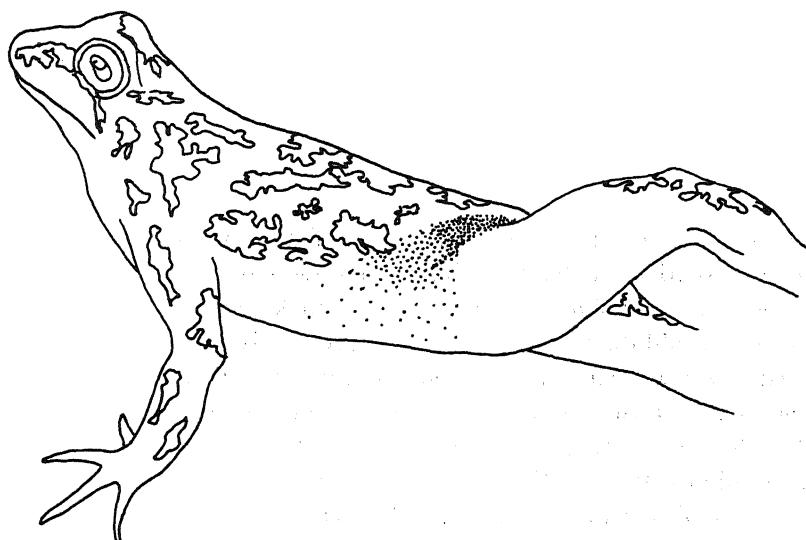
# APPENDIX G

## A Simple Criterion for Choosing Healthy Common Leopard Frogs (*Rana pipiens* Schreber)

The frog has long been a favorite experimental animal; yet the wide variations between their natural habitat and the new environments in which these animals usually find themselves during transportation and storage often creates damage which might elude an observer who is not well acquainted with the illnesses of frogs. In our experience, intracellular K<sup>+</sup> ion and adenosinetriphosphate (ATP) concentrations are particularly sensitive to this type of elusive damage. Data from analyses performed in 1955 (January, February, May, June, and December) of cellular constituents were studied statistically. The frogs used at this time were the best we could then select from among a large number of frogs supplied by Chicago, Wisconsin, and Vermont dealers. They showed no "red-leg," swelling of ankles, permanent blanching, or indeed any of the other visible signs usually used as criteria for rejection of unhealthy frogs. A total of 36 determinations (using flame photometry) on paired small muscles (*sartorius*, *semitendinosus*, *tibialis anticus longus*, and *iliofibularis*) from 18 frogs gave a K<sup>+</sup>-ion concentration of  $70.3 \pm 7.1$  millimoles per kilogram of fresh tissue. The average variation between values from paired muscles (difference/mean) was 5.8 per cent. ATP analyses by the method of Appendix D on 16 muscles from 8 different frogs (May and December) gave an average value of  $3.61 \pm 0.82$  millimoles per kilogram of fresh tissue.

In 1955, the author began to suspect a relationship between the K<sup>+</sup>-ion content of small frog muscles and the presence of a greenish-yellow (chartreuse) coloration on the back, flanks, and thighs of the frogs (see Figure G.1). With the use of this additional criterion, two similar batches of analyses were performed in October and December of 1956 (winter frogs). From a total of 16 paired small-muscle groups from eight different frogs, all of which showed distinct chartreuse coloration, the average K<sup>+</sup>-ion concentration was found to be  $79.3 \pm 6.1$  millimoles per kilogram; this average is about 13 per cent higher than that of the best frogs chosen early in 1955 before the establishment of this color criterion. The average variation between pairs was reduced to 3.1 per cent. Similarly, ATP analyses on the same paired muscles gave  $5.46 \pm 0.52$  millimoles per kilogram, the average

being over 50 per cent higher than the previously determined values. The differences between the mean values for both the ATP- and K<sup>+</sup>-ion contents are highly significant (both *p* values less than 0.001). Frogs chosen on the basis of this color criterion demonstrated better consistency both between paired muscles from the



**Figure G.1. LOCAL SKIN COLORATION OF *RANA PIPIENS*.** Dotted area indicates the region of a chartreuse (yellow-green) coloration which has been found to correlate with a higher degree of uniformity of physiological variables (for example, K<sup>+</sup>-ion content of tissues) among individual frogs.

same frog and among different frogs. The higher mean values are also entirely in agreement with the best published values known to the author. The high values we observed on tissues from winter frogs chosen on the basis of this coloration leads one to suspect that the large "physiological" variations observed sometimes in winter frogs are the results of the more severe damage suffered during transportation and during storage in the cold season.

# APPENDIX H

## Composition of Two Ringer's Solutions for Frog Tissues

Column 3 of Table H.1 gives the composition of the stock solutions used to make the Ringer's solutions; different volumes of these stock solutions and water are mixed to give a total volume of 1.182 liters. In making either Ringer's solution, it is imperative to add the  $\text{CaCl}_2$  solution last, preferably mixed with a large portion of the distilled water which is to be added, and after the rest of the solution has been thoroughly mixed. For the Ringer's-bicarbonate solution a 3%  $\text{CO}_2$ —27%  $\text{O}_2$ —70%  $\text{N}_2$  mixture (or 3%  $\text{CO}_2$ —97%  $\text{O}_2$ , or simply 5%  $\text{CO}_2$ —95%  $\text{O}_2$ ) should be bubbled through the solution for a half an hour before the addition of  $\text{CaCl}_2$ . On a hot day, some turbidity may be seen. In this case, keeping the solution

	Stock solutions		Ringer's-bicarbonate		Ringer's-phosphate	
	Concen- tra- tion, <i>M</i>	g/l	Concen- tra- tion, mM	Volume of stock solution, ml	Concen- tra- tion, mM	Volume of stock solution, ml
NaCl	$5 \times 0.118$	34.486	80.9	161.8	92.7	185.3
KCl	0.118	8.797	2.5	25	2.5	25
CaCl <sub>2</sub>	0.0845	9.378	1.0	10	1.0	10
MgSO <sub>4</sub> ·3H <sub>2</sub> O	0.118	20.583	1.2	12	1.2	12
NaHCO <sub>3</sub>	$5 \times 0.118$	49.572	17.3	34.6	17.3	13.28
NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	0.118	16.29	2.0	20	2.0	20
Na <sub>2</sub> HPO <sub>4</sub> ·12H <sub>2</sub> O	0.118	42.27	1.2	12	1.2	12
Glucose	$5 \times 0.236$	212.60	24.0	24	24.0	24
Water				882.5		880.4
Total volume				1182		1182

**Table H.1. COMPOSITIONS OF RINGER'S-BICARBONATE AND RINGER'S-PHOSPHATE SOLUTIONS USED IN OUR LABORATORY.** The second and third columns give compositions of stock solution; the fourth through seventh columns give ionic composition of Ringer's solutions as well as volumes of stock solutions needed to prepare it. For details, see text.

in a refrigerator will usually clear the Ringer's solution. The Ringer's-phosphate solution is more convenient to use for short-term experiments. In long-term experiments, where it is desirable to keep frog tissues alive for as long as possible, the Ringer's-bicarbonate should be used. In this case it is convenient to keep the tissue in a type of culture flask that provides a large air surface. The  $\text{CO}_2\text{--N}_2\text{--O}_2$  mixture is then allowed to flow slowly through the air space but is not actually bubbled through the Ringer's solution containing the tissues. For short-term experiments, of course, the Ringer's-bicarbonate can also be used if a closed system can conveniently be employed.



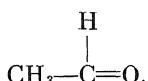
# GLOSSARY OF SELECTED TERMS AND CHEMICAL COMPOUNDS\*

Å See Angstrom unit.

Abscissa In a graph, the horizontal axis.

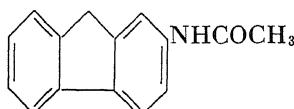
Absolute temperature The temperature of the Kelvin scale which has its zero at  $-273.16^{\circ}\text{C}$  (the temperature at which a gas would show no pressure if the general gas law held for all temperatures) and has degree units of the same magnitude as the centigrade scale.

Acetaldehyde



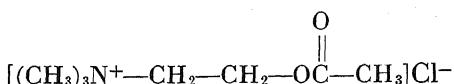
Acetic acid  $\text{CH}_3\text{—COOH}$ , a weak acid found in vinegar.

2-Acetylaminofluorene (AAF) N-2-fluorenylacetamide,



a carcinogen.

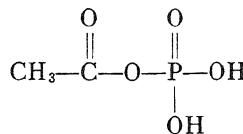
Acetylcholine chloride



\* Some definitions are too lengthy for inclusion in the Glossary. In these cases, the reader is referred to the Index where he will find the number of the page on which the definition or full explanation appears in boldface print.

a parasympathomimetic agent serving an important physiological function as the chemical agent transmitting nerve impulses across synapses such as the neuromuscular junction.

**Acetylphosphate**



a metabolic intermediate which plays an important part in the synthesis of lipids, carbohydrates, and amino acids.

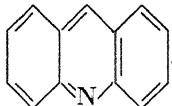
**Acid dissociation constant ( $K$ )** The equilibrium constant for the ionization of a particular acid. Thus, for acetic acid,  $\text{CH}_3\text{COOH} \rightleftharpoons \text{CH}_3\text{COO}^- + \text{H}^+$ , the acid dissociation constant may be represented,

$$K = \frac{[\text{H}^+][\text{CH}_3\text{COO}^-]}{[\text{CH}_3\text{COOH}]}$$

where  $[\text{H}^+][\text{CH}_3\text{COO}^-]$  and  $[\text{CH}_3\text{COOH}]$  represent the concentrations of the  $\text{H}^+$  ion, the acetate ion, and the unionized acetic acid, respectively.

**Acid group** A functional group in an organic molecule which can yield one or more protons  $\text{H}^+$  to, say, an aqueous medium; with this ionization, the acid group is left with one or more negative charges. Examples are the carboxyl group  $-\text{COOH}$ , sulfonic group  $-\text{SO}_3\text{H}$ .

**Acridine** Dibenzo[*b,e*]pyridine,



**ACTH** See  $\beta$ -Corticotropin.

**Actin** A major protein of muscle occurring in the myofibrils of muscle cells.

**Action potential** The transient local electrical potential variation which causes an active locus to be negative with respect to quiescent regions of a nerve or muscle fiber. The action potential, to the best of our knowledge, is tantamount to the nerve or muscle impulse. For the profile of the time course of variation, see Figure 10.3.

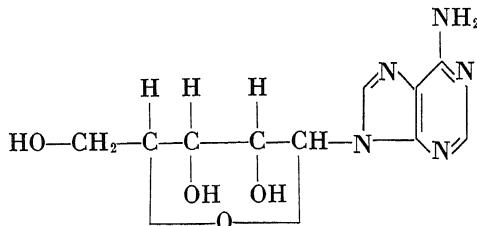
**Activated complex** When two reactant molecules possessing the necessary energy of activation come together, they first form a transition molecule or activated complex which decomposes to yield the products of the reaction.

**Activation energy** The energy barrier that reactants must be able to overcome before a reaction can be completed. For illustration, see Figure 11.8 where the activation energy is represented by  $\epsilon_{t,i}$ .

**Active transport** Movement of ions or molecules against a concentration and/or electrical gradient. The term, as generally used, is based on the membrane-pump concept of living cells according to which transport is effected by a continuous expenditure of metabolic energy, hence the term "active" transport.

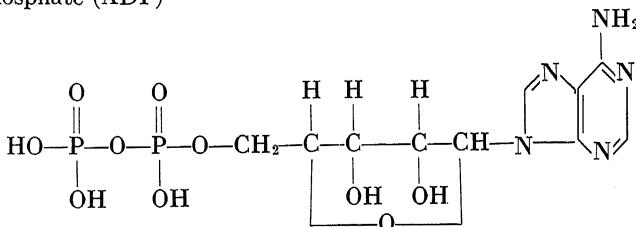
**Activity coefficient** A factor, usually represented by  $f$ , introduced by G. N. Lewis, to correct for other than ideal behavior of substances in solution. Thus the relation of the chemical potential  $\mu$  to the concentration of the substance  $c$  for the ideal case is  $\mu = \mu_c^0 + RT \ln c$ ; for other than ideal behavior,  $\mu = \mu_c^0 + RT \ln fc$ , where  $\mu_c^0$  is the chemical potential in the reference state.

**Adenosine** Adenine riboside,



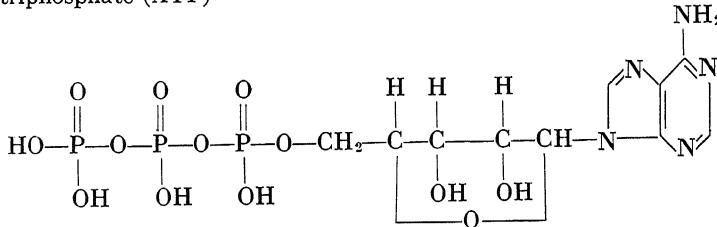
one of the basic components of ATP, ADP, and AMP; also found in nucleic acids.

**Adenosinediphosphate (ADP)**



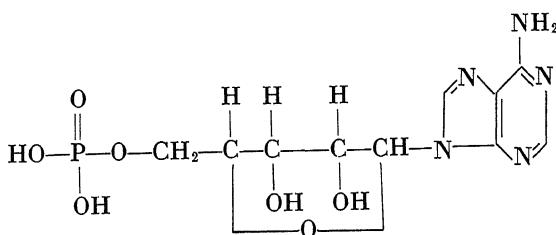
see 5-Adenylic acid and Adenosinetriphosphate (ATP).

**Adenosinetriphosphate (ATP)**



an important nucleotide compound occurring in many cells. Its ready decomposition to adenosinediphosphate (ADP) makes it important in many biochemical reactions.

**5-Adenylic acid** Adenosine-5-monophosphate (AMP),



known as muscle adenylic acid in contrast to yeast adenylic acid which is 3-adenylic acid. Important physiological component of many living cells and a breakdown product of adenosinediphosphate (ADP).

**5-Adenylic acid deaminase** An enzyme which hydrolytically deaminates adenylic acid according to the reaction, 5-adenylic acid + H<sub>2</sub>O ⇌ inosinic acid + NH<sub>3</sub>.

**ADP** See Adenosinediphosphate.

**Adrenaline** Epinephrine; a hormone produced by the adrenal medulla which has widespread physiological effects on organs and tissues innervated by sympathetic nerves, for example, constriction of blood vessels and hyperglycemia.

**Adrenocorticotropic hormone** See  $\beta$ -Corticotropin.

**Adsorption energy** The energy needed to separate an adsorbed entity from its adsorption site to a position an infinite distance away. It is negative in value, for example, -5 kcal/mole.

**Adsorption isotherm** A graph showing the variation of the concentration of an adsorbed species with the concentration of the same species in the free state within the same system at a constant temperature.

**Adsorptive induction** A change in the over-all electronic configuration of a molecule or a functional group created by the adsorption or exchange adsorption of a specific entity at some locus; in contrast to the type of inductive effect commonly referred to in organic chemistry which involves substitution or addition of a covalently linked group to the molecule.

**Aerobic** Pertaining to any organism which grows only in the presence of molecular oxygen; active only in the presence of molecular oxygen.

**Agglutination** The clumping together of cells or bacteria as a result of the interaction of the cells or bacteria with the corresponding immune serum.

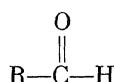
**AIB** See  $\alpha$ -Aminoisobutyric acid.

**Alanine** An  $\alpha$ -amino acid found in protein hydrolysates (see Table 0.1).

**Albumin** Any protein which is readily soluble in water and coagulable by heat.

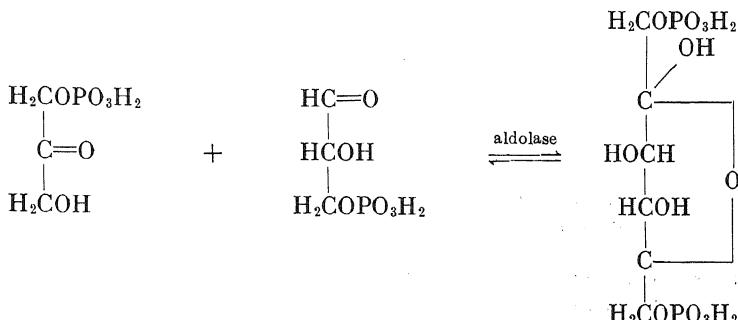
**Alcoholic group** A hydroxyl group —OH, on an aliphatic carbon atom of which other valences are satisfied by hydrogen or hydrocarbon radicals. Depending on other substituents —OH can be primary, RCH<sub>2</sub>OH; secondary, R<sub>2</sub>CHOH; or tertiary, R<sub>3</sub>C—OH.

**Aldehyde** Organic compound of general structure,



where R stands for a hydrocarbon radical.

**Aldolase** Any one of a class of enzymes which catalyze the formation of certain pentose and hexose phosphates from dihydroxyacetone phosphate and a 2 or 3 carbon aldehyde, for example, the reaction



Dihydroxyacetone phosphate     $\alpha$ -Glyceraldehyde-3-phosphate    Fructose-1,6-diphosphate

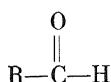
**Algae** A group of plants which may be unicellular, colonial, or multicellular and which possess no true root, leaf, or stem. They are found in water and damp places and include seaweeds and pond scum.

**Aliphatic** Pertaining to an open-chain carbon compound, fundamentally a derivative of methane.

**Alkali-metal ions** The alkali metals to which reference is made in this volume are the elements lithium, sodium, potassium, rubidium, and cesium in the first group of the periodic table. They are all very reactive, easily giving up one electron to become positively charged particles or ions.

**Alkaline-earth metals** The elements calcium, strontium, barium, and radium, all divalent metals in the second group of the periodic system.

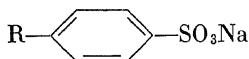
**Alkylaldehydes** A general term for an aliphatic aldehyde



where R represents any organic group not containing a ring structure.

**Alkylammonium chloride**  $\text{RNH}_3\text{Cl}$ , where R is an aliphatic hydrocarbon.

**Alkylbenzene sulfonate** Salt of sulfonated alkylbenzene,

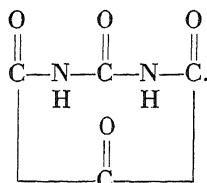


where R stands for an alkyl group (hydrocarbon chain).

**Allantoic** Pertaining to the allantois, an embryonic diverticulum of the hind gut which takes part in the formation of the umbilical cord and the placenta.

**Allergen** Any substance which is capable of inducing an allergy or allergic reaction.

**Alloxan diabetic** Diabetes mellitus caused by the destruction of the islet cells of the pancreas by alloxan (mesoxalyl urea);



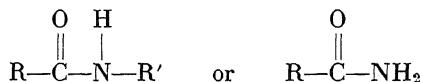
**Alloy** One of a large number of substances having metallic properties and consisting of two or more metals in a homogeneous mixture.

**Aluminum silicates** Approximately,  $\text{Al}_2\text{O}_3 \cdot 3\text{SiO}_2$ ; insoluble compounds which form glasses; used in dental cement and ceramics.

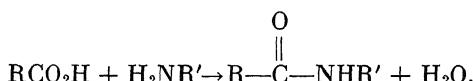
**Amberlite IRA-410** The trade name of a strongly basic anionic exchange resin of the quaternary amine type, sold by Rohm and Haas, Co.

**Ambient temperature** Temperature of the surrounding medium.

**Amide**

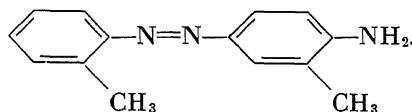


a group of compounds derived from an organic acid and an amine or ammonia,

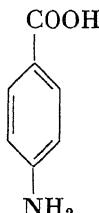


**Amino acid** Any one of a class of organic compounds containing the amino ( $\text{NH}_2$ ) group and the carboxyl ( $\text{COOH}$ ) group. Proteins are made up of amino acids joined by peptide linkages. For a list of the common amino acids, see Table 0.1.

**2-Amino-5-azotoluene**



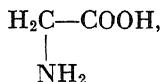
**p-Aminobenzoic acid**



an essential nutrient for a variety of microorganisms.

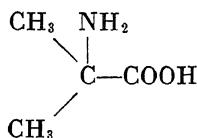
$\gamma$ -Aminobutyric acid  $\text{NH}_2\text{—CH}_2\text{—CH}_2\text{—CH}_2\text{—COOH}$ ; this compound is present in extracts of brain and spinal cord; it has inhibitory actions on nerve cells and is thought to play the role of a transmitter of inhibitory nervous impulses.

$\alpha$ -Amino group An amino group ( $—\text{NH}_2$ ) which is substituted on an  $\alpha$ -carbon atom or the first carbon atom adjoining a functional group in an aliphatic hydrocarbon chain. Thus glycine,



has an  $\alpha$ -amino group.

$\alpha$ -Aminoisobutyric acid (AIB)



a metabolically nonutilizable amino acid.

Amoeboid Any movement like that characteristic of an amoeba, due to the constant changing of shape of the moving object.

AMP See 5-Adenylic acid.

Amphoteric Capable of reacting either as an acid or as a base.

Amplification factor A number which estimates the extent to which a biological signal has been magnified in its total effects.

$\alpha$ -Amylase Any one of a class of enzymes which act hydrolytically on the  $\alpha$ -glucosidic 1,4-linkages of starch, glycogen, and polysaccharides producing glucose, maltose, fructose, etc.

Anaerobe Any microorganism that lives and grows only in the complete or nearly complete absence of molecular oxygen.

Anaplastic A condition of tumor cells in which there is a loss of normal differentiation, organization, and specific function.

Angstrom unit ( $\text{\AA}$ ) A unit of length equal to  $10^{-8}$  centimeter, named after a 19th century Swedish physicist, A. J. Ångström.

Animalizing agent Any agent which causes presumptive endodermal tissues (which arise from the vegetal pole) to develop into ectodermal structures.

Animal pole That pole of the egg which contains most cytoplasm and least yolk.

Anion In an electrolyte, the ion which carries the negative charge and thus migrates to the anode or positive electrode under the influence of an applied electromotive force.

Anionic charge The electrical charge borne by a negative ion.

Anode The positive pole of a galvanic battery or other electric source; the positive electrode through which current enters an electrolytic cell or a thermionic valve from an external source of electromotive force.

Anoxia Oxygen deficiency.

Antagonism Opposition or contrariety as between two muscles of the same limb, between two drugs, etc.

Anti-B agglutinin An antibody present in certain normal serums which agglutinates type B red blood cells; see Blood groups.

Antibody A serum protein synthesized by an animal in response to the presence of foreign substances, bacteria, toxin, etc., referred to as antigens. The function of an antibody is to react with these antigens and thus neutralize their untoward effects.

Antidiuretic Tending to check the secretion of urine, as of drugs which decrease urinary secretion.

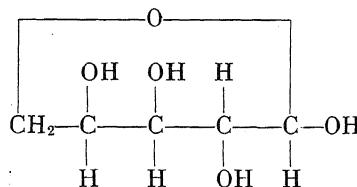
Antiferromagnetism A number of ferromagnetic substances contain two interlocking types of atoms or atom groupings, referred to as sublattices, which tend to magnetize oppositely. Such substances tend to show a cancellation of the total magnetization. This phenomenon is referred to as antiferromagnetism.

Antigen Any substance which, when introduced into the blood or tissue of an organism, elicits the formation of an antibody.

Antiserum A serum that contains an antibody or antibodies. It is obtained from animals that have been subjected to the action of an antigen either by injection into the tissues or bloodstream or by infection.

Apyrase An enzyme, present in muscle, potatoes, etc., which is capable of hydrolyzing both pyrophosphate bonds of ATP:  $\text{ATP} + 2\text{H}_2\text{O} \rightarrow \text{AMP} + 2\text{H}_3\text{PO}_4$ .

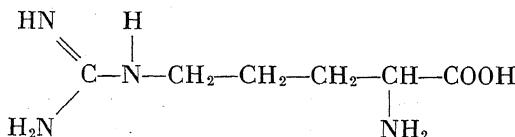
Arabinose Pectin sugar,



a pentose widely distributed in plants, usually in the form of complex polysaccharides.

*Arbacia punctulata*, *Arbacia pustulosa*, *Arbacia lixula* Species of sea urchin.

Arginine 1-Amino-4-guanidovaleric acid,



an important trifunctional amino acid which bears a residual positive charge when contained in a polypeptide chain (at neutral or low pH); see Table 0.1.

Aromatic molecule Any compound containing one or more ring structures with resonating conjugated double bonds.

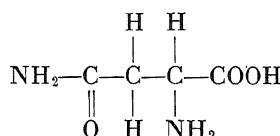
**Arsenite** Metarsenite; any salt of  $\text{HAsO}_2$ .

**Arthropoda** A phylum of invertebrate animals with jointed legs, an exoskeleton, and a segmented body. Lobsters and crayfish (crustaceans), spiders, and insects are all arthropods.

**Ascidian** Any simple or compound tunicate of the order Ascidiacea, a sea squirt.

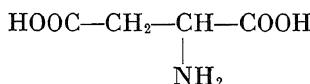
**Ascites cells** Cells from the ascites tumor of mice, a tumor which grows in the peritoneal cavity causing the production of a large amount of fluid exudate containing suspended tumor cells.

**Asparagine**



the  $\beta$ -amide of aspartic acid; an  $\alpha$ -amino acid.

**Aspartic acid**



an important trifunctional amino acid which bears a residual negative charge when contained in a polypeptide chain (at neutral or high pH); see Table 0.1.

**Association energy** See Adsorption energy, also Index.

**Astacina** The red carotenoid pigment of lobster and salmon.

**Asymmetric ionic distribution** At equilibrium, the concentration of a particular ion in one phase may not be equal to that in a second contiguous phase; this is referred to as an asymmetric ionic distribution.

**Atmospheric pressure** The pressure exerted by the atmosphere at any point; the mean atmospheric pressure at sea level, 760 mm Hg.

**ATP** See Adenosinetriphosphate.

**Auricle** The atrium of the heart, a chamber connecting the afferent blood vessels and the ventricle.

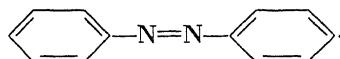
**Autocooperative interactions** Interactions in which a primary event favors subsequent events of similar nature. See Index.

**Avertin** A proprietary solution of tribromoethanol in amylyene hydrate; used as an anesthetic.

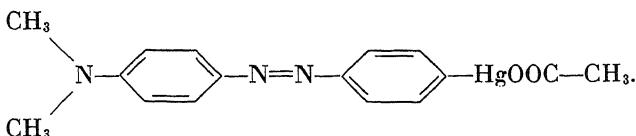
**Avogadro's number** The number of atoms or molecules in one mole of a given substance, equal to  $6.023 \times 10^{23}$ .

**Axon** A long process extending from and continuous with a nerve cell; it serves to conduct impulses to or from the cell body of the neuron and may or may not possess a myelin sheath.

**Azo dyes** A group of organic synthetic coloring materials derived from azobenzene,



**Azomercurial acetate** 4-*p*-Dimethylaminobenzeneazophenylmercuric acetate,



**Bacillus cereus** A species of rod-shaped spore-forming soil bacteria which are nonpathogenic and aerobic.

“Backbone” See Polypeptide backbone.

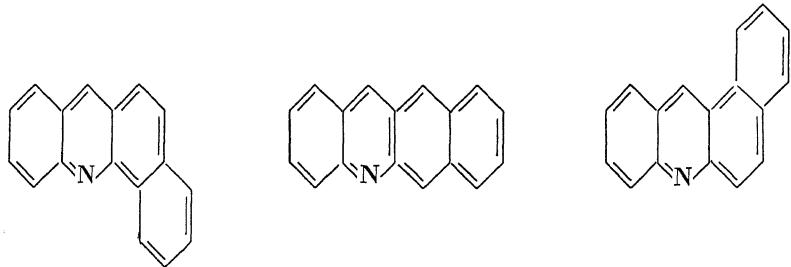
**Bacteriostatic** Referring to any agent which inhibits the growth or multiplication of bacteria but does not actually kill them.

**Bacterium (Bacteria)** A phylum of typically one-celled microorganisms which possess no chlorophyll.

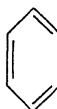
**Bacteroides vulgaris** A species of fusiform anaerobic bacteria often found in necrotic tissue.

**Bathochromic effect** A displacement of the absorption band of a compound toward longer wavelengths.

**Benzacridine** 1,2-, 2,3- and 3,4-benzacridines, in this order,

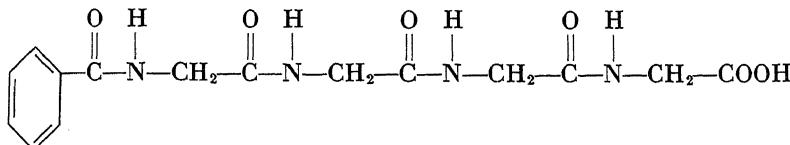


**Benzene**

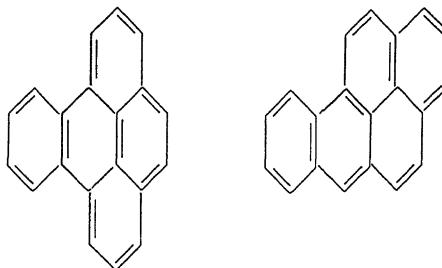


an organic solvent produced from coal.

**Benzoyltetraglycine**



benzoic acid derivative of tetraglycine.

**Benzpyrene**

hydrocarbon found in coal tar.

**Bessel function** One of a group of related functions which arise as solutions of certain differential equations commonly encountered in physical problems, as for example the diffusion problem in Chapter 11.

**Bioregulants** Biologically active agents such as vitamins, drugs, hormones, etc. See Index.

**Blastopore** In embryology, the small opening produced by invagination of the blastula; it communicates with the archenteron.

**Blastula** An early stage in the development of the embryo from an egg during which a central cavity, the archenteron, is formed in a mass of living cells.

**Blood group** One of several types into which human blood is classified on the basis of the compatibility of its corpuscles and serum with the corpuscles and sera of other persons; in the Landsteiner grouping four types designated AB, A, B, and O are characterized by different combinations of two agglutinable factors or antigens, A and B, on the red blood cells and two agglutinins or antibodies  $\alpha$ (anti-A) and  $\beta$ (anti-B), in the serum.

Group	Antigen on RBC surface	Antibody in serum
A	A	$\beta$
B	B	$\alpha$
AB	A and B	none
O	none	$\alpha$ and $\beta$

**Boltzmann constant** A universal constant,  $k = 1.380 \times 10^{-16}$  erg per degree. It is the ratio of the mean total energy of a molecule to its absolute temperature.

**Boltzmann distribution** A statistical function which describes the distribution of particles in a population; thus, if the total number of particles in the population is  $N$ , the number of particles  $N_i$  possessing kinetic energy equal to  $E_i$  at absolute temperature  $T$  is  $N_i = N \exp(-E_i/RT)$ , where  $R$  is the gas constant and  $E_i$  is the energy per mole.

**Born charging method** A simplifying method for estimating the dissociation energy of an ion pair; the medium is considered to be a continuous dielectric and the work performed in bringing a member of the pair from its associated state to a location at an infinite distance away, but still within the same dielectric medium, is integrated over this distance. The method was first used by Born.

**Born repulsion constant** The Born repulsion energy is represented by  $A/r^n$  where  $r$  represents the distance between the two ions or atoms in close association and  $n$  is usually given the values, 9, 12, etc;  $A$  is the Born repulsion constant. It is obvious that  $A$  expresses the parameter usually referred to as the size of the ion or atom.

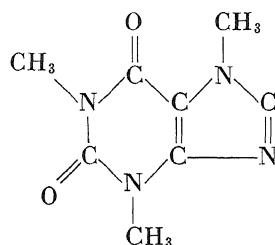
**Born repulsion energy** As two ions or atoms are brought close together, a characteristic repulsive force becomes operative when their outer electron shells begin to overlap. This repulsive potential or energy falls off very rapidly with distance and Born suggested that it be approximated by an inverse power of  $r^n$ . In the calculations presented herein,  $n$  has been given the value of 9.

**Bovine** Of a cow or ox.

**Bulk phase** A macroscopic object such as a drop of water or a glass bead can always be considered as consisting of a surface and all that is not surface; the latter is the bulk phase.

**Bulk-phase limited** A process such as diffusion or exchange diffusion which has its rate-limiting step in the bulk phase rather than at the surface.

**Caffeine** Methyltheobromine, 1,3,7-trimethylxanthine,



a diureide found in coffee, tea, and cola nuts.

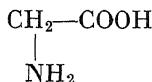
**Capillaries** The minute blood vessels which connect the arterioles and venules, forming a network in nearly all parts of the body.

**Carapace** Bony or chitinous shield covering the back or part of the back of an animal; found in crayfish, lobsters, etc.

**Carbon tetrachloride**  $\text{CCl}_4$ , an organic solvent which has a toxic action on the liver when ingested.

**Carboxyl hemoglobin** Carboxy hemoglobin, a stable combination of hemoglobin with carbon monoxide.

**$\alpha$ -Carboxyl group** Carboxyl group ( $-\text{COOH}$ ) as substituted on an  $\alpha$ -carbon atom, the first carbon atom in an aliphatic hydrocarbon chain. Thus glycine,



has an  $\alpha$ -carboxyl group.

**Carcinogen** A chemical substance which gives rise to cancer when administered to susceptible animals.

**Carcinogenesis** The production and development of cancer.

**Carcinoma** A malignant neoplasm originating in epithelial tissue.

**Cardinal adsorbent** An adsorbent on a protein or protein complex that critically controls the metastable cooperative state of a gang of sites in a protein system.

**Cardinal salt bridge** A salt linkage between fixed ionic groups on different protein chains which by changing its configuration due to an *F*-effect along one protein molecule initiates an *F*-effect along the other molecule.

**Cardinal site** A locus on a protein composed of one or more closely placed side-chain groups; it critically controls the metastable cooperative state of a group of neighboring sites (a gang).

**Carnivorous** Existing on a diet of meat.

**Catalyst** Any substance which causes the acceleration of a chemical reaction without undergoing any permanent chemical change itself.

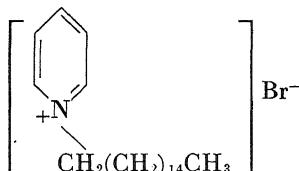
**Cathode** The negative electrode or pole of a galvanic cell; the electrode through which current leaves a phase.

**Cation** A positively charged ion; thus, a particle which, in an electrochemical decomposition, migrates toward the negative pole or cathode.

**Cationic charge** The charge borne by a positive ion.

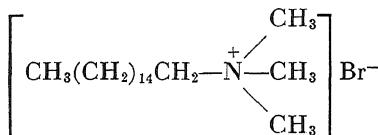
**Cellular potential** See Resting potential and Action potential.

**Cetylpyridinium bromide**



a cationic detergent with antiseptic properties.

**Cetyltrimethylammonium bromide**



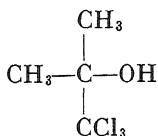
a long-chain quaternary ammonium salt which acts as a cationic detergent.

**Charge fixation** Charge fixation refers, strictly speaking, not to the fixation of a charge, but to the fixation in space of a charge-bearing ion or ionic group.

**Chelating agent** Any substance which is capable of forming a more or less stable heterocyclic ring compound with a metal cation.

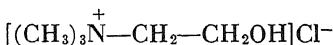
**Chemoreceptor** A receptor portion of a nerve cell adapted for excitation by chemical substances, for example, olfactory and gustatory receptors.

**Chloretone** Chlorobutanol,



an anesthetic used on laboratory animals; its germicidal properties make it useful as a preservative for certain drugs.

**Choline chloride**



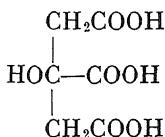
a nitrogenous compound important as a constituent of lecithin, acetylcholine, etc. Choline chloride can often be substituted for NaCl in Ringer's solution without changing biological activity.

**Choline acetylase** An enzyme which catalyzes the formation of acetylcholine by the reaction, choline + acetyl CoA  $\rightleftharpoons$  acetylcholine + CoA.

**Choline esterase** An enzyme which catalyzes the reaction, acetylcholine  $\rightarrow$  acetic acid + choline.

**Chordata** The phylum of animals possessing a notochord at some stage in their development.

**Citrate** Citric acid,

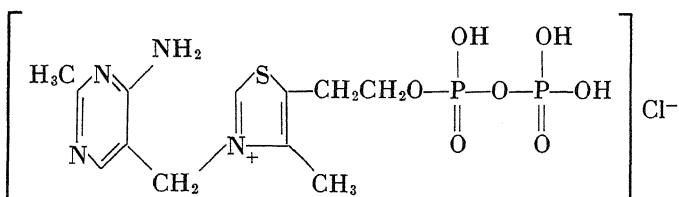


a salt of citric acid which is an important metabolic intermediate in the oxidative breakdown of glucose.

**Clostridium welchii** *Bacillus perfringens*, an anaerobic, spore-forming soil bacterium which is capable of causing severe wound infections. It produces several toxic substances among which are a lecithinase, a hemolysin, and a collagenase.

**CoA** See Coenzyme A.

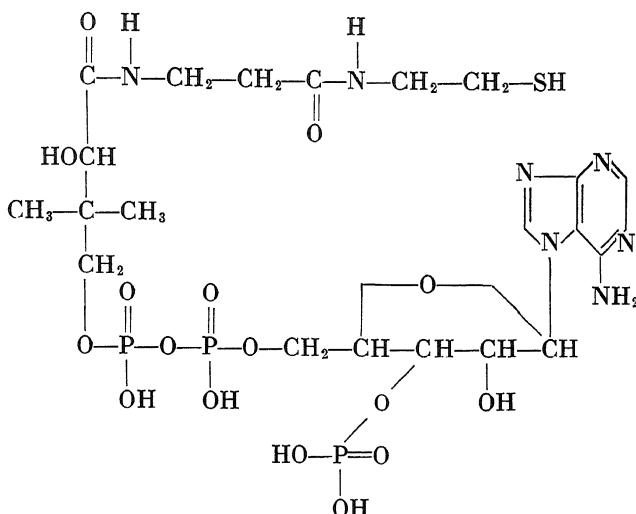
**Cocarboxylase** Diphosphothiamine,



a vitamin-B derivative which must be present for the enzymatic decarboxylation of many  $\alpha$ -keto acids.

**Codeine** Methylmorphine, an alkaloid narcotic derived from opium.

## Coenzyme A(CoA)



a compound made up of pantothenic acid, cysteamine, phosphoric acid, and adenosine which acts as a co-factor in enzymatic acetyl-transfer reactions.

**Collagen** A protein substance which makes up the supportive portions of the connective tissue of skin, tendon, etc. It is converted to gelatin by boiling.

**Collodion** A partially nitrated cellulose (nitrocellulose) which is soluble in alcohol-ether mixtures and forms transparent films on drying.

**Colloid** A multiphase system in which there is at least one finely divided dispersed phase (consisting of particles of roughly  $2 \times 10^{-6}$  to  $5 \times 10^{-7}$  cm) distributed through a continuous phase. In the present work, such systems are often referred to as semifix-chARGE systems.

**Colon** That part of the large intestine which extends from the caecum to the rectum.

**Competence** The state of reactivity of a part of an embryo enabling it to react to a given morphogenetic stimulus by differentiation in a given direction. An embryonic cell possesses as many competences as it has prospective potencies or possible morphogenetic fates.

**Competitive activation** An enzymatic site may be inactivated by interacting with another fixed site in its proximity. If an agent is added to the system which has a higher affinity than the enzymatic site for the second site, this agent will interact with the second site, freeing the enzymatic site to act on the substrate.

**Competitive inhibition** Inhibition of enzyme activity or other phenomena by an agent which competes with the substrate for the active site.

**Complement** A thermolabile, nonspecific protein complex found in normal blood serum which, together with an antibody, causes lysis of cells, destruction of bacteria, etc.

**Complexion** An *a priori*, equally probable, distinct, quantum-mechanically allowable state of an assembly of particles. See Index.

**Conductance** The reciprocal of the resistance of a substance, solution, or circuit to the passage of a current.

**Configurations 0, I, II, III** In the linear model for ion association presented in this work, a pair of ions with no water between them is in the 0-configuration; a pair of ions with one water molecule between them is in the I-configuration, etc. See Index.

**Configurational entropy** The entropy or degree of randomness arising from the number of different ways in which particles of a localized assembly may occupy different loci. See Index.

**Conformation** The spatial relation of groups in a chemical compound or in a portion of a large molecule.

**Connective tissue** The tissue which binds together and forms the support for the various structures of the body. It possesses abundant extracellular material (fiber, ground substance, etc.) in contrast to the other tissues.

**Contractile protein** Any protein which, under specified conditions, contracts reversibly; usually refers to actomyosin, the contractile element of muscle.

**Cooperative phenomenon** Fowler first used the term "cooperative transition" to describe changes of state of a system in which the interaction among individual atoms or molecules tends to increase in importance with the progress of the change under consideration. This type of cooperative phenomenon cannot be understood in terms of the properties of individual constituent atoms or molecules alone, but is the result of their interaction.

**Coronary veins** The veins of the heart.

**$\beta$ -Corticotropin** Adrenocorticotropic hormone (ACTH), a hormone of the anterior pituitary that specifically stimulates the secretory activity of the adrenal cortex.

**Cortisone** Compound E, 17-hydroxy-11-dehydrocorticosterone; a hormone from the adrenal cortex which plays a part in regulating carbohydrate metabolism. In therapeutic doses, it has an anti-inflammatory action.

**Coulombic force** Electrostatic force between two electric charges,  $q'$  and  $q$ ; it varies directly with the product of the two charges and inversely as the square of  $r$  the distance between them. Expressed by Coulomb as law of the force  $F$  between charges

$$F = \frac{qq'}{\epsilon r^2}$$

where  $\epsilon$  is the dielectric constant of the medium between the charges.

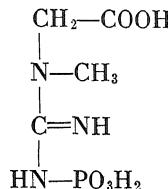
**Counterelectric field** When an electric field is imposed on a material containing dipolar molecules, these tend to orient themselves within the field; such orientation sets up another field, opposed to the original one and known as the counterelectric field.

**Counterion** An ion opposite in sign to another, more fixed in space, with which it may associate.

**Covalent bond** A type of chemical bond resulting from the sharing of two electrons by two atoms.

**Cozymase** See Diphosphopyridine nucleotide.

**Creatine phosphate (CrP)** Phosphocreatine,



a compound abundant in many living cells (see Table 9.2), which contains a labile phosphate bond.

**Cross-linking agent** A substance, such as divinylbenzene, possessing two or more reactive groups which make it useful for the formation of bridges between long-chain polymers. Used in making three-dimensional polymers such as the exchange resins.

**CrP** See Creatine phosphate.

**C-terminal residue** Protein molecules are formed by joining the  $\alpha$ -carboxyl group of one amino acid to the  $\alpha$ -amino group of another in a peptide linkage. The end of the protein molecule containing the free carboxyl group is referred to as a C-terminal residue.

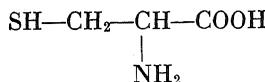
**Culture tube** A tube containing a nutrient medium which is used for the growth or culture of bacteria, etc.

**Curare**  $d$ -tubocurarine, an alkaloid of botanical origin used to block neuromuscular transmission.

**c-value** See Index.

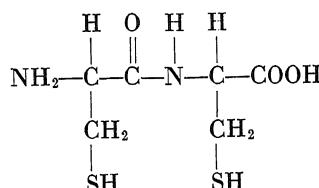
**Cyanide** Salt of cyanic acid, HCN; a metabolic poison which specifically interferes with biological oxidation reactions.

**Cysteine**  $\beta$ -Mercaptoalanine,

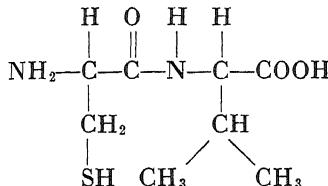


an  $\alpha$ -amino acid found in many protein hydrolysates; this residue contributes masked and unmasked SH groups to proteins.

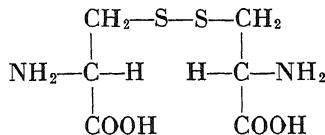
**Cysteinyl cysteine**



a dipeptide of cysteine.

**L-Cysteinyl-(D or L)-valine**

a dipeptide of cysteine and valine.

**Cystine**

a dicysteine formed by oxidation of cysteine.

**Cytochemical changes** Changes in the chemical organization and activity of the cell.

**Cytochrome oxidase** An enzyme widely distributed in cells, containing iron and a porphyrin; the iron undergoes reversible oxidation-reduction from the ferrous to the ferric state as the enzyme participates in electron-transfer reactions.

**Cytology** The branch of biology dealing with the structure, function, pathology, and life history of cells.

**Cytoplasm** The protoplasm of a cell exclusive of the nucleus.

**DAB** See *p*-Diaminoazobenzene.

**Deaminase** An enzyme which causes the removal of the amino group from organic compounds. Thus 5-adenylic acid deaminase converts 5-adenylic acid to inosinic acid (5-inosinic acid).

**Decylamine hydrochloride**  $\text{CH}_3(\text{CH}_2)_8\text{CH}_2\text{NH}_2 \cdot \text{HCl}$ , a cationic detergent.

**Dedifferentiation** Loss of the characteristics which specifically differentiate one cell or tissue from another; regression to a more primitive type of cell or tissue.

**D-effect** The microscopic direct electrostatic effect. See Index.

**Degrees of freedom** Number of different modes of movement, translational, rotational, or vibrational, available to an atom or other particle; the number of variables defining the state of a system (for example, pressure, temperature, composition) which may be fixed at will.

**Denaturant** Agent causing denaturation of proteins.

**Denaturation** A change in the nature of a protein brought about by changes in its physical or chemical environment. For complete definition and mechanism, see Index.

**Dendrite** Dendritic process, a short process of a nerve cell, generally having many branches; most neurons have several dendritic processes.

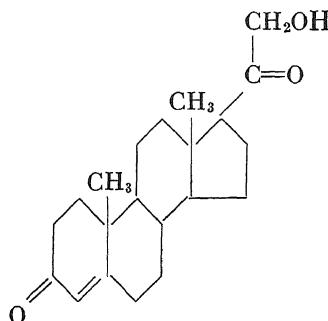
**Deoxyribonucleic acid (DNA)** Deoxyribonucleic acid, a nucleic acid especially abundant in the nuclei of animal and plant cells; it yields adenine, guanine, cytosine, thymine, phosphoric acid, and deoxyribose upon hydrolysis.

**Dephosphorylation** A reaction, catalyzed enzymatically, which involves the loss of a phosphate group.

**Depolarization** The process of reducing the resting potential of a nerve or muscle cell to zero.

**Desorption** Dissociation.

**Desoxycorticosterone** 11-desoxycorticosterone, Reichstein's substance *Q*, Kendall's desoxy compound B,



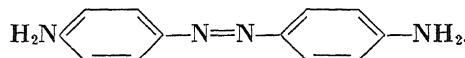
a hormone from the adrenal cortex which influences  $\text{Na}^+$ -ion excretion by the kidney.

**Detergent** A cleansing and emulsifying agent, usually a long-chain organic compound bearing an anionic (for example, carboxyl) or a cationic (for example, amino) group.

**Deutacetic acid** Acetic acid in which the carboxyl hydrogen is replaced by deuterium,  $\text{CH}_3\text{COOD}$ .

**Diabetes** A deficiency condition of animal or human, marked by a habitual discharge of a large quantity of urine. In particular it usually refers to a common disease, diabetes mellitus, a disorder involving failure of pancreatic secretion of insulin and, consequently, a profound disturbance of carbohydrate metabolism.

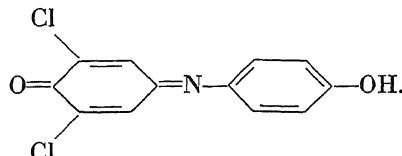
***p*-Diaminoazobenzene**



**Diaphragm** A large flat voluntary muscle which divides the thorax from the abdomen in mammals; it is used in breathing.

**Dicarboxylic acid** Dibasic acid, an organic acid bearing two carboxyl groups.

**2,6-Dichlorophenol** Indophenol,



**Dichromate** A salt containing the divalent bichromate radical,  $\text{Cr}_2\text{O}_7^{2-}$ .

**Dielectric constant** Inductivity, specific inductive capacity; a constant  $\epsilon$  which depends upon the nature of the medium and is given by

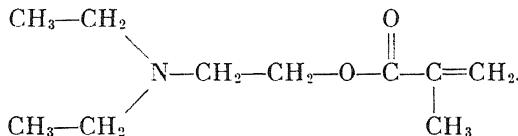
$$\epsilon = \frac{qq'}{Fr^2}$$

where  $F$  is the force of repulsion between two point charges  $q$  and  $q'$  which are a distance  $r$  apart in a uniform medium. This constant has meaning only in a macroscopic system.

**Dielectric medium** A medium whose constituent atoms and/or molecules undergo rotation, translation, or charge distortion in an electric field. A consequence of this is that a charge in one part of a dielectric is not communicated to other parts; dielectrics thus serve as electrical insulators.

**Dielectric saturation** In the intense electric field near an ion, particles of the medium become oriented so that the effective dielectric constant can no longer have the value usually given but is greatly reduced; this is the phenomenon of dielectric saturation. See Dielectric constant.

**Diethylaminoethylmethacrylate**



**Differential permeability** A membrane or the surface of a bulk phase may allow various substances to pass through at different rates. This is referred to as a differential permeability to these substances.

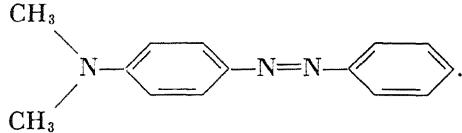
**Digilanid C** One of the glycosidic precursors which may be isolated from the leaves of the plant *Digitalis lanata*; digilanid C, on hydrolysis, yields the glycoside, digoxin. Digoxin, together with a number of other glycosides, including the more familiar digitoxin, are known for their therapeutic value in the treatment of congestive heart failure.

**Digitalis** The plant or the leaves of *Digitalis purpurea* (fox glove), used medicinally to combat congestive heart failure. Many different glycosides and active principles have been isolated from it.

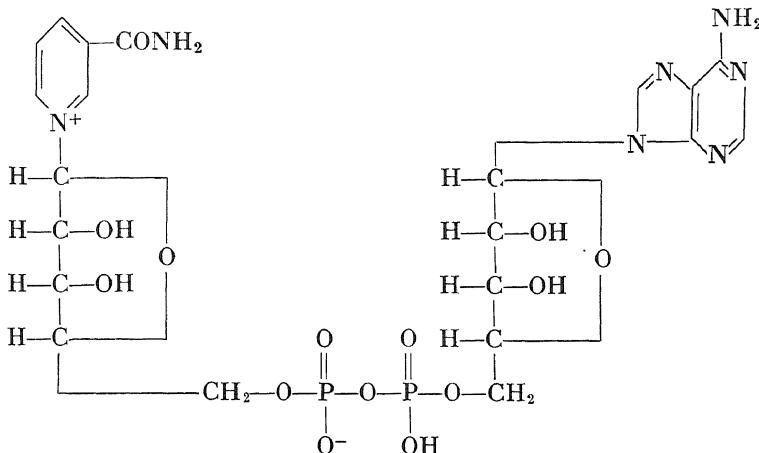
**Digitonin** A glycoside found in crude extracts of digitalis plants; it is a saponin with little of the effect on the heart characteristic of the cardiac glycosides such as digitoxin of which it is a close relative.

**Digitoxin** A glycoside assumed to be the chief active principle in digitalis.

**p-Dimethylaminoazobenzene (DAB)**



Diphosphopyridine nucleotide (DPN) Cozymase, coenzyme I;



the coenzyme of apozymase, necessary for the alcoholic fermentation of glucose; acts as hydrogen acceptor in various enzymatic oxidation-reduction reactions.

**Dipole** A molecule in which the effective centers of the positive and negative charge are separated.

**Dipole moment** Product of the electronic charge and the distance between the positive and negative electrical centers of a dipole.

**Dissociation constant** The ratio of the product of the concentrations of the molecules or ions resulting from a dissociation to the concentration of the nondissociated molecule at equilibrium. For the reaction,  $\text{AB} \rightleftharpoons \text{A}^+ + \text{B}^-$ , the dissociation constant  $K$  is given by

$$K = \frac{[\text{A}^+][\text{B}^-]}{[\text{AB}]}$$

where the bracketed quantities represent the respective concentrations.

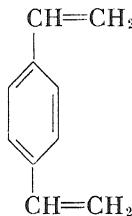
**Dissociation energy** The difference between the energy possessed by two entities when they are associated and the energy they possess at infinite separation from each other. It is equal in magnitude to the association (or adsorption) energy, but opposite in sign; that is, dissociation energy is a positive value.

**Distortion polarization** Formation of a dipole due to distortion of the electron cloud of a molecule; an induced dipole is produced.

**Distribution ratio** The ratio of the concentrations of a substance found in two contiguous phases (usually at equilibrium) is referred to as the distribution ratio of the substance in these two phases.

**Disulfide linkage** —S—S—; example, the linkage between two cysteine groups to form cystine.

## Divinylbenzene (DVB)



a chemical used as a cross-linking agent in the manufacture of exchange resins.

DNA See deoxyribonucleic acid.

Dodecyltrimethylammonium bromide [CH<sub>3</sub>(CH<sub>2</sub>)<sub>11</sub>—N<sup>+</sup>(CH<sub>3</sub>)<sub>3</sub>]Br<sup>-</sup>, a cationic detergent.

**Donnan equilibrium** The conditions which exist at equilibrium when two different solutions are separated by a membrane which is permeable to some of the ions of the solutions but not to all of them. Because there is an asymmetric distribution of the ions between the two solutions, an electrical potential develops between the two sides of the membrane; the two solutions vary also in osmotic and in hydrostatic pressure.

**Donnan ratio** The ratio of the concentrations of any one permeant ion in two solutions separated by a semipermeable membrane at equilibrium. It may be represented,

$$r = \left[ \frac{[C_i]_1}{[C_i]_2} \right]^{\frac{1}{n}} = \left[ \frac{[A_j]_2}{[A_j]_1} \right]^{\frac{1}{m}}$$

where [C<sub>i</sub>]<sub>1</sub> is the concentration of the *i*th permeant cation of valence *n* in solution and [A<sub>j</sub>]<sub>2</sub> is the concentration of the *j*th permeant anion of valence *m* in solution 2.

**Dopa oxidase** 3,4-Dihydroxyphenylalanine oxidase; an enzyme which oxidizes tyrosine and dihydroxyphenylalanine (dopa) to melanin.

**Dorsal lip** In the development of amphibian and other ova, the liplike structure at the dorsal border of the blastopore during gastrulation.

**Doublet migration** A possible mechanism by which an ion can move across a fixed site at the surface or within a fixed-charge system. This process involves the occupation of the site by the ion followed by its desorption without the participation of a second free ion.

**Dowex 1, Dowex 2** Commercial ion exchange resins (Dow Chemical Co.) bearing positively charged amino groups.

**Dowex 50** A commercial ion exchange resin compound of polystyrene sulfonate cross-linked with divinylbenzene.

**DPN** See Diphosphopyridine nucleotide.

**DVB** See Divinylbenzene.

**ɛ°** See Oxidation-reduction potential.

**Echinodermata** A phylum of the animal kingdom including starfish, sea urchins, etc.

**Ectoderm** The outermost of the three germ layers of classical embryology.

**Edestin** A globulin protein from hemp seed which may be obtained in crystalline form.

**EDTA** See Ethylenediaminetetraacetic acid.

**Effective negative charge** The effective negative charge of an atom is equivalent to its *c*-value.

**Effector site** One of a cluster or gang of reactive sites which produces a physiological response to an agent adsorbed at another, the receptor, site.

**Egg albumin** Ovalbumin, a protein which constitutes 20 per cent of the white of hens' eggs.

**Electrode** An electrical conductor through which an electric current enters or leaves a conducting medium.

**Electroencephalogram** A graphic record of the electrical potential fluctuations (brain waves) between electrodes placed on various parts of the surface of the head or brain.

**Electrokinetic phenomenon** Any phenomenon associated with the movement of charged particles through a continuous medium or with the movement of a continuous medium over a charged surface. The four principle electrokinetic phenomena are, electrophoresis, electroosmosis, streaming potential, and sedimentation potential. These phenomena are related to one another through the zeta potential  $\zeta$  of the electrical double layer which exists in the neighborhood of the charged surface (see Figure 10.6).

**Electrolysis** The separation of the anionic and cationic components of a salt by the application of an electric field.

**Electrolyte** Any substance which, upon being dissolved in a suitable medium or melted, dissociates into ions, thus forming a conductor of electricity. Acids, bases, and salts are electrolytes.

**Electromotive force (emf)** The driving force which tends to cause a movement of electricity around an electric circuit.

**Electromyogram** A record of the changes in the electrical potential of a muscle; recorded with electrodes external to the muscle or inserted into the bulk of the muscle, but not intracellularly.

**Electron** An elementary particle which is the negatively charged constituent of ordinary matter.

**Electron affinity** A number of (electronegative) elements form stable singly charged negative ions (for example, F, Cl, Br, I, O, S). The energy difference between the anionic and the neutral states is called the electron affinity.

**Electronegativity** The tendency of an atom or group in a molecule to draw electrons to itself.

**Electroneutrality** The state of possessing equal numbers of positive and negative charges.

**Electronic partition function** The way in which a set of electronic energy levels is populated at a given temperature. See Partition function.

**Electron micrograph** A photograph of an object taken through an electron microscope, thus magnified up to about 100,000 times.

**Electrophilic** Electron deficient, electron attracting; as, a substituent or adsorbent on a molecule which draws electrons from the rest of the molecule to itself.

**Electrophoresis** The migration of colloidal particles suspended in a fluid medium, under the influence of an electric field.

**Electrophoretic boundary** The boundary formed by colloidal particles migrating under the influence of an electric field.

**Electrophoretic mobility** The velocity of migration of a colloidal particle under the influence of an electric field.

**Electrostatic bond** A stable association formed between two ions of opposite charge due entirely to electrical attraction between the ions.

**Electrostatic effect** Any effect due to Coulombic interaction between two or more electrical charges.

**Electrostatic energy** The work necessary to separate two associated charged particles to an infinite distance from each other.

**Electrostatic field strength** In a dielectric medium (dielectric constant =  $\epsilon$ ) the force between two charges  $q$  and  $q'$  a distance  $r$  apart is

$$\frac{qq'}{\epsilon r^2}$$

If  $q'$  has the magnitude of unity, the force  $q/\epsilon r^2$  is referred to as the electrostatic field strength at the point at which  $q'$  is located.

**Electrostatic interaction** Interaction between particles due to their charges; it is described by Coulombs' Law. The interaction can involve either attraction or repulsion. See Coulombic force.

**Electrostatics** The study of electric charges at rest under conditions where the positive and negative charges are separated from each other.

**Elution** The separation of material by rinsing with a volume of flowing fluid.

**Emf** See Electromotive force.

**Endoderm** The innermost of the three germ layers of classical embryology.

**End plate** Motor plate, a specialized organ formed at the termination of motor nerve fibers on striated muscle (see Figure 13.4).

**End-plate potential** A local partial or complete depolarization of the muscle cell surface at an end plate seen as a graded response to stimulation of the nerve supplying it or as the result of electrical or chemical stimulation at that locus.

**Enthalpy** Heat content, a thermodynamic quantity given by  $H = E + PV$ , where  $E$  is the internal energy of the system;  $P$ , pressure; and  $V$ , volume.

**Entropy** That portion of the energy of a substance which is unavailable for the performance of useful work. It is due to the various modes of motion of the molecules and may be considered a measure of the degree of randomness of the molecules. Entropy  $S$  is directly proportional to the heat content  $H$  and inversely proportional to the absolute temperature,  $S = H_{\text{rev}}/T$ . In statistical mechanics, entropy is related to the number of complexions  $\Omega$  by the relation,  $S = kT \ln \Omega$ , where  $k$  is the Boltzmann constant.

**Enzymatically active site** A locus on a molecule or cell which possesses enzymatic activity.

**Enzyme** A biological catalyst; enzymes are proteins or protein complexes.

**Enzymology** The branch of science dealing with the study of enzymes.

**Eosin** Tetrabromofluorescein, a red fluorescent pigment used in histology to stain cellular cytoplasm.

**Epithelial cells** Those cells which make up the covering of the internal and external surfaces of the body, including the linings of cavities and mucous membranes.

**Equilibrium** See Thermodynamic equilibrium.

**Equivalent conductance** A measure of the quantity of electricity transferred across a unit area, per unit potential gradient, per unit time, per number of equivalents of an electrolyte per unit volume of solution.

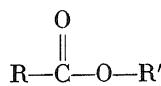
**Erythrocyte** Red blood cell (RBC).

**Erythrocyte ghost** After the action of certain hemolytic agents on red blood cells, dim colorless outlines of the cells may be seen; these are called ghosts. They represent the incompletely destroyed framework or stroma of the cell.

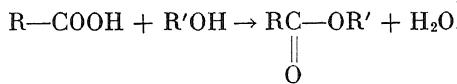
**Erythrosin** Sodium salt of tetraiodofluorescein, a reddish dye.

***Escherichia coli*** Colon bacillus, a motile, flagellated gram-negative organism normally present in the intestine.

**Ester**



a class of organic compounds resulting from the combination of an organic acid with an alcohol,

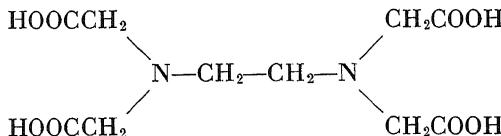


**Estradiol** A steroid hormone thought to be the main estrogenic hormone secreted by the ovary. It is important in control of the development of the secondary sexual characteristics of the female.

**Ether Group**  $\text{R}-\text{O}-\text{R}'$ , any group in which an oxygen connects two aliphatic hydrocarbons.

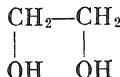
**Ethylamine hydrochloride**  $\text{C}_2\text{H}_5\text{NH}_2\text{HCl}$ .

**Ethylenediaminetetraacetic acid (EDTA)**



a chelating agent.

Ethylene glycol



the simplest polyhydroxyl alcohol.

Ethyl ether  $\text{CH}_3-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_3$ , a volatile liquid used as an organic solvent as well as an anesthetic.

Ethyl mercaptan  $\text{CH}_3-\text{CH}_2\text{SH}$ , a liquid with a powerful onionlike odor.

Etiology The science or demonstration of causes, especially the causes of disease.

Evocation The action of a chemical substance in eliciting a morphogenetic effect.

Evocator A chemical substance emitted by an organizer which is active in the control of morphogenesis.

Exchange energy According to wave-mechanical theory, the attractive energy between a pair of atoms includes a term which depends upon the exchange of electrons between the two nuclei. This exchange energy in a covalent linkage is practically equal to the total bond energy.

Exchange resin A synthetic resinous material which carries fixed ions whose counterions can be exchanged. It is used for purification of solutions, etc.

Excitation In physiology, the evocation of the normal response of an excitable cell or tissue; for example, excitation of nerve brings about impulse generation; of muscle, contraction.

*Extensor digitorum* muscle A muscle of the leg and foot of the frog which serves to extend (straighten) the toes.

Faraday constant ( $F$ ) The amount of electricity (96,490 absolute Coulombs) which, when passed through a solution, will liberate one gram equivalent of an electrolyte component, sodium from NaCl, for example.

Fast muscle Twitch muscle, a muscle which, when stimulated by a single shock above threshold in intensity, responds with a twitch, in contrast to slow fibers which respond by a slow and sustained shortening.

Fatty acid One of a group of organic monobasic acids derived from the hydrocarbons by the equivalent of the oxidation of a methyl group,  $\text{R}-\text{CH}_3 \rightarrow \text{RCH}_2\text{OH} \rightarrow \text{RCHO} \rightarrow \text{RCOOH}$ .

*F*-effect The combined inductive and direct electrostatic effect. See Index.

Ferret A member of the weasel family.

Ferricyanide  $\text{Fe}(\text{CN})_6^{4-}$ , salt of ferricyanic acid; a mild oxidizing agent.

Ferrihemoglobin Methemoglobin, a transformation product of oxyhemoglobin produced by poisoning with nitrates, chlorates, and other substances; it contains a true oxide of heme ( $\text{Fe}^{+++}$ ), thus differing chemically from oxygenated hemoglobin.

Ferromagnetism The property of matter that permits it to become an induced magnet; especially the property of becoming strongly magnetic as iron, nickel, and cobalt.

**Fixed anion** A negatively charged functional group which is part of a large molecule or fixed-charge system and, hence, whose position in space is determined by the position of the large molecule or the fixed-charge system.

**Fixed cation** A positively charged functional group which is part of a large molecule or fixed-charge system and, hence, whose position in space is determined by the position of the large molecule or the fixed-charge system.

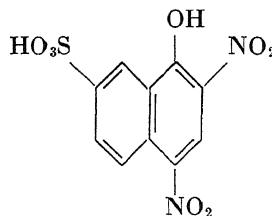
**Fixed-charge system** A three-dimensional system consisting of a fixed framework onto which are fixed, more or less regularly, a large number of charge-bearing groups. A typical fixed-charge system contains a substantial volume of water.

**Fixed polar group** Any polar functional group whose position in space is determined by the large molecule (usually a protein) or fixed-charge system to which it is attached.

**Flame photometry** A method of spectrochemical analysis in which samples in solution are excited to luminescence by introduction into a flame. It is based on the principle that different substances emit luminescence of different wavelengths and that the intensity of the radiation is proportional to the concentration of the substance. The method is useful for analysis of small amounts of the alkali metals, Li, Na, K, Rb, and Cs.

**Flatworm** *Platyhelminthes*, the lowest phylum of worms; they possess soft, flattened bodies. Examples are planaria, flukes, tapeworms.

**Flavianic acid** 2,4-Dinitro-1-naphthol-7-sulfonic acid,



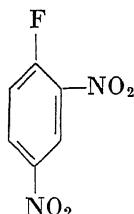
a yellow compound used in dyeing wool.

**Flemming's fluid** A solution used to fix tissues; made of 3 parts glacial acetic acid, 12 parts 2 per cent osmic acid solution, and 50 parts 1 per cent chromic acid solution.

**N-2-Fluorenylacetamide** See 2-Acetylaminofluorene.

**Fluorescent dyes** Dyes which emit radiation when exposed to light; the light emitted may be of the same or different wavelengths as the impinging light.

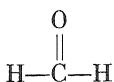
**Fluorodinitrobenzene**



a reagent which reacts with terminal  $\alpha$ -amino groups on proteins.

**Fluorodinitrobenzene method** A method of labeling the terminal amino-acid group in a polypeptide with 1-fluoro-2,4-dinitrobenzene; useful for identifying the sequence of amino acids in a polypeptide.

### Formaldehyde



a gas formed by incomplete combustion of organic substances; in 40 per cent aqueous solution, it finds wide use as a disinfectant and preservative for biological materials, and is known as formalin.

**Fractionation** The separation of a system into its components; the separation and isolation of the various proteins composing a tissue.

**Free energy** Gibbs free energy, the work which a system is able to perform in a given isothermal process at constant pressure; a thermodynamic function given by

$$F = H - TS$$

where  $H$  is enthalpy or heat content;  $S$ , entropy; and  $T$ , absolute temperature (See also Helmholtz free energy).

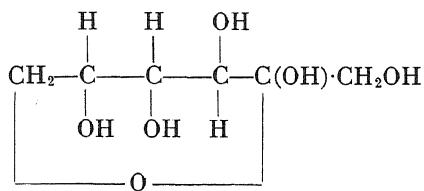
**Free solution** A solution in which each solute or solvent particle, over an infinite length of time, has equal probability of being found at a particular locus, as opposed to a fixed-charge system where some ions are localized directly or secondarily to a more or less rigid framework fixed in space.

**Free volume** Total volume available to a particle for translational motion.

“Freezing in” of water molecules See Dielectric saturation.

Frequency modulation Coding of a message in terms of the number of impulses per second.

### Fructose Levulose, fruit sugar,



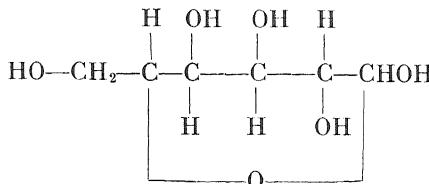
a hexose found widely in fruits.

**Fumarase** An enzyme which catalyzes the reaction,  $\text{fumarate} \rightleftharpoons \text{malate}$ .

**Functional group** Any reactive group on a molecule, for example, carboxyl, hydroxyl, nitro, amide.

**Fungi** A phylum of plants whose members are devoid of chlorophyl or any other pigment capable of photosynthesis; therefore, they are parasitic.

Galactose



a hexose constituent of lactose, or milk sugar, as well as of many oligosaccharides and polysaccharides occurring in nature.

Gang of sites A number of sites, polar and/or H-bonding that are linked to each other by a polypeptide chain the length of which does not exceed the maximum distance of transmission for a direct *F*-effect which can bring about an effective exchange of adsorbents at the successive sites.

Gas constant (*R*) The proportionality constant which relates the product of the pressure *P* and volume *V* of one mole of a perfect gas to its absolute temperature *T*,

$$R = P/VT.$$

Gastric mucosa The lining of the stomach.

*Gastrocnemius* muscle The calf muscle; a large muscle which originates on the distal end of the femur or thigh bone and inserts, through the tendon of Achilles, onto the bone of the heel.

Gastrula In embryology, the stage following the blastula and formed by the invagination of the archenteron.

Gel A colloid which is firm in consistency although containing much liquid.

Gelatin A protein obtained from animal tissues containing collagen (bones, ligaments, and skin) by boiling them with water.

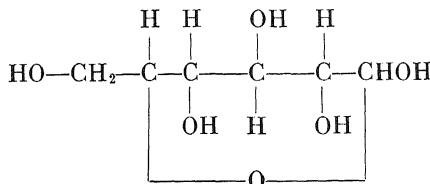
Gene A supermolecule or micelle considered to be the unit of heredity. Most recent researches suggest that the genes are actually the DNA molecules which are found in chromosomes.

Geometric progression Any sequence of numbers,  $a, ar, ar^2, ar^3, \dots, ar^n, \dots$ , in which each term, except the first, is obtained by multiplying the immediately preceding term by the number, *r*.

Gestation age Age of an embryo starting with fertilization of the ovum.

G-F Transformation The transformation of a protein (actin, for example) from the globular to the fibrous form. See Index.

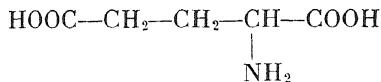
Glucose *d*-Glucose, dextrose, grape sugar,



a hexose widely distributed in fruits and other parts of plants.

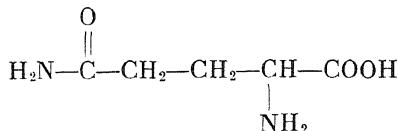
**$\beta$ -Glucuronidase** An enzyme found in the liver and spleen where it catalyzes the hydrolysis of conjugated glucuronides.

**Glutamic acid**



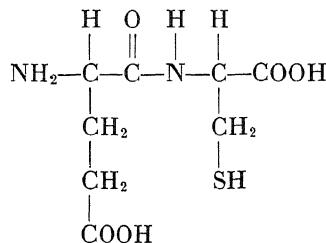
an important amino acid found in most protein hydrolysates; it bears a residual negative charge when contained in a polypeptide chain at neutral and high *pH*. See Table 0.1.

**Glutamine**



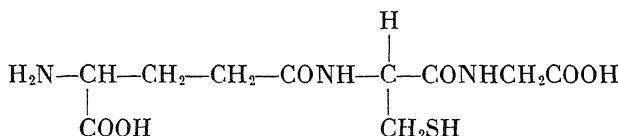
an amino acid found in protein hydrolysates.

**L-Glutamyl-L-cysteine**



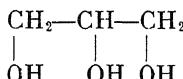
a dipeptide of glutamic acid and cysteine.

**Glutathione**  $\gamma$ -L-glutamyl-L-cysteinylglycine,



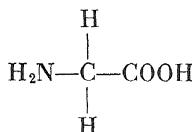
a tripeptide which is widely found in animal and plant cells and has a powerful reducing action.

**Glycerol**



a syrupy liquid, miscible with water; used as a preservative of biological material, an emollient in hand lotion, etc.

## Glycine



an  $\alpha$ -amino acid found in protein hydrolysates.

**Glycogen** Animal starch; the reserve carbohydrate of animals; a polysaccharide made up of glucose units.

**Glycolytic metabolism** The hydrolytic decomposition of sugars in metabolism; in particular, those decompositions that lead to the formation of lactic acid through an anaerobic process.

**Glycosides** A class of compounds which represent the combination of an aglycon with from one to four molecules of a sugar. Aglycons are complex ring compounds related to the steroids, for example, digitoxin, a cardiac glycoside, is a combination of three molecules of the sugar, digitoxose, with the aglycon, digitoxigenin.

**Gram-negative organisms** Organisms which become decolorized and take on the contrast stain when treated with the method devised by a Danish physician, H. C. J. Gram.

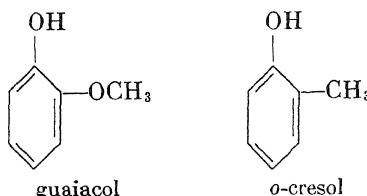
**Gram-positive organisms** Organisms which are stained deep violet by a method developed by a Danish physician, H. C. J. Gram.

**Group A, Group B, Group O, Group AB** See Blood groups.

**Group of sites** Several or many sites on a protein molecule, each separated from its nearest neighbor by a distance no greater than the maximum distance of transmission of the direct *F*-effect; these sites react to external changes in a cooperative fashion.

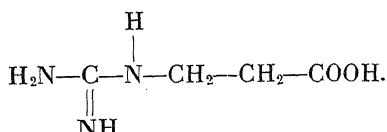
**Growth hormone** Somatotropic hormone, a hormone secreted by the anterior hypophysis which promotes general growth of the animal.

## Guaiacol-cresol mixture

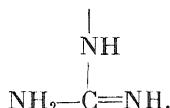


A potential may be measured at the interface between an aqueous solution and a guaiacol-cresol mixture; for this reason, such a mixture has sometimes been used as a model of a cell membrane.

**Guanidine hydrochloride**  $\text{HN}=\text{C}(\text{NH}_2)_2\text{HCl}$ , a freely soluble salt.

 $\beta$ -Guanidopropionic acid

Guanidyl group



At *pH* values below its acid dissociation constant, the guanidyl group bears a net positive charge obtained by addition of a proton.

**Heat of dissociation** The change in heat content of one mole of a substance undergoing a dissociation reaction at constant temperature and pressure.

**Heat of neutralization** The change in heat content of the reacting substances when acids or bases are neutralized at constant temperature and pressure.

**Heavy-metal ions** Ions of metals of high atomic weight such as  $\text{Pb}^{++}$ ,  $\text{Ag}^+$ , etc.

**Heavy water ( $\text{D}_2\text{O}$ )** Water in which hydrogen of mass one (H) has been replaced by its isotope deuterium (D), which is hydrogen of mass two.

**Helix** A spiral; the curve assumed by a straight line drawn on a plane when that plane is wrapped around a cylinder in a single layer.

**Helmholtz free energy** Work function; a thermodynamic function given by  $A = E - TS$ , where  $E$  is the internal energy;  $S$ , the entropy; and  $T$ , the absolute temperature. The maximum reversible work that can be made available in a given isothermal process.

**Hemoglobin** The pigment-protein complex of red blood cells by means of which they are able to transport oxygen.

**Hemolymph** The nutrient fluid or blood of insects and some other invertebrates.

**Hemolysis** The modification of red blood cells in such a manner as to cause the release of hemoglobin; this may be accomplished by heating, freezing, chemicals, biological agents, hypotonic solutions, etc.

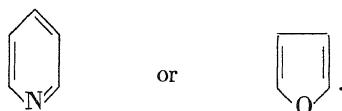
**Hepatoma** A tumor of the liver.

**Herbivorous** Feeding entirely or chiefly on plants.

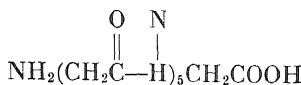
**Heterochromy, Heterochromous** The phenomenon and property of staining so that the parts of a whole may be distinguished by their different colors.

**Heterocooperative interactions** Those interactions of a gang of sites which are made less probable by an earlier similar interaction. See Index.

**Heterocyclic compound** A ring compound containing both carbon atoms and atoms of other elements in the ring; for example,



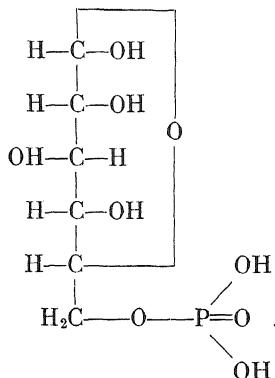
**Heterogeneous catalysis** Any catalytic reaction between gases, solutes, etc., occurring at the surface of a solid.

**Hexaglycine**

the hexapeptide of glycine.

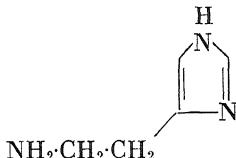
**Hexose** Any sugar containing six carbon atoms.

**Hexosephosphate** Any combination of hexose with phosphate, for example, glucose-6-phosphate,

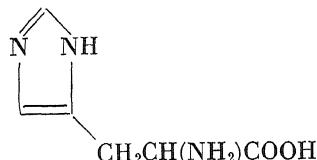


**High-energy phosphate bond** Labile pyrophosphate bond, any bond between a phosphate group and an organic molecule which is particularly labile; compounds possessing such a bond are particularly useful in biological synthetic reactions. Early measurements of the bond energy of these compounds indicated high bond energies ( $-12$  to  $-16$  kcal/mole), hence their name; recent studies have indicated, however, that the energy of these bonds is actually in the range of  $-4$  to  $-5$  kcal/mole.

**Hill's reaction** The evolution of oxygen from water in the presence of certain extracts of plant leaves; thought to be a part of the photosynthetic reaction.

**Histamine**

the product of the decarboxylation of histidine; it is released by animal tissues in allergic reactions.

**Histidine**

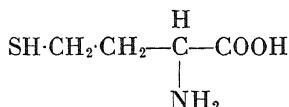
an amino acid which carries a net positive charge in an acid medium when joined into a polypeptide chain.

**Histogenesis** In embryology, formation and differentiation of a tissue.

**Histology** The branch of anatomy dealing with the structure, composition, and function of the tissues.

**Hofmeister series** See Lyotropic series.

**Homocysteine**



an  $\alpha$ -amino acid which is a metabolic intermediate.

**Homogeneous acid catalysis** Any catalytic reaction occurring in a solution where an acid serves as the catalyst; for example, hydrolysis of an *o*-ester.

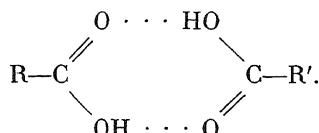
**Hormone** A chemical substance secreted into the body fluids by an endocrine gland which has a specific effect on the activity of other organs.

**Hydrated ion** An ion which possesses associated water molecules.

**Hydration energy** Free energy of hydration; the difference between the free energy of a particle in a vacuum and the free energy of a particle in an infinitely dilute aqueous solution.

**Hydrazinium** Any salt of hydrazine; for example, hydrazine sulfate,  $\text{H}_2\text{N}-\text{NH}_2-\text{H}_2\text{SO}_4$ .

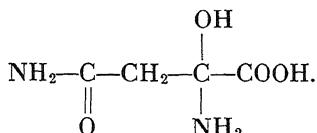
**Hydrogen bond (H-bond)** A weak bond ( $-2$  to  $-8$  kcal/mole) largely ionic in nature, between a strongly electronegative atom (O,F,N) of one molecule and another electronegative atom on a second molecule through a hydrogen atom which is linked to one of the electronegative atoms by a covalent bond; for example,



**Hydrolysis** Destruction or decomposition of a chemical substance by water, usually according to the scheme,  $\text{R}-\text{R}' + \text{H}_2\text{O} \rightleftharpoons \text{RH} + \text{HOR}'$ ; heat, acids, or bases must often be added to make the reaction proceed with observable speed.

**Hydrolytic products** Substances formed as the result of hydrolysis.

**$\alpha$ -Hydroxyasparagine**



**Hypophysectomy** Surgical removal of the pituitary body, a small endocrine gland at the base of the brain.

**Hypophysis** The pituitary gland; an endocrine gland at the base of the skull which is divided into two or three parts; the posterior hypophysis secretes oxytocin and anti-

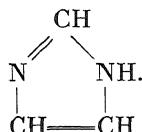
diuretic hormone; the anterior hypophysis secretes hormones which influence growth (growth hormone) and the secretion of other endocrine glands (ACTH, thyrotropic hormone, etc.); an intermediate lobe secretes a melanocyte-stimulating hormone.

**IAA** See Iodoacetate.

**I-effect** Inductive effect. See Index.

**Iliofibularis** muscle A fusiform muscle on the ventral aspect of the thigh of the frog; originates from the pelvic girdle and inserts just under the *sartorius* on the fibula.

**Imidazole** Glyoxaline,



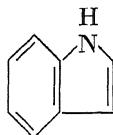
**Immunity** The power which an individual sometimes acquires to resist or overcome an infection to which most or many members of its species are susceptible.

**Immunology** The branch of biology dealing with the study of immunity; especially dealing with studies of antigens, antibodies, and their reactions.

**Impermeant substance** Any substance which is unable to enter a given phase from another phase in which it is dissolved or to pass through a given membrane.

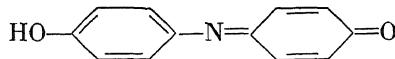
**Indirect F-process** Indirect *F*-effect, a cooperative perturbation on a protein molecule in which linearly arrayed sites successively change their counterions. This chain reaction is suggested as the primary mode of transmission of biological signals. See Index.

**Indole** 2,3-benzopyrrole,



occurs in coal tar, some plants, and in feces.

**Indophenol**



a dye sometimes used as an indicator for oxidation-reduction reactions.

**Induction** The morphogenetic effect brought about by an organizer, inductor, or evocator acting on competent tissue.

**Inductive effect** See Index.

**Inductive factor** The effectiveness of a particular adsorbent or reactant in producing an effect through the induction mechanism can be expressed as an inductive factor. For the simplest adsorbent this factor is equal to the adsorption energy of the entity.

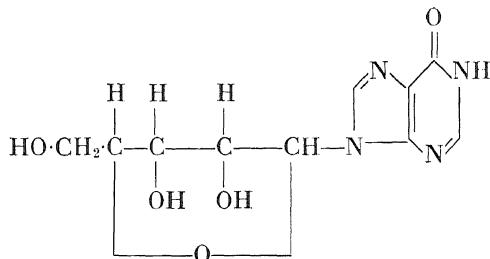
**Inductorium** An apparatus for generating induced currents, often used in electrophysiology to apply a short, measured electrical stimulus to a muscle or nerve.

**Inertia** That property of matter which manifests itself as a resistance to any change in the motion of a body; thus, with no external force acting, a body at rest remains at rest and a body in motion continues moving with a uniform speed.

**Influenza virus** Any member of a large group of viruses which cause acute respiratory-tract infections; four immunological types are known, A, B, C, and D.

**Inhibition** The action to prevent or arrest a physiological activity, especially any action which tends to prevent depolarization of an excitable cell (muscle or nerve).

**Inosine**



a riboside which can be obtained by the deamination of adenosine.

**Insulin** A protein hormone secreted by the cells of the islets of Langerhans of the pancreas which is essential for the maintenance of proper carbohydrate metabolism. See Figure 0.4 for structural formula.

**Internal energy** The energy content of a system including all forms of energy other than those resulting from the position of the system in space, which is assumed to remain constant.

**Interface** Boundary between two phases.

**Interstitial ions** The ions which do not occupy regular lattice sites in ionic crystals or in a fixed-charge system.

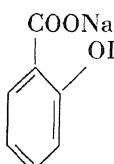
**Intrinsic *c*-value** See Index

*In vitro* In glass; hence isolated from the system in which it occurs in nature.

*In vivo* In the living system; hence, *in situ*; observed under the conditions normally prevailing.

**Iodoacetate** Salt of iodoacetic acid,  $\text{ICH}_2\text{COOH}$ ; a metabolic poison, specifically interfering with anaerobic glycolysis (see Figure 8.1).

***o*-Iodosobenzoate, sodium**



salt of *o*-iodosobenzoic acid; a mild oxidizing agent.

**Ion** An electrically charged atom, radical, or molecule formed by the loss or gain of one or more electrons.

**Ion cloud** Array of ions of one charge in the vicinity of an ion of opposite charge.

**Ionic mobility ( $u$ )** A measure of the ability of a charged particle to move in an electric field; thus  $u = v/x$ , where  $v$  is the drift velocity under an electronic field of intensity  $x$ .

**Ionic site** Any fixed ionic group in a true fixed-charge system or a semifixed-charge system.

**Ionic strength** A measure of the intensity of the electrical field due to the ions in a solution; given by

$$\mu = \frac{1}{2} \sum_i c_i z_i^2$$

where  $c_i$  is the molality of the  $i$ th ion and  $z_i$  is its valence.

**Ion-ion interaction** Interaction between one ion and another.

**Ionization potential** The work necessary to displace an electron from the lowest energy level in an atom to an infinite distance, so that a positively charged ion results.

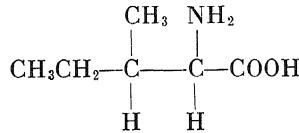
**Ionized molecule** A molecule which has lost or gained electrons and, for this reason, bears a charge.

**Ion selectivity** The ability of any substance, living or nonliving, to show different reactivities to different ions.

**Isoelectric point** The point of electric neutrality or zero potential; hence the pH value at which an amphoteric substance is neutral; below or above this pH value it acts, respectively, as a base or as an acid.

**Isohemagglutinin** Any substance found in the blood serum of an individual which agglutinates the red blood corpuscles of other members of the same species; examples are the blood-group agglutinins  $\alpha$  and  $\beta$ .

**Isoleucine**



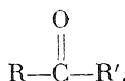
an  $\alpha$ -amino acid which is neutral when joined into a polypeptide; see Table 0.1.

**Isopiestic measurements** Measurements of the properties of a solution by methods involving the vapor pressure.

**Isotonic solution** A solution possessing the same osmotic concentration as the plasma of a given animal.

**$\beta$ -Keratin** A protein found in the dead outer corneal layer of the skin and variously modified into hair, horn, feathers, or hoofs.

**Ketone** Organic compound of general structure,



**Kilocalorie** The amount of heat required to raise one kilogram of water from 15° to 16°C.

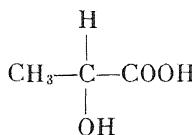
**Kinetic energy** The energy possessed by a body due to its motion, given by

$$\text{K.E.} = \frac{1}{2}mv^2$$

where  $m$  is the mass of the body and  $v$  is its velocity.

**K-Region** A locus in polynuclear hydrocarbons. See Table 18.7 for illustration.

**Lactic acid**



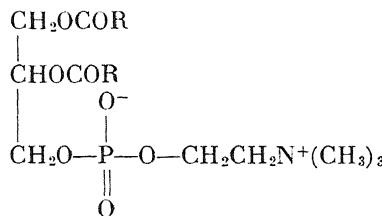
an organic acid found in sour milk as a result of the action of lactic-acid bacteria; formed in cells as a result of anaerobic glycolysis.

**Lactic dehydrogenase** An enzyme which catalyses the reduction of pyruvic acid by reduced DPN to yield lactic acid.

**$\beta$ -Lactoglobulin** The form of globulin protein present in milk.

**Latent heat of vaporization** Heat of vaporization, the difference in the heat content of one mole of a substance in liquid and vapor forms at the same temperature and pressure.

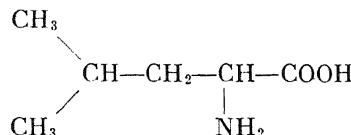
**Lecithin**



a naturally occurring phosphatide especially abundant in nerve tissue, semen, and the yolk of egg.

**Lecithinase** Any enzyme which splits lecithin.

**Leucine**



an  $\alpha$ -amino acid which is neutral when joined to a polypeptide. See Table 0.1.

**Light scattering** The scattering that occurs when a beam of light encounters a population of particles; the electrically charged nuclei and electrons in the particles vibrate. The oscillation of these charged particles sets up light beams of the same wavelength as the excitatory beam but in all directions. These transmitted beams can be photographed and used to gain information about molecular structure.

**Linear polyelectrolyte** A linear polymer bearing many dissociable (charged) groups such as carboxyl, sulfonic, or amino.

**Linear polymer** A long-chain molecule built of many similar units, referred to as monomers.

**Lipid** A general term applied to a group of compounds characterized by solubility in organic solvents and insolubility in water; includes neutral fats, sterols, phospholipids, etc.

**Lipochrome** A class of fat-soluble hydrocarbon pigments, carotene and xanthophyll, for example.

**Living state** The unique state shared by all living cells, plant and animal alike, in contrast to death, is called the living state.

**Log-log plot** A graph of the relation of any two variables in which the logarithm of one variable is plotted on the abscissa and the logarithm of the other variable on the ordinate. For the specific use of log-log plots for plotting adsorption isotherms, see Section 5.5.

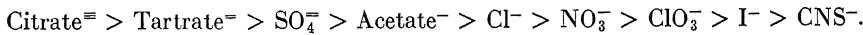
**London dispersion energies** Nonspecific attractive forces between molecules resulting from momentary electronic asymmetries.

**Lone pair of electrons** Unshared electron pair of an atom in a molecule.

**Lyophilic series** Hofmeister series, lyotropic order; the arrangement of a series of ions in order of their decreasing ability to remove lyophilic substances (starches, proteins, etc.) from colloidal solutions; classically, the order of cations is



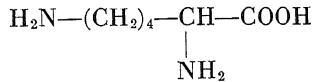
and that of anions is



**Lyotropic orders** See Lyophilic series.

**Lysin** An antibody which disintegrates cells.

**Lysine**



an amino acid found in most protein hydrolysates, which bears a residual positively charged group when it is fixed into a polypeptide chain at neutral and low pH.

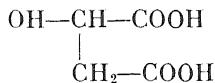
**Lysis** The dissolution or destruction of cells as the result of the action of a lysin; the decomposition of a substance.

**Lysozyme** An enzyme which has a destructive action on various bacteria; found in tears, egg albumin, leucocytes, etc.

**Macroscopic** Usually applied to any system of larger than molecular dimensions.

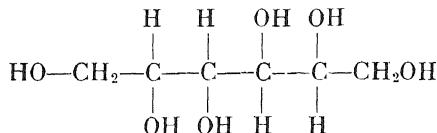
**Macroscopic dielectric constant** See Dielectric constant.

**L-Malate**



salt of 1-hydroxysuccinic acid, intermediate product in the biological oxidation of glucose.

**Mannitol** Mannite, manna sugar,



a hexose widely distributed in nature.

**Mass action, law of** The rate of a chemical reaction is proportional to the active masses of the reacting substances. If this law be applied to the reaction,  $\text{A}^+ + \text{B}^- \rightleftharpoons \text{AB}$ , the rate of the reaction to the right will be proportional to  $[\text{A}^+]$  and  $[\text{B}^-]$  while the rate of the reaction to the left will be proportional to  $[\text{AB}]$ , where the quantities in brackets are concentrations. When the rates in both directions are equal we say equilibrium has been reached. At this point  $[\text{AB}]/[\text{A}^+][\text{B}^-]$  is constant.

**Mechanoreceptor** A receptor or nerve-end organ which is excited by mechanical events such as pressure (touch), sound waves, or stretching of a muscle.

**Melanoma** A tumor arising from melanin-pigmented cells usually developing from a nevus (mole); the cells of such tumors may, in time, lose their ability to make melanin, at which time they are referred to as amelanotic.

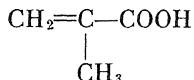
**Mesodermal** Pertaining to or derived from the mesoderm or middle layer of the three primary germ layers of the embryo. Thus derived are the connective tissue, bone and cartilage, muscles, kidney, etc.

**Mesomeric effect** See Resonance.

**Metabolism** The sum total of chemical reactions occurring in a living cell or organism by which food is transformed into living protoplasm and by which protoplasm is broken down into simpler compounds with the exchange of energy.

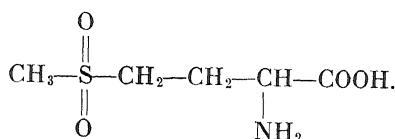
**Metastable state** A definite equilibrium state of a system which is not, however, the most stable equilibrium state at the given temperature; for example, a supercooled liquid is in a metastable equilibrium state.

**Methacrylic acid**



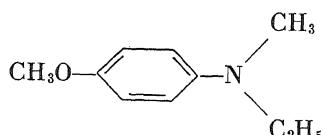
a compound which polymerizes easily; it is used in the manufacture of resins and plastics.

Methionine sulfone Oxidized methionine,

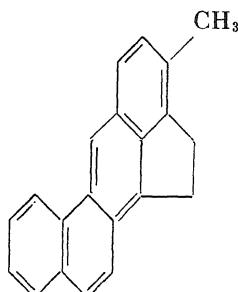


Methoxyl group  $\text{CH}_3\text{O}$ , a substituent group of organic molecules.

*p*-Methoxyphenylethylmethylamine

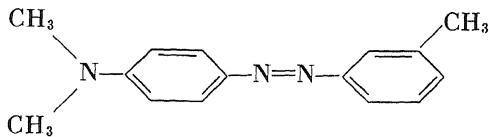


Methylcholanthrene

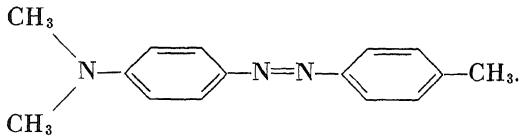


a carcinogenic polycyclic compound.

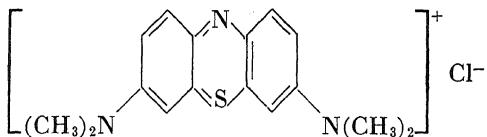
3'-Methyl-4-dimethylaminoazobenzene



4'-Methyl-4'-dimethylaminoazobenzene

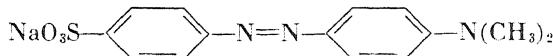


Methylene blue



a dark blue-green dye used as a bacterial stain and as an indicator for oxidation-reduction reactions.

Methyl orange



a dye used as a pH indicator.

**Micellar aggregate** Aggregation of linear long-chain molecules (such as soaps) in solution into clusters of several molecules.

**Microcell** See Index.

**Microscopic** Anything dealing with particles and systems of atomic or molecular size.

**Microsome** One of the fine, granular elements in protoplasm.

**Milieu** Surroundings, environment.

**Mitochondria** Granules and filaments in the cytoplasm which may be separated from it by techniques of ultracentrifugation. They are thought to be the site of many metabolic reactions.

**Mole fraction ( $X_i$ )** The number of moles ( $C_i$ ) of one component of a solution divided by the total number of moles of all components of the solution,

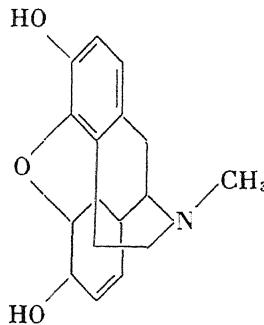
$$X_i = \frac{C_i}{\sum_i C_i}.$$

**Monomer** A small molecule which is linked with other molecules of the same kind to make a polymer; it must have at least two functional groups.

**Mono-monovalent** Pertaining to two particles, each bearing a single charge.

**Mono-multivalent** Pertaining to two particles, one singly charged and another bearing more than one charge.

**Morphine**



the most effective alkaloid contained in opium; a narcotic used to alleviate pain.

**Morphology** The science which treats the external configuration or the structure of animals and plants.

**Mosaic egg** An egg which possesses specific areas destined to give rise to definite portions of the larva; division of such an egg results in the development of incomplete embryos.

**Moving-boundary method** A method for determining the transference numbers of ions,  $M^+$  and  $A^-$ , of salt MA; a solution of  $M^+A^-$  is placed between solutions of salts  $M'A'^-$  and  $M'^+A^-$  such that sharp boundaries are preserved between the solutions. When the electric field is applied across the entire system, the rates of movement of  $M^+$  and  $A^-$  may be determined by the rate of movement of the boundaries between the solutions.

**Mucopolysaccharide** A group of polysaccharides containing hexosamine; they may be combined with protein. Mucins are water dispersions of mucopolysaccharides.

**Mucosa** A mucous membrane, a membrane lining the internal surface of an organ.

**Muscle spindle** A small bundle of specialized muscle fibers found in voluntary muscles which acts as a stretch receptor initiating a reflex which tends to maintain a particular degree of tension.

***Muskelsaft*** Muscle juice, a crude extract of muscles.

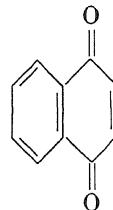
**Myelinated** Referring to a nerve which is covered by a many-layered sheath of a fatty substance called myelin.

**Myofibril** One of the fine, longitudinal filaments composing a muscle fiber. See Figure 2.8.

**Myokinase** Adenylic acid kinase, an enzyme which catalyzes the reaction,  $2 \text{ ADP} \rightleftharpoons \text{ATP} + \text{adenylic acid (AMP)}$ .

**Myosin** A globulin present in muscle; believed to be one of the major contractile proteins.

**1,4-Naphthoquinone**  $\alpha$ -Naphthoquinone,



substituted naphthoquinones occur in nature, various pigments and vitamin K, for example.

**Narcotic** Any drug that produces sleep or stupor and at the same time relieves pain.

**Natural frequency** The frequency with which an object will vibrate if struck or set to vibrating in any way.

**Natural logarithm (ln)** Logarithm of a number  $N$  to the base  $e = 2.71828 \dots$ ; thus  $x = \ln N$ , if  $N = e^x$ .

**Neoplasia** The formation of a neoplasm.

**Neoplasm** Any new and abnormal growth, such as a tumor.

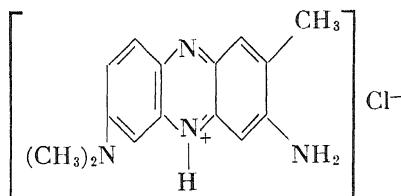
**Neural tube evocator** An evocator which evokes the development of a neural tube.

**Neuroblast** An embryonic nerve cell.

**Neuromuscular junction** The point of junction of a nerve fiber with the muscle fiber which it innervates; it is specialized for transmission of the impulse from the nerve which excites the muscle. See Figure 13.4.

**Neuron** A nerve cell.

**Neutral red**



a dye used as a *pH* indicator.

**Nissl bodies** Chromophile granules found within the nerve cell.

**Nitroprusside reaction** A color reaction used as a test for the presence of sulphydryl groups ( $-\text{SH}$ ) in a protein solution; saturated Na nitroprusside ( $\text{Na}_2[\text{Fe}(\text{NO})(\text{CN})_5] \cdot 2\text{H}_2\text{O}$ ) and ammonium hydroxide ( $2M$ ) are used as reagents. In the presence of SH groups a dark red color develops.

**Nocuous agents** Harmful agents.

**Nonelectrolyte** Any water-soluble substance which possesses no charged functional groups; for example, sugars, ureas, etc.

**Notochord** A longitudinal elastic rod of cells which, in lowest vertebrates and the embryos of higher vertebrates, forms the supporting axis of the body.

**Novocain** See Procaine.

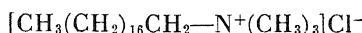
**Nucleic acids** A group of compounds in which one or more molecules of phosphoric acid are combined with carbohydrate (pentose or hexose) molecules; these are, in turn, combined with bases derived from purines (guanine and adenine) and pyrimidines (thymine, cytosine, and uracil).

**Nucleophilic reagent** An electron donor in chemical reactions.

**Nucleus** A spheroid body within a cell, distinguished from the rest of the cell by its denser structure.

**Obligate anaerobes** A microorganism which can grow only in the absence of free oxygen.

**Octadecyltrimethylammonium chloride**



a cationic detergent.

**Oligochaeta** An order of segmented worms having no distinct head, the earthworm, for example.

**Ommatidium** One of the elongated units in the compound eye of arthropods.

**Oncology** The sum of knowledge concerning tumors; the study of tumors.

**Onium ions** A group of organic compounds of the type  $\text{RXH}_n$  which are isologs of ammonium and contain the element X in its highest positive valency; examples, oxonium  $\text{R-OH}_2^+$ , ammonium  $\text{RNH}_3^+$ , sulfonium  $\text{RSH}_2^+$ , phosphonium  $\text{RPH}_3^+$ .

**Ontogeny** The complete developmental history of an individual organism.

**Optical isomers** Two compounds which are identical in all respects except the rotation of polarized light; this difference arises from a difference in the steric configuration of the groups linked to one or more asymmetric carbon atoms.

**Optical rotation** The rotation of a beam of polarized light on passing through a particular compound due to asymmetry in the molecule.

**Orange II** *p*-(2 Hydroxy-1-naphthylazo) benzenesulfonic acid, sodium salt; a common dye, occasionally used as pH indicator.

**Order-disorder transitions** In alloys such as that of copper and gold, slow cooling from a high temperature produces an ordered crystalline compound while rapid cooling produces a random solid solution. Transformations between these conditions are referred to as order-disorder transitions.

**Organizer** A living part of an embryo which exerts a morphogenetic stimulus upon another part or parts, bringing about its determination and the resultant histological and morphological differentiation.

**Orientation polarization** Polarization due to orientation of a permanent dipole in electric field (see Distortion polarization); in an electric field a permanent dipole rotates so that its positive end points, on the average, toward the negative electrode. This rotation is called orientation polarization as opposed to distortion polarization, which results from the actual distortion of the molecule due to the electric field.

**Osmium tetroxide** Osmic acid, an oxidizing agent, used as a fixative for biological preparations and a stain in electron microscopy.

**Osmotic pressure** The pressure necessary to equalize the rates of diffusion of solvent across a semipermeable membrane separating two solutions containing differing concentrations (correctly, activities) of nonpermeable substances.

**Osmotic work** The work done in moving a substance against a concentration gradient.

**Overshoot** At the height of the action potential, the nerve or muscle surface usually reverses its polarity so that the inside becomes positive with respect to the outside. The potential difference at this point is called the overshoot.

**Ovum** An egg; the female reproductive cell which, after fertilization, develops into a new member of the same species.

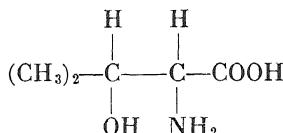
**Oxidation-reduction potential ( $E^{\circ}$ )** Standard oxidation-reduction potential, the potential established at an inert electrode when dipped into a solution containing equimolar amounts of an ion or molecule in two states of oxidation. See Index.

**Oxidative metabolism** Oxidative conversion, that part of the breakdown of glucose and other foodstuffs that involves reactions directly or indirectly with oxygen.

**Oxyhemoglobin** An easily reversible complex of oxygen and hemoglobin.

**Oxytocin** Pitocin, the principle uterus-contracting and milk-ejecting hormone of the posterior pituitary gland. For structure, see Figure 0.3.

Oxyvaline Hydroxyvaline,



an amino acid.

Paper chromatography A method of chemical analysis in which a solution to be analyzed is placed at one end of a strip of paper and allowed to diffuse along the paper for a specified length of time in the presence of a specified solvent. At the end of the diffusion period, the paper is treated with chemicals which produce color reactions with the solutes in the unknown solution. From the position of the colored areas on the paper, using appropriate standards, the compounds present in the original solution may be deduced.

*Paracentrotus lividus* A species of sea urchin.

Paramecia A group of one-celled, elongated animals having a large mouth in a fold at the side and moving by means of cilia.

Parameter Any quantity whose value varies with the circumstances of its application.

Parthenogenesis The stimulation of an egg to more or less complete development by means not involving the introduction of spermatozoa.

Partition function (p.f.) In statistical mechanics, the number of different ways in which the energy of a system can be partitioned among the species of the system. See Index.

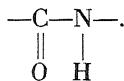
Pectinesterase An enzyme which removes the ester groups from pectins, a class of polysaccharides of botanical origin which form strong gels.

Penicillinase An enzyme, produced by certain bacteria, which antagonizes the antimicrobial action of penicillin.

Pentose A five-carbon sugar.

Peptide A polymer containing amino acids in peptide linkages, usually a relatively small molecule as opposed to a protein.

Peptide linkage Linkage between amino acids in proteins,



It is the basic unit of the protein polypeptide chain.

Perchloric acid  $\text{HClO}_4$ , an extremely strong acid which is useful, in dilute solution, for leaching ions from tissues.

Peristalsis The wormlike movement by which the alimentary canal and other tubular organs which have both circular and longitudinal muscle fibers propel their contents. It consists of a wave of contraction passing along the tube.

Permeability The specific properties of a membrane or surface which determine the rate of entry of a particular ion or molecule.

**Permeability constant (*P*)** A constant relating to the rate of passage of a substance through a surface or membrane. See Index.

**Permeable** Any phase which allows a particular substance to enter or pass through is said to be permeable to that substance.

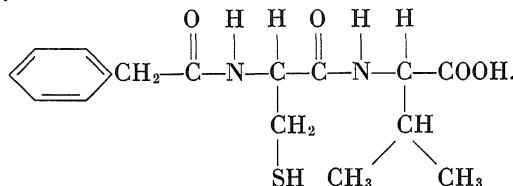
**Permutation** Any one of the combinations or changes in position possible within a group; the permutations of 1, 2, and 3 are 123, 132, 213, 231, 312, 321.

**Permutite** A silicate which acts as an ion exchanger, exchanging  $\text{Na}^+$  ion for  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  ions. It is often used as a water softener.

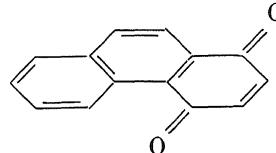
**pH** The negative logarithm of the hydrogen-ion concentration,

$$\text{pH} = -\log [\text{H}^+].$$

**Phenylacetylcysteinyl-D-valine**



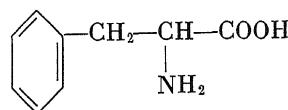
**Phenanthrenequinone**



an oxidizing agent.

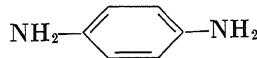
**Phenolic group** A hydroxyl group on an aromatic ring, the  $-\text{OH}$  of phenol, for example.

**Phenylalanine**



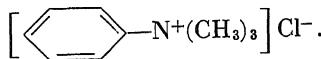
an  $\alpha$ -amino acid often found in protein hydrolysates.

***p*-Phenylenediamine**



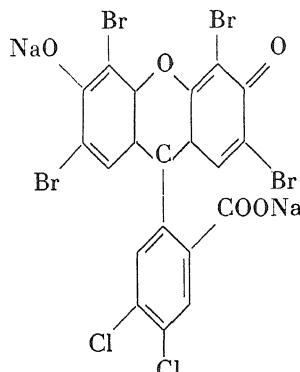
a black or brown dye.

**Phenyltrimethylammonium chloride**



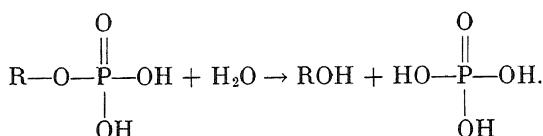
***Pheretima*** A genus of earthworm.

**Phloxine** Tetrabromodichlorofluorescein, sodium salt,



a purple aniline dye.

**Phosphatase** One of a large group of enzymes which hydrolyzes esters of phosphoric acid, for example,



**Phosphocreatine** See Creatine phosphate.

**Phospholipase** Any enzyme which catalyzes the breakdown of a phospholipid, lecithinase, for example.

**Phospholipid** Ester of fatty acids, such as lecithin, containing nitrogen and phosphorus radicals.

**Phosphorylase** An enzyme which catalyzes the reaction, glycogen + phosphate  $\rightleftharpoons$  glucose-1-phosphate.

**Phosphotransacetylase** An enzyme which catalyzes the reversible acetyl transfer reaction involving CoA,



**Photoactivation** Activation of a molecule or reaction by light.

**Photoreceptor** A nerve end organ or receptor which is excited by light.

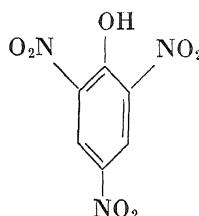
**Photoreduction mechanism** A reduction reaction activated by light.

**Photosynthesis** A chemical combination caused by the action of light; specifically, the formation of carbohydrates from carbon dioxide and water in the chlorophyl-containing tissue of plants under the influence of light.

**Phylum (phyla, pl.)** One of the primary divisions of the animal or vegetable kingdom.

**Physostigmine** Eserine, an alkaloid from the Calabar bean which produces constriction of the pupils, increased gut motility, increased salivary secretion, etc., by inhibiting the action of choline esterase, thereby prolonging the action of the acetylcholine.

Picric acid 2,4,6-trinitrophenol,



an organic acid which is used as a fixative for biological specimens. It is a protein denaturant.

Pith cells Cells from the loose spongy tissue occupying the center of the stem of dicotyledonous plants.

*Pithophora* A genus of algae.

Pitressin See Vasopressin.

Pituitary See Hypophysis.

pK The negative of the logarithm of the dissociation constant  $K$  of an acid or base,

$$pK = -\log K.$$

*Planaria* Any of the numerous small leaf-shaped flatworms belonging to the class Turbellaria.

Planck's constant A universal constant defined as the ratio of a quantum of radiant energy of a particular frequency to the frequency,

$$h = 6.547 \times 10^{-27} \text{ erg/sec.}$$

Named after Max Planck, German physicist.

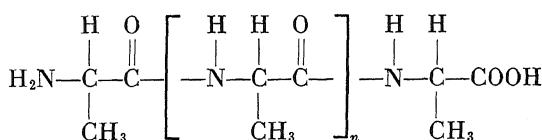
Plasma The fluid portion of the blood in which the corpuscles are suspended.

Pluteus The free-swimming larva of the sea urchin.

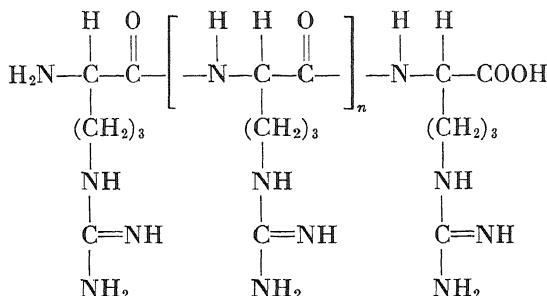
Polar groups The chemical groups on a molecule which are able to acquire a negative or positive charge,  $-\text{COO}^-$ ,  $-\text{NH}_3^+$ , etc., also includes dipolar groups and multipolar groups which may or may not have net charges.

Polarizability The dipole moment produced by a unit electric field acting on a molecule. In the absence of permanent dipoles, the polarizability of a molecule is the sum of separate electronic polarizabilities of its constituent atoms with slight corrections due to the bond structure. When a moment  $m$  is induced by a field of intensity  $F$ , the polarizability of the molecule  $\alpha$  is  $m/F$ .

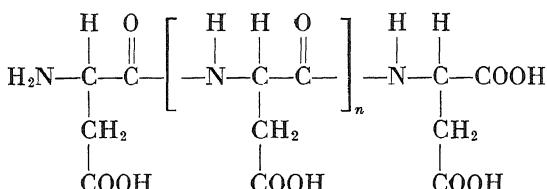
Poly-DL-alanine



polymer of alanine.

**Poly-DL-arginine**

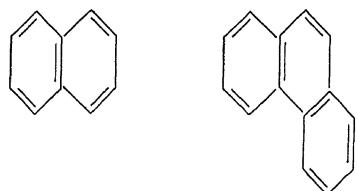
a polymer of arginine which bears excess positive charges in neutral solution.

**Poly-L-aspartic acid**

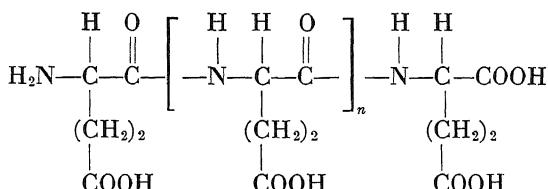
a polymer of aspartic acid; it bears many anionic groups in neutral solution.

**Polyatomic** Any particle containing more than one atomic nucleus.

**Polycyclic hydrocarbon** A hydrocarbon containing two or more rings, for example, naphthalene, phenanthrene,

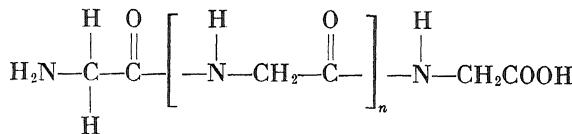


**Polyelectrolyte** A polymer containing many dissociable (charged) groups such as carboxyl, sulfonic, amino, etc.

**Poly-D-glutamic acid**

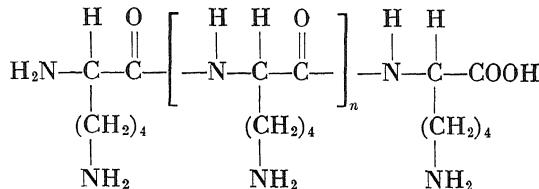
a polymer of glutamic acid; a highly negatively charged compound in neutral solution.

## Polyglycine



a polymer of glycine; a neutral compound.

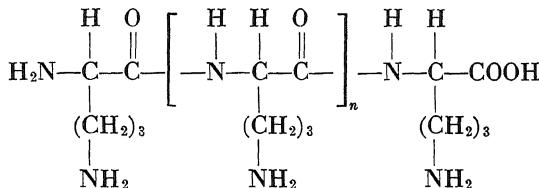
## Poly-L-lysine



a polymer of lysine; a highly cationic compound with profound effects on the surface properties of cells.

**Polymerization** The linking of small molecules with other similar molecules to make larger, usually long, chainlike molecules.

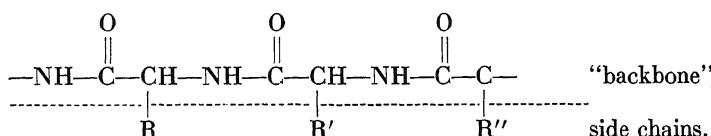
## Poly-DL-ornithine



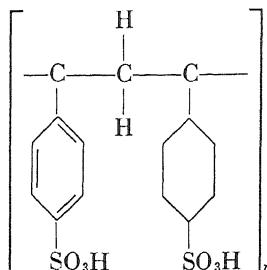
a polymer of ornithine; an  $\alpha$ -amino acid not usually found in animal protein. The polymer bears excess cationic charges in neutral solution.

**Polypeptide** A compound composed of a number of amino acids joined by peptide linkages.

**Polypeptide "backbone"** Refers to the linked portions of a polymeric linear chain containing several peptide linkages (exclusive of side chains, that is, R groups),

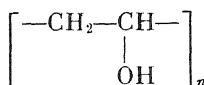


**Polystyrene sulfonate**



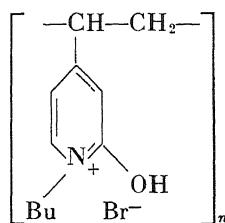
a polymer of vinylbenzene sulfonate; when the linear polymers are combined with a cross-linking agent such as divinyl benzene a cationic exchange resin results.

**Polyvinyl alcohol**



polymer of vinyl alcohol.

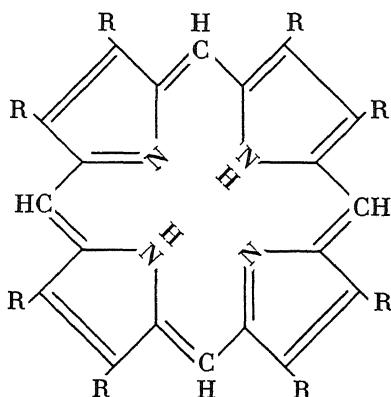
**Poly-*p*-vinyl-*N*-*n*-butylpyridonium bromide**



**Porcine** Of a pig.

$\pi$  electrons Mobile or delocalized electrons; in double and triple bonds, electrons occupying the external or  $\pi$ , molecular orbital; they have unit components of angular moment along the interatomic axis.

**Porphyrin** Any of several compounds in which four pyrrole nuclei are linked in a cyclic structure by methine radicals,



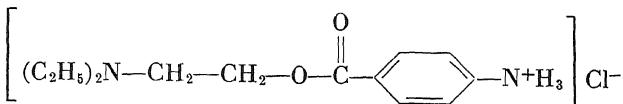
Various porphyrins are found in hemoglobin and chlorophyll.

Positive-inductive effect *I*-effect. See Index.

Potato apyrase See Apyrase.

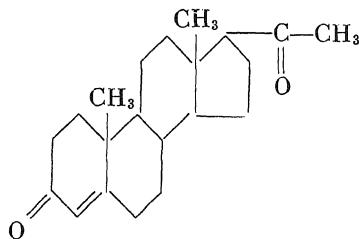
Potential energy The capacity to do work which a body possesses by virtue of its position or configuration.

Procaine Novocain®,



hydrochloride used as local anesthetic.

Progesterone



a steroid hormone elaborated by the corpus luteum of the ovary which acts on uterus, preparing it for the reception and development of the fertilized ovum by a glandular proliferation of the endometrium.

Proliferation The production, by cellular division, of new parts, buds, or offspring.

Proprioceptors Proprioceptive receptors, sensory nerve terminals which give information concerning movements and position of the body. They occur chiefly in the muscles, tendons, and semicircular canals.

Protamine Any member of a class of strongly cationic proteins found in combination with nucleic acids. See Figure 0.2.

Protein Any one of a group of complex organic nitrogenous compounds, widely distributed in plants and animals, which form the principal constituents of the cell protoplasm. They are essentially linear combinations of  $\alpha$ -amino acids, possessing a polypeptide chain.

Protein hydrolysate Mixture of  $\alpha$ -amino acids resulting from treatment of protein with HCl or NaOH at elevated temperatures.

Proteolytic enzyme Any enzyme which digests proteins.

Proton Hydrogen nucleus, hydrogen ion, H<sup>+</sup> ion.

Proton acceptor Any substance which has a tendency to gain a proton (H<sup>+</sup> ion) in a chemical reaction or association; for example, bases are proton acceptors.

Proton donor Any substance which has a tendency to lose a proton (H<sup>+</sup> ion) in a chemical reaction or dissociation; for example, acids are proton donors.

**Protoplasm** All matter of which life is a manifestation. The ubiquitous and essential material of animal and plant cells.

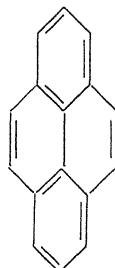
**Protozoa** The phylum of one-celled animals, usually microscopic, which forms the lowest division of the animal kingdom.

**Psoas** The larger *psoas* muscle, *psoas major*, is a large fusiform muscle of the loin whose origin is the side of the vertebral column and which inserts into the top of the humerus, or thigh bone.

**Purkinje fibers** Specialized muscle fibers which form a network in the septum and sub-endocardial tissue of the ventricle of the heart. They are thought to be concerned with the rapid propagation of stimuli from the atria to the ventricles.

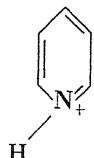
**PVBB** See Poly-*p*-vinyl-*N*-*n*-butylpyridonium bromide.

**Pyrene**

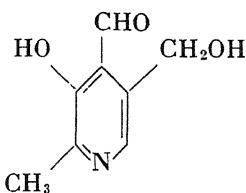


polycyclic hydrocarbon found in coal tar.

**Pyridinium** A cation

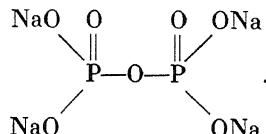


**Pyridoxal**



the alcohol derivative of this compound is Vitamin B<sub>6</sub>.

**Pyrophosphate linkage** The linking of two or more phosphate groups together, as in sodium pyrophosphate,



**Pyrrole** A heterocyclic ring compound; see Figure 7.3.

**Pyruvate kinase** Pyruvate phosphokinase, pyruvic phosphoferase, phosphopyruvate, transphosphorylase; a phosphate-transferring enzyme which catalyzes a reaction important in the metabolic degradation of glucose.  $\text{Phosphoenolpyruvate} + \text{ADP} \rightleftharpoons \text{pyruvate} + \text{ATP}$ .

**Pyruvate phosphoferase** See Pyruvate kinase.

**Quantum mechanics** The study of energetic physical processes in terms of discrete quantities of energy called quanta. See Index.

**Quinone** 1,4-benzoquinone; used as an oxidizing agent.

**Radioactive tracer** A small amount of a radioisotope of a substance added to the non-radioactive substance so that the path, reaction, or position of the substance may be followed.

**Radioisotope** Radioactive isotope; an isotope of an element which is unstable, producing  $\alpha$ ,  $\beta$ , or  $\gamma$  radiation as it decays.

**Receptor site** A chemical group or ensemble of chemical groups which functions as a receptor for biological signals.

**Rectus abdominus** muscle A flat muscle extending the length of the abdomen from the xiphoid process of the sternum to the pubis. In the frog it is a slow muscle, responding to a stimulus by graded contracture.

**Refractive index** Index of refraction, the ratio of the velocity of light in a vacuum to the velocity  $v$  of light of a particular wavelength in any substance,  $n = c/v$ .

**Regulative egg** An egg in which all portions are totipotential; divisions of such an egg are able to give rise to complete embryos.

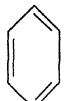
**Relaxing factor** Any substance which, when applied to a contractile system such as actomyosin threads or a muscle fiber, either allows it to lengthen or opposes the action of a substance which causes the system to shorten.

**Renal tubule** Minute canals which make up the substance of the kidney; as the glomerular filtrate passes through these tubules it is modified to become urine.

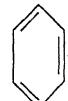
**Repulsion constant** See Born repulsion constant.

**Residual charge** Any electric charge retained by an amino acid after both its  $\alpha$ -amino and  $\alpha$ -carboxyl groups have formed peptide linkages.

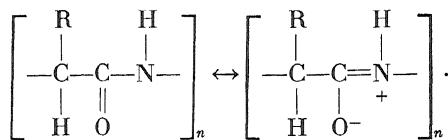
**Resonance** A representation of certain molecules as existing in two or more arrangements in which the positions of the atoms are identical but in which the positions of certain electrons differ. The actual arrangement of the electrons is intermediate between the alternatives; for example, benzene is often represented as resonating between the two structures,



and



**Resonating chain** Any length of a linear molecule containing several resonating bonds; in the present monograph it most often refers to the polypeptide backbone of a protein molecule which resonates according to the scheme,



**Respiration** Internal respiration, the exchange of gaseous constituents between the blood and the cells of the body.

**Resting potential** Electrical potential difference between the inside and outside phases of cells of resting excitable tissues such as nerve and muscle.

**Retina** The innermost coat of the back part of the eyeball; made of a layer of cells sensitive to light.

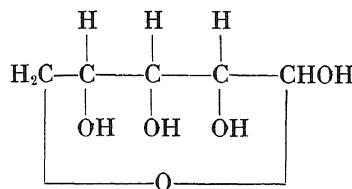
**Reversible work** Work done by a thermodynamically reversible process which is a process carried out in such a manner that all changes occurring in any part of the process are exactly reversed when it is carried out in the opposite direction.

**rH** A measure of the oxidation-reduction potential of a system. It is defined as the negative logarithm of the hydrogen-gas pressure which would exist at an electrode in equilibrium with the solution containing the oxidation-reduction system. This nomenclature is seldom used now.

**Rhodopsin** A purple pigment believed to be a conjugated protein containing a carotenoid pigment. Found in the light-sensitive rods in the retinas of animals.

**Ribonuclease (RNAase)** An enzyme which catalyzes the depolymerization of ribonucleic acid. This is one of the few proteins for which the complete structure has been worked out.

**Ribose**



a pentose sugar which is one of the basic constituents of ribonucleic acid.

**Ringer's solution** A solution resembling the blood serum in its salt constituents; first devised by and named after S. Ringer. See Appendix H for examples.

**Rodent** A member of an order of mammals including rats, mice, and beavers.

**Rotational entropy** The entropy possessed by a substance due to the rotatory movements of its molecules.

**Rotational freedom** The number of modes of rotational movement available to a particle.

**Rotational partition function** The partition function in relation to the various modes of rotational movement available to a particle. See Partition function.

**Salmine sulfate** The sulfuric acid salt of a protamine derived from the spermatozoa of the salmon; a strongly cationic protein. See Figure 0.2.

**Saltatory migration** A mode of migration of ions in fixed-charge systems in which the ion penetrates the energy barrier between fixed sites without undergoing an adsorption-desorption process.

**Salt linkage** The ionic bond between fixed ions of opposite charges on proteins due to Coulombic interaction.

**Sarcoma** Any of the various malignant tumors originating in tissues of mesodermal origin.

**Sartorius muscle** A thin, flat muscle extending, in the frog, from the pubis to the lateral aspect of the knee. It has very little connective tissue on its inner surface, making it suitable for the study of the resting potential, diffusion of ions, etc., in muscle cells.

**Saturated hydrocarbon** A hydrocarbon with no free valence and in which there are neither double nor triple bonds.

**Scalar quantity** Any numerical measure of a property of matter which has no direction.

**Schulze-Hardy rule** The ion which is effective in bringing about coagulation is the one of opposite sign to that of the colloidal particle, and that the coagulating power increases with increasing valence of the ion; these conclusions concerning the effect of sign and valence on coagulation and related phenomena are referred to as the Schulze-Hardy rule.

**Sciatic nerve** A large nerve trunk which arises in the lower part of the spinal column and supplies motor and sensory innervation to the back of the leg and the foot.

**Scintillation counter** A device for the quantitative detection of radioactivity; it measures the light emitted by a material (the scintillator) when a particle passes through it.

**Sea urchin** An echinoderm consisting of a rounded body with no appendages and covered with many movable spines.

**Selective accumulation** The accumulation of one substance (ion or nonelectrolyte) within a cell to a greater concentration than another substance, although both are in equal concentration in the external phase.

**Selectivity** The ability of any material to differentiate between two species of ion or two different molecules; it is given as the ratio of the effect of one ion to that produced by the same concentration of the second ion. For example, glass electrodes for measuring pH show a high  $H^+$ -ion/ $Na^+$ -ion selectivity; that is, changes in  $H^+$ -ion concentration produce a greater change in the potential across the electrode than do changes in  $Na^+$ -ion concentration. Resting muscle cells show a high selectivity for  $K^+$  ion over  $Na^+$  ion, accumulating the former and rejecting the latter.

**Semifixed-charge system** A charge-bearing linear or three-dimensional polymer, not of macroscopic dimensions. The primary difference between this and a true fixed-charge system is in the greater entropy of dissociation for ions dissociating from charged sites on the semifixed-charge system. Examples are proteins in solution. See Index.

**Semilogarithmic plot** A graph of two variables where one variable is plotted on a logarithmic scale and the other on an arithmetic scale.

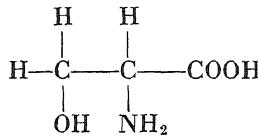
**Semipermeable membrane** A membrane which allows some constituents of a given solution to pass through it but not others.

**Semitendinosus muscle** A small muscle on the dorsal part of the thigh which, in the frog, originates by two heads from the pelvic girdle and inserts at the knee.

**Sensory cell** Any nerve cell specialized for the reception of the stimuli which give rise to sensation.

**Sepia** The cuttlefish; a cephalopod and close relative of the squid.

**Serine**



an  $\alpha$ -amino acid.

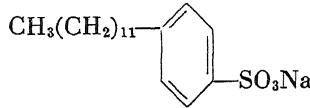
**Serum albumin** A water-soluble protein which forms the chief protein of the blood plasma.

**Silk fibroin** The protein of silk.

**Slow fibers** Muscle fibers which respond to stimulation by a gradual contracture dependent on the strength of stimulus instead of a twitch; excitation of these fibers does not give rise to a propagated action potential. Such fibers are found in the *rectus abdominus* and certain leg muscles of frogs as well as in certain invertebrates.

**Smooth muscle** Unstriated or involuntary muscle; the type of muscle found in the walls of the intestines, blood vessels, etc.

**Sodium dodecylbenzenesulfonate**



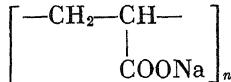
an anionic detergent.

**Sodium dodecylsulfate**



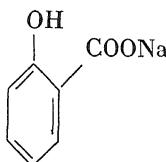
an anionic detergent.

**Sodium polyacrylate (SPA)**



the sodium salt of polymerized acrylic acid,  $\text{CH}_2=\text{CH}-\text{COOH}$ , a linear polyelectrolyte.

Sodium salicylate

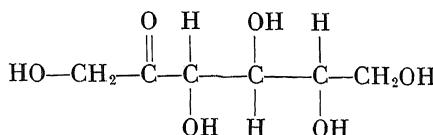


sometimes used medically to relieve pain.

**Solid solution** A homogeneous solid containing two or more substances.

**Solid state** A state of aggregation of matter, usually crystalline in nature, that possesses rigidity and the tendency to maintain both its shape and volume; distinct from the gaseous and liquid states.

Sorbose



a hexose sugar.

**Sorption** Retention of material at or on a surface; in molecular terms, adsorption of a substance to a molecule; for example, hydration of a protein molecule by water-binding.

**SPA** See Sodium polyacrylate.

**Specific gravity** The ratio of the density of any material to the density of water under specified conditions (4°C and atmospheric pressure).

**Spermatophyte** Any seed-bearing plant.

**S—S bond** See Disulfide linkage.

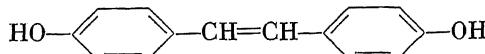
***Staphylococcus aureus* (*Staph. aureus*)** A species of spherical bacteria which forms golden colonies on agar plates due to a lipochrome pigment. They are extremely pathogenic, giving rise to many kinds of infections.

**Statistical mechanics** The branch of physics which endeavors to interpret and predict the macroscopic properties of the microscopic systems (atoms, molecules, ions, electrons, etc.) of which these aggregates are composed.

**Steady state** A condition of a system where no changes are observable in a particular parameter due to the fact that any process occurring in one direction is exactly balanced by an equivalent process in the opposite direction. The shape of the flame of a steadily burning candle represents such a steady state.

**Steric hindrance** Hindrance to a chemical reaction due to bulkiness or spatial configuration of a substituent group.

**Stilbesterol**



a synthetic compound with estrogenic activity.

**Stirling's theorem** Mathematical approximation for obtaining  $\ln N!$ :

$$\ln N! \sim \ln N - N$$

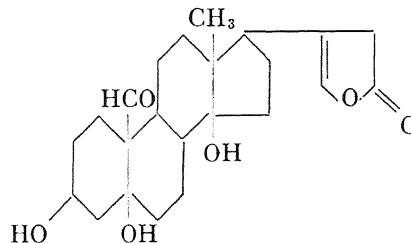
valid for the very large values of  $N$  in statistical mechanics.

**Stokes' law** G. G. Stokes found that if a small sphere of radius  $r$  travels at a velocity  $u$  through a fluid, liquid, or gas, having a coefficient of viscosity  $\eta$ , the force which must be applied to maintain a constant velocity against the frictional force due to viscosity is given by the formula,

$$F = 6\pi r\eta u.$$

***Streptococcus fecalis*** A species of gram-positive spherical bacteria which are normally found in feces.

**Strophanthidin**



A digitalis-like compound from *Strophanthus kombé*.

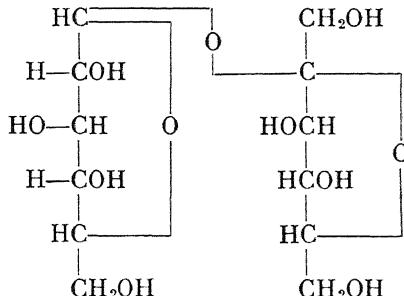
**Strychnine** Alkaloid present in seeds of *Strychnos nux vomica* and other species of *Strychnos* genus; a nerve poison, causing intense convulsions.

**Sublimation** The process whereby a substance passes directly from the solid to the vapor state.

**Substituent group** Any atom or group of atoms which may be substituted for a group present on a molecule.

**Substrate** The substance acted upon by an enzyme.

**Sucrose** Cane sugar,



a disaccharide of glucose and fructose.

**Sulphydryl group** —SH.

**Sulfonamides** Generic name for derivatives of *p*-aminobenzenesulfonamide (sulfanilamide); some of these compounds are medically important bacteriostatic agents.

**Supercooled liquid** A fluid which remains liquid though cooled to below its freezing point.

**Supernatant** The liquid remaining when a substance has been precipitated from a solution.

**Superprecipitation** An intense form of protein precipitation, especially of actomyosin, as described by Szent-Györgyi in "Muscular Contraction."

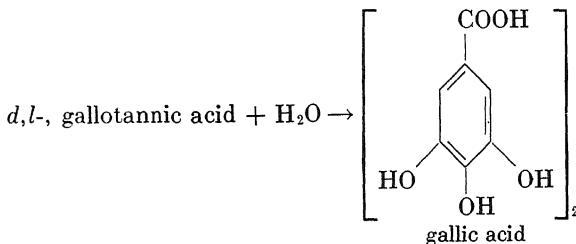
**Surface limited** If the slowest step in a diffusion process from one phase (say a solid) to another phase (say a liquid) is across the boundary between the phases, this process is surface limited.

**Synergism** Cooperative action of two agents in such a manner that the total effect is greater than the sum of the two effects taken independently.

**Synthesis** The building up of a compound from smaller components.

**Synthesizing template** A mold which serves as a matrix for an ordered polymerization and synthesis.

**Tannic acid** Any one of a variety of naturally occurring polymers of hydroxybenzoic acid. The one commonly referred to as tannic acid is gallotannic acid, the internal ester of gallic acid;



**Taste buds** Epithelial end organs occurring on the surface of the tongue; sensitive to various ions, sugars, etc.

**Taurine**  $\text{H}_2\text{N---CH}_2\text{---CH}_2\text{---SO}_3\text{H}$ , a  $\beta$ -amino acid found in bile.

**TCA** See Trichloroacetic acid.

**Template** A pattern or mold.

**Tetanus** An acute infectious disease caused by a toxin produced in the body by the bacterium *Clostridium tetani*; ionic contraction of a skeletal muscle induced by rapid tetanic stimulation.

**Tetanus toxin** A powerful ptomaine exotoxin produced by *Clostridium tetani*; it specifically attacks nerve cells, producing tetanus or lockjaw.

**Tetradecanedioic acid**  $\text{HOOC---(CH}_2\text{)}_{14}\text{---COOH}$ , dibasic carboxylic acid with 14  $\text{CH}_2$  groups intervening between the two carboxyl groups.

**Thermal energy** That part of the energy of a system due to the motion of the molecules; it is a function of temperature.

**Thermal entropy** The entropy possessed by a particle due to its translational, rotational, and vibrational motion in contrast to configurational entropy which it possesses as a result of its spatial configuration.

**Thermodynamic equilibrium** A state in which all influences tending to cause a change in a system are balanced so that no macroscopic change can be observed with time.

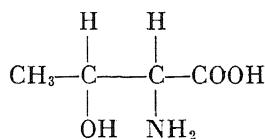
**Thermodynamics** The science that deals with the relationship of heat and mechanical energy and the conversion of one into the other.

**Thiocyanate** Salt of thiocyanic acid, HSCN.

**Thioglycolic acid** HSCH<sub>2</sub>—COOH, a reducing agent which is also a sensitive reagent for iron, molybdenum, silver, and tin.

**Thiol** A compound containing the monovalent radical —SH.

**Threonine**



an  $\alpha$ -amino acid commonly found in protein hydrolysates.

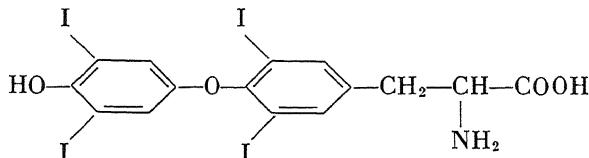
**Threshold** That value at which a stimulus is just sufficient to produce an effect; a stimulus of lower value (strength, duration, etc.) produces no detectable effect.

**Thymus** An endocrine gland located in the superior mediastinum and lower part of the neck in mammals; it is a structure of early life.

**Thyroid gland** An endocrine gland in the neck which accumulates iodide and secretes thyroxin.

**Thyrotropin** A hormone produced by the anterior lobe of the pituitary gland which influences the development and activity of the thyroid gland.

**Thyroxin**



a hormone secreted by the thyroid gland which exerts a stimulating effect on metabolism.

**Tibialis anticus longus** muscle In the frog, a double-headed muscle which originates at the front of the knee by a long thin tendon and inserts by long tendons into either side of the foot.

**Time constant** The time needed for a condenser to discharge to  $1/e$  of its initial charge, where  $e$ , the base of the natural logarithm, has a value equal to 2.71828.

**Tissue culture** Cells of animal tissues which have been isolated and are growing *in vitro*.

**Transference number of an ion** The fraction of the total current carried by a given ion in a solution through which an electric current is passed.

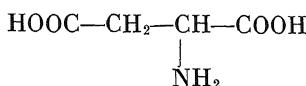
**Translational degrees of freedom** Number of modes of translational movement available to a particle.

**Translational partition function** The partition function in relation to the various modes of translational movement available to a particle. See Partition function.

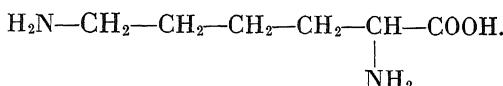
**Transmissivity factor** Transmission factor, describes the ability of a chemical group to transmit an inductive effect. See Index.

**Trichloroacetic acid (TCA)**  $\text{Cl}_3\text{C}-\text{COOH}$ , a strong acid and protein denaturant.

**Trifunctional amino acid** An amino acid which possesses polar groups (amino or carboxyl groups) beyond the  $\alpha$ -amino and  $\alpha$ -carboxyl groups; examples are aspartic acid,



or lysine,



**Trigger adsorption** An adsorption onto a cardinal site that changes the adsorbents on a gang of sites in a cooperative and all-or-none fashion.

**Trimethylalkylamine** Trimethyloctylamine, trimethylnonylamine, trimethyldecylamine, etc., a class of cationic detergents where R is an aliphatic hydrocarbon.

**Trimethylammonium nitrate**  $[(\text{CH}_3)_3\text{N}^+\text{H}]^+\text{NO}_3^-$ .

**Triode electronic tube** A device consisting of an evacuated glass tube containing a cathode, a grid, and a plate. When placed in an appropriate circuit, electrons pass from the cathode to the plate, setting up an electric current. This flow of electrons which must pass through the grid is controlled by the amount of charge on the grid. Such a tube may be used as an amplifier, small changes in the charge on the grid producing large changes in the amount of current flowing through the tube.

**Triplet migration** A mechanism by which an ion can move across a fixed site at the surface or within a fixed-charge system. This process involves the occupation of the site by the ion followed by its desorption with the participation of a second free ion.

**Tropomyosin** A protein extracted from the water-insoluble residue of muscle.

**True fixed-charge system** A three-dimensional fixed lattice which contains water and has more or less regularly distributed fixed ionic sites. In such a system, a large majority of the counterions are associated with the fixed sites due to the greatly decreased entropy of dissociation.

Tryptophan An  $\alpha$ -amino acid found in protein hydrolysates. See Table 0.1.

Tryptophanase An enzyme which catalyzes the change of tryptophan into indole.

*d*-Tubocurarine See Curare.

Tyrosinase A copper-containing enzyme, widely distributed in animals and plants which oxidizes tyrosine to orthoquinone.

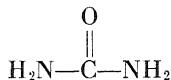
Tyrosine An aromatic  $\alpha$ -amino acid found in protein hydrolysates.

Univalent ion Any ion bearing only one positive or negative charge.

Unmasking of groups Any procedure in which specified groups become reactive to some reagent toward which they were originally inert.

Uranyl chloride Uranium (VI) dioxydichloride,  $\text{UO}_2\text{Cl}_2$ .

Urea



a product of protein metabolism; in high concentration it acts as a protein denaturant.

Urease A colorless crystalline globulin enzyme which catalyzes the breakdown of urea into carbon dioxide and ammonia and of hippuric acid into benzoic acid and glycine.

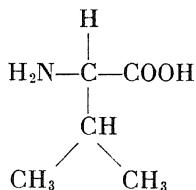
Uterus The womb, the hollow muscular organ of mammals in which the impregnated ovum develops into the fetus.

Vacuole A cavity within a cell or tissue, generally containing fluid.

Vagus nerve Tenth cranial nerve, a long nerve, originating in the brain, which supplies parasympathetic (motor) innervation to the organs of the neck, thorax, and abdomen. It also carries fibers of visceral sensation from these areas.

Valence An expression of the combining capacity of an element as measured by the number of hydrogen atoms or their equivalents with which one atom of the element can combine.

Valine



an  $\alpha$ -amino acid.

van der Waals' forces Interatomic or intermolecular attractive forces due to the interaction between fluctuating dipoles, which result from momentary dissymmetries in the negative and positive charges on atoms and their neighbors.

Vasopressin Pitressin, antidiuretic, a hormone of the posterior lobe of the pituitary gland which controls the toxicity of the urine; it also causes an increase in blood pressure.

**Vector sum** A sum of quantities taking into account both their magnitudes and their directions.

**Vegetalizing agent** Agent causing the shifting of the presumptive fate of tissues which normally develop into ectodermal tissues such that they become endodermal in character.

**Vegetal pole** In the developing ovum, the hemisphere which contains more yolk and in which segmentation is slow. The vegetal pole gives rise to endodermal tissues.

**Ventricle (of the heart)** One of the pair of cavities constituting the lower portion of the heart; that on the left side propels the blood through the systemic arteries; that on the right side propels blood through the pulmonary artery.

**Veratridine** An alkaloid of botanical origin; it is a component of veratrine, which has long been used by physiologists to produce a tetanus of skeletal muscle.

**Vibrational degrees of freedom** Number of modes of vibrational movement available to a particle.

**Vibrational partition function** The partition function in relation to the various modes of vibrational movement available to a particle. See Partition function.

**Virus** An obligate-parasitic pathogen, too small to be seen by ordinary microscope methods. Chemically, viruses are composed of nucleoprotein.

**Viscosity** The internal fluid resistance of a substance, caused by molecular attraction which makes it resist a tendency to flow or the passage of particles through it.

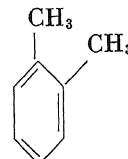
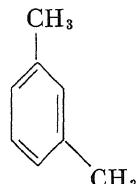
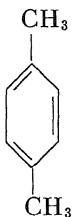
**Vitamin** Any of a number of unrelated, complex organic substances found variously in most foods and essential, in small amounts, for the normal functioning of the body.

**Vitreous state** A condition in which certain substances, notably glasses, can exist, lying between the solid and liquid states; thus while glasses are hard and rigid, they are amorphous, possessing no definite external structure and no crystal lattice; under stress they may change their shape.

**Voluntary muscle** Striated muscles, except heart muscle which is striated but involuntary.

**Wavelength** The distance in space occupied by one complete oscillation of a wave.

**Xylol** Xylene, one of three isomers of dimethylbenzene,



a liquid used as an organic solvent and in preparing tissue for microscope slides.

**Xylose (D- and L-)** Wood sugar; a pentose widely distributed in plant materials.

**Zero-point energy** The energy possessed by a particle at 0°K.

**Zeta potential ( $\zeta$ )** That portion of the surface potential at the boundary between two phases that determines the electrokinetic properties of a particle or surface. See Figure 10.6.

**Zygote** The fertilized ovum.

**Zygotic ontogeny** The development of the fertilized ovum.

**Zwitterion** A complex molecule which can bear both positive and negative charges and does so at its isoelectric point; for example, glycine  $\text{NH}_3^+—\text{CH}_2—\text{COO}^-$ .

# SYMBOLS AND ABBREVIATIONS

The entries in this list are grouped according to the nature of the symbol: 1, numerical symbols; 2, species and group symbols; 3, units and abbreviations; and 4, operators. In general, roman letters are used for species and groups to distinguish them from numerical symbols, which are italicized.

## 1. NUMERICAL SYMBOLS

*A* area

*A* moment of inertia

$A_{ij}$  specific repulsive constant between *i*th and *j*th entities

*A*,  $A'$  specific repulsive constants used in Chapter 4

$A_{p_i}$ ,  $A_{\tilde{x}}$ ,  $A_{Na}$ , etc. a constant in the expression relating the association energy of the subscripted species to the *c*-value of the adsorbing site

*B* moment of inertia

$B_{p_i}$ ,  $B_{\tilde{x}}$ ,  $B_{Na}$ , etc. a constant in the expression relating the association energy of the subscripted species to the *c*-value of the adsorbing site

*C* capacitance

*C* moment of inertia

$C(0)$ ,  $C(t)$ ,  $C(\infty)$  concentration at indicated time

$C_f$  concentration of fixed ionic sites

$C_{ads}$ ,  $C_{ins}$  adsorbed, interstitial concentrations

$C_j^{in}$ ,  $C_j^{ex}$  intracellular, extracellular concentrations of *j*th ion

$C_i^I$ ,  $C_i^{II}$  concentration of *i*th species in phases I, II

$C_+$ ,  $C_-$  ionic concentrations

*D* diffusion constant

- D* dielectric constant  
*D*<sub>1</sub> effective dielectric constant  
*D<sub>r</sub>* dielectric constant at distance *r* from center of ion  
*D(r)* dielectric constant as function of distance from center of ion  
*E* internal energy  
*E*<sub>Na</sub> work done against concentration gradient per mole of Na<sup>+</sup> transported  
*E*<sup>0</sup> oxidation-reduction potential  
*E*<sub>intr</sub><sup>0</sup> intrinsic oxidation-reduction potential  
*E*<sub>mask</sub><sup>0</sup> oxidation-reduction potential in the masked state  
*E*<sub>thres</sub><sup>0</sup> oxidation-reduction potential in the threshold reactive state  
*F* Helmholtz free energy  
*F*<sub>A</sub> physiological effect of pharmacon A  
*F*<sub>f</sub>, *F*<sub>s</sub> free energy of fixed-charge phase, solution phase  
*F*<sub>i</sub><sup>I</sup>, *F*<sub>i</sub><sup>II</sup> free energy of *i*th species in phases I, II  
*F* Faraday constant  
*H* enthalpy (heat content)  
*I*<sub>ii(p)</sub> inductive factor defined in Chapter 5  
*J*<sub>0</sub>( $\zeta$ ) Bessel function of zero order  
*J*<sub>Na</sub><sup>in-ex</sup>(*t*) rate of "pumping" in units of moles of Na<sup>+</sup> ion exchanged per kilogram of fresh muscle per hour, at time *t*  
*K* equilibrium constant, usually an association constant  
*K*<sub>A</sub> reciprocal of "affinity" of pharmacon A  
*K*<sub>D</sub> distribution coefficient  
*K*<sub>I</sub> Michaelis constant of enzyme-inhibitor complex  
*K*<sub>S</sub> Michaelis constant of enzyme-substrate complex  
*K'* empirical constant in Chapter 5  
*K'* selectivity coefficient  
*K'*<sub>A</sub> dissociation constant of protein-anion complex  
*K*<sub>i</sub> association constant for *i*th ion  
*K*<sub>i</sub> dissociation constant for *i*th ion  
*L* Avogadro's number  
*L*<sub>y</sub> over-all length of fixed-charge system in *y* dimension  
*M*<sub>s</sub>, *M*<sub>ads</sub>, *M*<sub>ins</sub>, *M*<sub>ads</sub><sup>A</sup>, *M*<sub>ins</sub><sup>A</sup>, etc. symbols representing the total number of particles of a particular species or group of species in indicated phase or state  
*N*, *N'* number of particles in a system  
*N* number of binding sites  
*N*<sub>A</sub>, *N*<sub>B</sub>, *N*<sub>S</sub>, etc. number of particles of a particular type in a system or phase

- $N_{\text{dissoc}}$ ,  $N_{\text{assoc}}$  number of dissociated, associated particles
- $N_y$  number of serially arranged gangs in a fixed-charge system
- $N_{\pm}$  number of ions, negatively charged and positively charged, in a system
- $N_A^0$  total number of A in a system in any form
- $P_j$  permeability constant of  $j$ th species
- $P(t)$ ,  $P(\tau)$  expressions defined in Chapter 11
- $Q(t)$ ,  $Q(\tau)$  expressions defined in Chapter 11
- $R$  molar gas constant
- $R$  specific resistance
- $\mathfrak{R}$  an expression defined in Chapter 2
- $S$  entropy
- $\mathfrak{S}$  an expression defined in Chapter 2
- $\mathfrak{S}_{\pm}(\Delta E_{ij} - \Delta E_i)$  an expression defined in Chapter 11
- $T$  absolute temperature
- $\mathfrak{U}$  total potential energy of a system
- $U_{\text{elect}}$  electrostatic energy of proteins
- $U_i$ ,  $U_w$ ,  $U_+$ ,  $U_-$  zero-point energy of subscripted entity
- $\mathfrak{U}_i^s$  total potential energy of configuration  $s$  with counterion  $p_i$
- $V$  potential difference
- $V$  rate of enzymatic activity
- $V$  volume
- $V_f$ ,  $V_s$  volume of fixed-charge phase, solution phase
- $(V_i)^{\text{inward}}_{\text{dou}}$  rate of inward movement of  $i$ th ion via doublet dissociation
- $(V_i)^{\text{inward}}_{\text{sal}}$  rate of inward movement of  $i$ th ion via saltatory migration
- $(V_i)^{\text{inward}}_{\text{tri}}$  rate of inward movement of  $i$ th ion via triplet dissociation
- $V_i^{\text{inward}}$  observed rate of entry of  $i$ th ion
- $(V_i^{\text{inward}})_{\text{max}}$  maximum rate of entry of  $i$ th ion
- $\mathfrak{V}_f$  free volume
- $W$  work
- $X_A$ ,  $X_B$ ,  $X_{AB}$  mole fraction of subscripted species in a system
- $X_{p^+}$  mole fraction of resin sites associated with an exchanging ion  $p^+$
- $X_x^{\text{fo}}$ ,  $X_j^{\text{f}_e}$ ,  $X_{\text{salt}\text{lk}}^{\text{f}_e}$ ,  $X_{\text{salt}\text{lk}}^g$ ,  $X_{\text{S}}^{\text{f}_e}$ ,  $X_{\text{ATP}}^{\text{f}_e}$ , etc. mole fraction of the super-scripted species adsorbing the subscripted species or in the state indicated by the subscript
- $\bar{X}$  amount of base or acid
- $Z$ ,  $Z_i$  valence of an ion
- $a$  distance of closest approach between interacting ions
- $a_{\text{in}}^{\text{H}}$ ,  $a_{\text{out}}^{\text{H}}$   $\text{H}^+$  activities within a glass and in the outside solution

- $b, b_i$  displacement of permanent dipole from the center of a molecule  
 $c_0, c_i$ , etc.  $c$ -value of site  $f_0, f_i$ , etc.  
 $(c_0)_0, (c_i)_0$ , etc.  $c$ -value of site  $f_0, f_i$ , etc. with no counterions on neighboring sites  
 $c'$   $c'$ -value  
 $d$  sum of ionic radii of interacting ions  
 $d$  a distance defined in Chapter 4  
 $e$  a distance defined in Chapter 4  
 $f$  a distance defined in Chapter 4  
 $f_C$  activity coefficient of species C  
 $f(A)$  a function defined in Chapter 7  
 $f_H^{\text{in}}, f_H^{\text{ex}}$  intracellular, extracellular  $H^+$  activity coefficients  
 $g$  gravitational acceleration  
 $g$  a distance defined in Chapter 4  
 $g$  a ratio defined in Chapter 10  
 $h$  Plank's constant  
 $h$  thickness  
 $i$  an integral index  
 $j$  an integral index  
 $k$  Boltzmann's constant  
 $k$  a variable defined in Chapter 4  
 $k$  an integral index  
 $k_1, k_2, k_3$  rate constants of reactions  
 $l$  an integral index  
 $l_1^y$  length of  $y$  dimension of a gang in extended form  
 $l_2^y$  length of  $y$  dimension of a gang in salt-linked form  
 $m, m', m''$ , etc. distribution numbers  
 $m_{ij}$  number of peptide linkages between sites  $f_i$  and  $f_j$   
 $n$  empirical constant defined in Chapter 5  
 $n_i$  refractive index at wavelength  $\lambda_i$   
 $n_i$  number of  $CH_2$  groups in side chain of site  $f_i$   
 $n_v$  number of molecules in vapor state  
 $n, n', n''$ , etc. distribution numbers  
 $n_i^s$  number of ions  $p_i$  in configuration  $s$   
 $p$  pressure  
 $p$  probability of finding a counterion at a given distance from an ion  
 $pH$  negative logarithm of  $H^+$ -ion concentration  
 $pK, pK(1), pK(2)$ , etc. negative logarithm of dissociation constant  
 $pK_a$  negative logarithm of acid dissociation constant

- (p.f.) partition function excluding microcell configurational term,  $\rho$  or  $\sigma$   
 (p.f.) partition function including microcell configurational term,  $\rho$  or  $\sigma$   
 $(p.f.)_{\text{trans}}$ ,  $(p.f.)_{\text{rot}}$ ,  $(p.f.)_{\text{vib}}$ ,  $(p.f.)_{\text{elect}}$  translational, rotational, vibrational, electronic components of the partition function  
 $(p.f.)_A^{\text{tr}}$ ,  $(p.f.)_{\tilde{x}}^{\text{ads}}$ ,  $(p.f.)_i^{\text{ins}}$ ,  $(p.f.)_{\text{assoc}}$ , etc. partition function of the indicated species in the indicated state  
 $q$  critical distance of separation that defines an ion pair  
 $r$  center-to-center distance between anion and cation  
 $r$  Donnan ratio  
 $r_A$  amount of adsorbent A taken up per mole of protein (Chapter 7)  
 $r_f$  a distance defined in Chapter 4  
 $r_H$  amount of  $H^+$  ion bound per mole of protein  
 $r_{ij}$  scalar distance from  $i$ th to  $j$ th entity  
 $r_i^s$  equilibrium distance in configuration  $s$  from center of ion  $p_i$  to "center of gravity" of fixed ionic group  
 $r_0$  radius of cylinder or particle  
 $r_1, r_2, r_3$  shell radii within a microcell (Chapter 2)  
 $s$  integral index referring to configurations 0, I, II, III  
 $t$  time  
 $t(n_0, n_1, n_2, n_3)$  number of complexions corresponding to distribution numbers  $n_0, n_1, n_2, n_3$   
 $t_{1/2}$  half time for a process  
 $t_+, t_-$  transference numbers  
 $u_+, u_-$  mobilities  
 $\Delta A$  change in optical absorption  
 $\Delta E$  standard change of internal energy for a process or equilibrium  
 $\Delta F$  standard change of free energy for a process or equilibrium  
 $\Delta H$  standard change of enthalpy (heat content) for a process or equilibrium  
 $\Delta H'$  apparent heat of ionization  
 $\Delta H_{\text{fusion}}$  heat of fusion  
 $\Delta V$  change of volume  
 $\Delta W$  work  
 $(\Delta E_{p_i})_i$  adsorption energy of ion  $p_i$  on site  $f_i$   
 $\Delta_{ij}(p), \Delta_{ij}(p_i)$  change of  $c$ -value of site  $f_i$  due to adsorption of ion  $p$  or  $p_i$  on site  $f_j$   
 $\Delta_{ij}(p|q), \Delta_{ij}(p_i|q_i)$  change of  $c$ -value of site  $f_i$  due to exchange of ion  $p$  for ion  $q$ , or ion  $p_i$  for ion  $q_i$ , on site  $f_j$   
 $\Pi_A, \Pi_K$  solution tension of anion, cation  
 $\Phi$  physiological effect

- $\Omega$  total number of complexions
- $\Omega_{\text{therm}}, \Omega_{\text{conf}}$  total number of thermal, configurational complexions
- $\alpha$  fraction of associated ion pairs
- $\alpha, \alpha_i, \alpha_+, \alpha_-, \alpha_w$  polarizability of subscripted species or entity
- $\beta_{ij}$   $\pm 1$ , in Chapter 5
- $\delta$  a distance
- $e$  electronic charge
- $\epsilon$   $\epsilon$ -potential
- $\epsilon$  molecular extinction
- $\epsilon_0, \epsilon_1, \epsilon_2, \epsilon_i, \epsilon_j, \epsilon'_0, \epsilon'', \text{etc.}$  energy levels
- $\epsilon_i$  activation energy in Chapter 10
- $\epsilon_{d_i}^\ddagger$  doublet activation energy for ion p<sub>i</sub>
- $\epsilon_{t_{ij}}^\ddagger$  triplet activation energy
- $\epsilon_s^\ddagger$  saltatory activation energy
- $\zeta$  "intrinsic activity" of a pharmacon
- $\zeta_n$  nth root of the Bessel function of zero order
- $\eta$  a positive number defined in Chapter 4
- $\eta, \eta', \eta'', \text{etc.}$  energy levels
- $\theta_{ij}(p)$  the direct electrostatic term
- $\kappa$  a constant defined in Chapter 15
- $\lambda$  equivalent conductance
- $\lambda$  wavelength
- $\mu$  ionic strength
- $\mu, \mu_{\text{ads}}, \mu_{\text{ins}}$  chemical potential
- $\mu_i$  scalar permanent dipole moment of i<sup>th</sup> entity
- $\mu_w$  permanent dipole moment of a water molecule
- $\nu$  an indicial integer
- $\nu, \nu_1$  frequency
- $\pi_i, \pi_0$  a constant specific to each polar group
- $\rho$  number of sites within a microcell at which a counterion may be said to be associated
- $\rho_i^s$  number of sites within a microcell at which ion  $\rho_i$  may be said to be associated in configuration s
- $\sigma$  a symmetry number
- $\sigma$  number of sites in a microcell at which a counterion may be said to be dissociated
- $\tau$  a time variable defined in Chapter 11
- $\tau_{ij}$  a term defined in Chapter 5
- $\varphi$  average energy barrier of interstices

- $\chi$  transmissivity factor for a CH<sub>2</sub> group  
 $\psi$  potential, cellular potential, surface potential  
 $\psi_{\text{gen}}$  generator potential  
 $\omega$  transmissivity factor for a CONHCH group  
 $\omega'$  a constant specific to a particular protein in a particular medium (Chapter 7)  
 $\omega, \omega', \omega'',$  etc. degeneracy of energy level

## 2. SPECIES AND GROUP SYMBOLS

- A, A', A<sup>+</sup>, B, B', B<sup>+</sup>, G, G', G<sup>+</sup>, J, J', J<sup>+</sup>, R<sup>-</sup>, X<sup>+</sup>, Y<sup>-</sup> mobile counterions  
A, B, AB free, combined species  
 $\mathfrak{X}, \mathfrak{X}'$  critical adsorbents  
C cation  
D, D' dye molecules  
X, X' oxidant molecules  
S solvent  
E enzyme  
S, S<sup>-</sup> substrates  
P product of enzymatic reaction  
I inhibitor of enzyme  
M a labile, metabolically generated cardinal adsorbent in contractile processes  
ES enzyme-substrate complex  
R receptor  
A drug  
RA receptor-drug complex  
 $f, f_0, f_i, f_c, f^-, f^+,$  etc. fixed ionic sites  
 $f_0$  "active" site  
 $f_c$  critical site  
 $f_E, f_E^+$  enzymatic sites  
g gang  
p, p<sub>i</sub>, q, q<sub>i</sub>, etc. mobile counterions  
p<sub>i</sub><sup>ex</sup>, p<sub>j</sub><sup>ex</sup> counterions from extracellular phase  
f<sup>+</sup> f<sup>-</sup> salt linkage  
f<sup>+</sup>Y<sup>-</sup>, f<sup>-</sup>X<sup>+</sup>, etc. fixed-site-adsorbed-counterion pairs  
p<sub>i</sub><sup>ex</sup> · f fixed-site-adsorbed-extracellular-counterion pair (doublet)  
p<sub>i</sub><sup>ex</sup> · f · p<sub>j</sub><sup>ex</sup> triplet of fixed site with two extracellular counterions  
d<sub>i</sub> doublet with ion p<sub>i</sub>  
t<sub>ij</sub> triplet with ions p<sub>i</sub> and p<sub>j</sub>

## 3. UNITS AND ABBREVIATIONS

Å	angstrom unit
‡	activated, activation
ads	adsorbed
assoc	associated
cal	calorie
cm	centimeter
conf	configurational
const	constant
deg	degree (centigrade)
dissoc	dissociated
elect	electronic, electrostatic
eq	equivalent
ex	extracellular
f	fixed-charge phase
fr	free
g	gram
gen	generator
in	intracellular
∞	infinity
ins	interstitial
ι	interstices
intr	intrinsic
°K	degrees absolute
kcal	kilocalorie
kg	kilogram
mask	masked
max	maximum
μF	microfarad
γ	microgram
μg	microgram
μ	micron
mg	milligram
ml	milliliter
mm	millimeter
mμ	millimicron
mM	millimolar
mM	millimoles
mM/l	millimoles per liter

mv millivolt  
 $M$  molar  
 $N$  normal  
 r resin phase  
 rel relative  
 rot rotational  
 s solution phase  
 salt lk salt linkage  
 S.E. standard error  
 sec second  
 surf surface  
 thres threshold  
 trans translational  
 v volt  
 vac vacant  
 vib vibrational

#### 4. OPERATORS

$d$  differential  
 $\frac{d}{d}$  derivative  
 $\frac{\partial}{\partial}$  partial derivative  
 $\left(\frac{\partial}{\partial}\right)_x$  partial derivative with  $x$  held constant

exp exponential function of

! factorial

> greater than

< less than

$\int_b^a$  definite integral

ln natural logarithm

log common logarithm

$\prod_i$  product over index  $i$

$\sum_i$  summation over index  $i$

(J|G) replacement of G by J on a fixed site

{ } surface concentration of enclosed species in moles per square centimeter

[ ] volume concentration of enclosed species in moles per cubic centimeter



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## A NOTE ON THE AUTHOR

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