

**Evidence Based Cell Biology,
with Some Implications for Clinical Research**

by

Harold Hillman

(Unity Laboratory of Applied Neurobiology)

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This book is dedicated to my unexcelled wife, Elizabeth, in whose eyes are truth.

Author of

Certainty and Uncertainty in Biochemical Techniques. (1972). Surrey University Press, Henley.

(With Sartory, P) *The Living Cell.* (1980). Packard Publishing, Chichester.

The Cellular Structure of the Mammalian Nervous System. (1985). MTP Press, Lancaster.

The Case for New Paradigms in Cell Biology and in Neurobiology. (1991). Mellen Press, Lampeter.

(With Jarman, D) *Atlas of the Cellular Structure of the Human Nervous System.* (1991). Academic Press, London.

Theories, like mist on spectacles, sometimes obscure vision. *Confucius*, 515-479 b.c., quoted by *Charles Chan*, 1943.

Primum non nocere. *Hippocrates*, 460-377 b.c.

He that hath ears to hear, let him hear. *Matthew*, 11, 15.

A truth, established by proof, does not gain in force from support of scholars; nor does it lose its certainty, because of popular dissent. *Moses Maimonides*, 1190.

But as soon as he began using his mind, the wisdom of heresy became strong in him again. *Nachman of Bratislav*, 1772-1811.

To tell the truth is dangerous, to listen is annoying. *Danish proverb*.

Nothing is too wonderful to be true - if it be consistent with the laws of nature. *Michael Faraday*, 1791-1867.

It is easier to ride on the back of a tiger than to climb off it. *Russian proverb*.

So rested he by the tum-tum tree, and stood awhile in thought. *Lewis Carroll*, 1872.

A tale so free of every doubt, all possible, probable shadow of doubt, all possible doubt, whatever. *William Gilbert*, 1889.

“Is there any point to which you would wish to draw my attention?”

“To the curious incident of the dog in the night time.”

“The dog did nothing in the night-time.”

“That was the curious incident,” remarked Sherlock Holmes.

Arthur Conan Doyle, 1891.

E pericoloso sporgesì. *Ferrovie Italiane*, 1936.

Double think means the power of holding two contradictory beliefs in one's mind simultaneously, and accepting both of them. *George Orwell*, 1949.

“And the people all said, “Sit down, sit down, you're rocking the boat” ”.

Frank Loesser, 1951.

Purification of enzymes may modify their properties, and it is, therefore, essential to investigate the behaviour of enzymes not only in the purified state, but also in the natural environment. *Sir Hans Krebs, 1962.*

The specificity of an inhibitor is inversely proportional to its familiarity.
Horace Davenport, (personal communication) 1962.

The history of medicine records many examples of the persistence of forms of therapy, which owed more to the attractiveness of the tenuous theories underlying them, than to any unequivocal demonstration of their effectiveness. *Harold Hewitt, 1979.*

Cave lacunam. *Vectis Londinii.*

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Foreword

I have been involved in research on cytology, neurobiology and resuscitation, for more than 45 years. During this time, I have thrown doubt on the use of subcellular fractionation, histochemistry and electron microscopy, for elucidating the biology of living cells, (Hillman, 1972). With the late Mr Peter Sartory, I have brought evidence that the Golgi apparatus, the endoplasmic reticulum, the nuclear pores, etc., are artifacts of preparation for histology and electron microscopy (Hillman and Sartory, 1977; 1980); anatomical synapses do not exist in the mammalian central nervous system (Hillman, 1985b); there are only neurons and microglia in the living nervous system (Hillman, 1986a,1996a); the chemical hypothesis of nervous transmission is untenable (Hillman, 1991b); histology and histochemistry can not be used to study the properties of living cells (Hillman, 2000). The ‘politics’ of presenting unpopular views, have been examined (Hillman, 1991a; 1996b). On this topic, please see Martin et al, (1986), Kohn, (1992), Martin, (1996) and Moran, (1998).

It is clear that lectures and publications throwing doubt on the consensus views pose considerable threats to some of one’s colleagues. If I am wrong, only my reputation has been damaged. If I am right, those colleagues proved wrong may have well been wasting their time and careers, and using public or charitable resources naively. They might have used their time and resources to carry out more productive research. However, we should not forget about our responsibilities to the queues of ill people in clinics waiting patiently for the results of our endeavours.

Truth in cytology may be defined as the structures, events and relationships of cells in living intact animals in their natural environments and in diseases. The real message of this book is that, in the evolution of biology so far, too little attention has

been paid to the effects of technical procedures. Research workers have: failed to obey the laws of geometry, physics and thermodynamics; they have avoided doing crucial control experiments; they have used inadequate calibration systems; they believe that large teams and more expensive procedures result in greater truths; they have given too little attention to the possibility that they were looking at artifacts; many give the impression that contradictions and difficulties within their beliefs will go away, if they ignore them; many have failed to distinguish between hypotheses and findings; there is a common view that the authority of textbooks, Nobel prize winners, and committees distributing research funds, are more persuasive than the evidence adduced in support of scientific hypotheses.

There are more research workers and more resources engaged in biological medical research than at any other epoch in history. This fact gives current beliefs an enormous inertia, which in my estimate would be unlikely to permit significant changes in parameters during the 21st century. It seems likely that another century will have to elapse, before such crucial changes may occur. On the other hand, the ‘noise - to - signal’ ratio may be so high by then, and the large scientific community may have invested so much effort and resources, that bad quality medical and biological research will continue indefinitely. There are plenty of examples in science, medicine and religion, of absurd views being accepted for centuries, without challenge, and without threatening the professionals or the systems, in which they flourished. John Stuart Mill pointed out that the victory of ‘truth’ over ‘untruth’ is by no means inevitable.

I will be pleased to enter into dialogue with any interested persons, as long as I am physically capable of doing so.

The monograph starts with a critical review of *methods* used in biology, to

emphasise that the value of all experimental findings is totally dependent on the quality of the methods used to elucidate them. Then, it examines the structures common to most cells, which have been summarised in the ‘generalised’ cell. Some of the commonly accepted structures are found to be artifacts produced by the procedures, used for examining them, especially by electron microscopy. Some ‘specialised’ cells, such as neurons and muscle cells, are examined in greater detail. The biology attributed to living cells, especially the concepts, such as active transport, signalling, apoptosis, etc., are given attention as they form such a large part of current understandings about the cell. Finally, I have indulged in the arrogance of age, in proffering advice to many categories of students, research workers, clinicians and philosophers, to whom the biology of the cell is important.

Throughout the text, any reference to either gender obviously applies to both. I have used tables extensively to summarise the findings of several schools of research workers. I have used key and usually original references to important points, about which cell biologists generally agree. A complete list of references would make the monograph too weighty for biologists with cardiac conditions.

This book is mainly addressed to those young biologists, before their ambition or necessity to obtain funds for their research, has induced them to make intellectual compromises.

Cell division and genetics have not been dealt with in this monograph.

Like most authors, I write, so that I may talk to people whom I can not meet, and so that I may continue expressing my opinions after I am dead.

I wish to thank my daughter, Miss Annie Rachel Hillman, and Dr Nick McKay of Surrey University, for drawing the computer graphics. Annie Rachel also helped with many pernickety corrections. I am indebted to the authors and publishers mentioned for permission to reproduce figures and tables. I wish to record my heartfelt thanks to Mrs Susan Shaw for typing the Carolingian manuscript so patiently. Miss Sophia Hillman, my other daughter, kindly guided me around the computer, while my son, Benedict, knew how to drive it.

I apologise to any author of a figure or table, whom I have inadvertently failed to acknowledge.

Chapter 1

A Few Milestones in Cell Biology

There have been many publications on the history of cell biology (please see, for example, Schleiden and Schwann, 1847; Nordenskjöld, 1928; Baker, 1948a,b, 1950a, 1954; Singer, 1957, 1959; Hughes, 1959; Hall, 1969; Rothschild, 1973; Bracegirdle, 1978; Norman, 1991; Teich, 1992; Ling, 1992; Finger, 1994; Von Dröscher, 1996). Research proceeded by various steps, for example: the description of the cell by Hooke; the generalisation that tissue was composed of cells; sectioning, staining and mounting of tissue; the achromatic lens; histopathology; tissue culture; phase contrast microscopy; fluorescence microscopy; electron microscopy; immunocytochemistry. Rather than studying the history in detail, I have drawn up a list of important advances, and would refer the reader to the original publications on these subjects (Table 1). The most important landmarks are the development of light microscopy and histology in the 19th century and the evolution of biochemistry, in the 20th century.

Important milestones in cell biology

<i>Discovery or concept</i>	<i>Reference with dates</i>
Inflammation described	Celsus, 2nd century; Galen, 130-201 Goldsby, Kindt and Osborne, 2000, page 9
Vaccination	Chinese 10 th century; Jenner, 1798
First use of compound microscope in biology	Various, early 1600's
Blood transfusion in animals described	Lower, 1660
Cells described	Hooke, 1667
Protozoa, red cells and bacteria seen	Leeuwenhoek, 1677
Supravital staining	Tremblay, 1744; Brandt, 1881
Nuclei and nucleoli seen	Fontana, 1781
Brain structure	Floureens, 1824
Brownian movement seen	Brown R, 1826, 1828
Achromatic objective described	Lister, 1830
Protoplasm described	Purkinje, 1838
Generalisation that tissue is composed of cells	Schleiden and Schwann, 1847
Microdissection carried out	Hannover, 1844
Neuroglia described	Von Virchow, 1846, 1854, 1859

<i>Discovery or concept</i>	<i>Reference with dates</i>
Diffusion described	Graham, 1861
Mitochondria seen	La Vallete St George, 1867
Stimulation of brain	Fritsch and Hitzig, 1870
Histological methods developed	Various, late 19th century
Cell division described	Strassburger, 1875
Cellular immunity observed	Von Behring and Kitasato, 1890
Immunisation against tetanus	Schutz, 1892
Phagocytosis described	Metchnikoff, 1892
Immunisation against diphtheria	Von Behring and Wernicke, 1892
Theory of receptors described	Ehrlich, 1897
Physiological synapses described	Sherrington, 1897
End-fusse stained	Held, 1897; Auerbach, 1898
Autommunity proposed	Ehrlich, 1901
Blood groups identified	Landsteiner, 1901
Tissues grown in culture	Harrison, 1907
Anaphylaxis described	Richet, 1913
Tissues sliced and incubated	Warburg, 1923
Concept of homeostasis described	Cannon, 1932
Phase contrast microscopy described	Zernike, 1934
Electron microscope applied to biology	Marton, 1934
Bilipid membrane proposed	Davson and Danielli, 1936
Subcellular fractionation	Claude, 1946; Tiselius and Kabat, 1939
Intracellular micropipette introduced	Graham and Gerard, 1946
Observation of nucleolonema	Estable and Sotelo, 1951
Description of DNA as a double helix	Watson and Crick, 1953
Tolerance to foreign cells	Billingham, Brent and Medawar, 1954
Sliding filament hypothesis of muscle contraction	Huxley and Hanson, 1955
Use of Cartesian diver	Lindeström-Lang, 1937: Zeuthen, 1961
Association - induction hypothesis	Ling, 1962
Development of hybridomas	Köhler and Milstein, 1975
Polychromatic light illumination	Hewlett, 1983; Oldfield, 1998
Quantum dot fluorescence	Michalet et al, 2003

Table 1. The dates are those of the discoveries or of the descriptions of the concepts.

Chapter 2

Rules of Engagement for Research

The rules of engagement, used in my own research and in this monograph, may be summarised as follows.

1. A hypothesis is a guess, extrapolation or supposition, whose only value is derived from the accuracy with which it can forecast the results of future experiments. Findings have power.
2. A hypothesis must be provable and disprovable by experiment, observation or reasoning.
3. All procedures, measurements, hypotheses and conclusions, imply their own portfolios of inherent assumptions, whether or not the practitioners identify them. These assumptions should be stated, and their warrantability tested by experiment, calculation or reason. Alternatively, one may demonstrate that ignoring an assumption would not make a significant difference to the conclusion.
4. If two different procedures yield different values for the same parameter, one or both of them must be wrong. A good research worker should not fail to resolve which of the results is likely to be correct.
5. Research workers should not be agnostic about important differences in their specialist disciplines. Either there is enough evidence to convince them of one view, which they should espouse, or there is not enough, in which case they should reject it.
6. In considering scientific viewpoints, a real objection to one piece of evidence that it ignores the natural laws, can not be gainsaid by any other evidence - however massive - that is compatible with the theory, or appears to support it, or does not contradict it.
7. Research workers who cite the findings of others take upon themselves the intellectual responsibility for the validity of the findings quoted. The person quoting must examine the validity in detail, including any challenges to it. It is not good enough in responding to doubts about quoted evidence to refer to the experience or authority of the person quoted.
8. An experiment is only as good as the calibrations of the measurements , the warrantability of the inherent assumptions, and the controls of the procedures, done in parallel with it.

9. The study of the cell is, itself, something of an approximation, because most cells have to be separated from the tissues with a variable risk of changing their entropy, in order to examine them.

10. Cells are governed by the laws of geometry, physics, physical chemistry, chemistry and thermodynamics.

11. A structure of which a two dimensional model can not be made, can not exist.

12. The refusal to answer proper questions at meetings, by correspondence, or in conversation, represents a serious derogation of the duty of an academic, which the academic community should not tolerate.

Chapter 3

The Second Law of Thermodynamics

According to the Second Law of Thermodynamics, in a closed system, any change in entropy must be accompanied by a change in free energy (Atkins, 1994). Free energy drives all chemical reactions, so that any change in entropy will change the rates of reactions, their equilibria and the concentration of the products. That is, changes in entropy must push the reactions one is studying away from those in their original states *in vivo*.

Living systems are generally open, so that these considerations are difficult to apply, but many experimental procedures in biology, biochemistry, biophysics and pharmacology are carried out partly or completely in closed conditions. For example, the tissue is homogenised in test tubes insulated by air, which is a poor conductor of heat; homogenates are centrifuged in evacuated cold centrifuges; washing, filtering, chromatography and electrophoresis, are also done in an air atmosphere, so that heat can not escape rapidly. The degree to which a reaction is open or closed depends upon the rate of the procedure, the concentrations of the tissue and reagents, the temperature, and the heat conductivities of the tissue, reagents, reaction vessels, and their environments. It is often impossible to determine to what degree complex reactions are open or closed, since many of these parameters are unknown (and often unknowable) in the precise conditions of a particular experiment.

However, in any system, which is partly or completely closed, it is useful to examine at what steps the entropy or the free energy is likely to be changed by the procedure. One may list those steps in any procedure which obviously change the entropy (E) or free energy (F) (Table 2). Few steps do *not* change the entropy of reactions under study. (A similar analysis may be made of any experiment in biology or chemistry).

Changes of entropy and free energy during biochemical procedures

<i>Step</i>	<i>Effect</i>	<i>Change in entropy (E) or free energy (F)</i>
Killing the organism	diffusion occurs; gradients across membranes and between adjacent tissues diminish	E
Tissue hypoxia	oxidative reactions diminish	E
Post mortem changes	denaturation of proteins; redistribution of solutes	E, F
Tissues cools	rates of reactions diminish	F
Tissue is sliced, homogenised, macerated or sonicated	it is subjected to pressure; concentration gradients are diminished; heat is generated; enzymes, substrates and activators diffuse from their sites <i>in vivo</i> to other locations	E, F
Powerful chemically active reagents are added	tissue is diluted; tissue constituents exchange with reagents and bind to them	E, F
Tissue is ‘washed’, rinsed, diluted, or eluted	it is diluted; soluble constituents are extracted	E
Tissue is centrifuged or stirred	friction occurs between particles and centrifugation medium; pressure rises; heat is generated	E, F
Enzyme activators and inhibitors added	change enzyme activities	E
Tissue is filtered, or triturated	it is diluted; may bind to filter paper or column; soluble constituents are extracted	E
Dialysis	small molecules and ions are removed	E
Constituents are concentrated	the affinity for other constituents is changed	E
Constituents are precipitated or suspended	they change their solubility and reactivity	E
Tissue is dried, dehydrated or blotted	concentrations of all soluble constituents increase; water and volatile substances are removed; proteins are denatured	E, F
Tissue is frozen or heated	reaction rates are changed; dehydration occurs; proteins are denatured	E, F
Crystallization	the solvent is extracted; all bonds with other tissue constituents are broken; the concentration of the substance increases to 100%	E, F
Tissue reacts with added constituents	exothermic or endothermic reactions occur	E, F

<i>Step</i>	<i>Effect</i>	<i>Change in entropy (E) or free energy (F)</i>
Sedimentation	reagents exceed their solubility products	F
Partition	reagents react with each species and phase	E
Lyophilisation	dehydration and concentration	E
Purification	(see chromatography and electrophoresis)	E, F
Mounting	subjection to xylol or propylene oxide and mountant	F
Staining	organelles coloured, which changes light absorption	E
Antigen - antibody reactions	plus other 'non-specific' reactions	E
Addition of fluorochromes	reaction not only with proteins	F
Subjection to x-rays	DNA 'damage'	F, E
Bombardment of electrons	liberation of heat and X-rays	E, F
Addition of non-isotonic reagents	accelerates the movement of soluble constituents	E
Tissues are dissolved in powerful reagents	proteins are denatured, pH changes	E
Substances are extracted	bonds are broken	
Tissue is fixed	enzyme activities are inhibited; proteins are denatured	E, F
Chromatography and electrophoresis	bonds are broken; tissue is heated	E, F
Preincubation and incubation	tissue exchanges with media	E
Contamination	substrates are used up; toxic products are produced	E
Microscopy	tissue is illuminated and absorbs energy	E
Permeabilisation	denatures proteins; opens membrane pores	E

Table 2. Effects of procedures on the entropy and free energy of the chemical reactions within tissues. *It should be noted that most manipulations change the entropy of a system, and many change the free energy.*

If the experimental procedures cause large changes in entropy and, or free energy, in those parts of the systems which are closed, they will change the rates of reactions and the equilibria of the reactions, from the natural ones in the living intact animals or plants (Hillman, 2002). The main problems are, firstly, that it is difficult

to know how open or closed an experimental system is, and, secondly, it would be a major problem to assess the total effects of all the changes in entropy and free energy in a particular complex experimental procedure.

One of the most fundamental problems in biochemistry is the conformation of proteins *in vivo*, in dilute complex solutions, compared with the situation in high concentration in pure solution, or in crystals. Very little attention has been paid to this so far (Jaenicke, 1987; Price, 1992).

The definition of biology given here implies that experiments *in vivo* give more accurate information about the state of the living intact organism than those *in vitro*. However, it does not mean that experiments *in vitro* are valueless. One can only assess the *rates* of reactions in intact tissues by experiments *in vitro*, if one measures, calculates or assesses, the effects of the procedure on the parameter one is measuring. However, one can examine the *mechanism* of a process which occurs in the intact organism, if the process still continues vigorously in an isolated tissue or fraction *in vitro*.

A list of the kinds of experiments which involve minimal changes in entropy or free energy is given (Table 3), to indicate the wide range of such procedures, as it is often asserted that there are no alternative to current high energy procedures. Non disruptive techniques are to be preferred (Bourne, 1964, 1967; Marsden, 1984; Foskett and Grinstein, 1990). An excellent review of the thermodynamics in pharmacology appears in Raffa (2001), although all the authors in this book studied simple systems.

Experiments involving minimal changes in entropy

- Experiments on living animals
- Epidemiological studies
- Radio active experiments
- Nutrition experiments
- Microscopical observations on free living unicellular organisms
- Telemetry of physiological parameters
- Natural behaviour of wild organisms
- Whole body movements
- Anaesthetised animals
- Nuclear magnetic resonance
- Positron emission tomography
- Implanted electrodes
- Transparent windows
- Isolated limbs and organs
- Injections into living cells and their environments
- Extracellular and intracellular micropipetting
- Examination of body fluids
- Biochemistry and physiology of uniform single cell populations

Table 3. This list is not comprehensive.

Section A

Measurements and Procedures

Chapter 4

Calibration of Measurements of Substances Originating from Living Tissue

Substances are extracted from tissue and the values of the constituents are read, or printed out from, spectrophotometers, photometers, spectrometers, spectrophotofluorimeters, pH meters, ion selective electrodes, etc. These measurements imply the following assumptions: firstly, that the extraction procedure does not alter the quantity of the material being measured, despite the many powerful chemical reagents used (Table 4); secondly, that the extraction and none of the reagents used throughout the procedure affect the amplitude of the signal recorded; thirdly, that the extraction is complete; fourthly, when calibrations are carried out with pure protein solutions, which represent only one of the many chemical species of protein in the tissues, the particular calibrating protein is an accurate measure of all the proteins in the biological tissue; fifthly, that a calibration made using pure solutions at the end of the experiment will give the same value as if the calibrating substance had been administered to the 'living' animal, before the experimental procedure - a 'recovery' calibration. The best way is to give graded quantities of the substance to the living animal or tissue section before the tissue is processed, and thus use the intact animal for bio-assay. The concentration in the intact tissue is then calculated from the slope of the curve of added graded quantities against the amplitude of the signal recorded from the instrument. Failure to use 'recovery' calibrations makes measurements qualitative rather than quantitative.

Widely used chemical agents

Fixatives	Reducing agents
Strong acids or strong alkalis	Reagents which cause exothermic or endothermic reactions when added
Dehydrating agents	Proteolytic enzymes
Non-isotonic aqueous solutions	Corrosive agents
Bleaches	Heavy metal salts
Oxidising agents	Carcinogens
Permeabilisers	Detergents

Table 4. Chemical reagents, which are likely to alter the rates of reactions, when added to tissues. *These are widely used in biochemical experiments, although most of them kill cells.*

Chapter 5

Parameters of Tissues Which Measured Concentrations Are Referred

In considering the chemistry of a cell, ideally, one would like to measure the chemical *activity* of a particular chemical species in a particular subcellular phase, or the *concentration* of the constituent in that phase in the living intact animal. This is only possible for stable substances, whose activity, or the equilibria of the reactions in which they are involved, are not affected by killing the animal, or any of the chemical reagents used in the procedures.

However, measurement of any substance has to be referred to a particular parameter of the tissue, and reference to each of them carries its own burden of assumptions. Some of them are unwarrantable (Table 5). The reasons for which it is difficult to measure the concentrations or activities in a particular organelle is because there are so many phases in a tissue, - even more than there are subcellular organelles or compartments (Table 7). Furthermore, these change when the animal is killed in order to make the measurements.

Parameters of tissue to which measurements are referred

<i>Measurement</i>	<i>Assumptions</i>	<i>Comments</i>
1. Fresh weight	<ul style="list-style-type: none"> (i) no significant exchanges between the organ and the circulation during dying. (ii) inhomogeneity of the tissue does not matter. (iii) manipulations, such as cutting out the tissue, compression, etc., do not make a significant difference to measurements 	intra- and extra-cellular concentrations are sought because the living tissues are heterogeneous, and most reactions occur in aqueous phases, while visible structures are insoluble in water
2. Dry weight	(i) drying to constant weight only extracts water.	this extracts all substances, whose boiling points are below 100 - 105°C; extraction may break down unstable substances
3. Per ml of tissue water	assumptions of 1 and 2	this is a real attempt to find out tissue concentrations relative to the tissue water, which would determine the chemical activity in the aqueous phases

<i>Measurement</i>	<i>Assumptions</i>	<i>Comments</i>
4. Per g or ml of intracellular space	(i) markers such as inulin, sucrose, xylose, or arabinose, do not penetrate the cell membranes. (ii) markers can be recovered completely from tissue (iii) they do not themselves affect metabolism (iv) they should all show the same intracellular volume	unproven assumption they can not untested assumption they do not. inulin from different sources has different molecular weights
5. Per g of incubated weight	(i) there is no significant exchange of tissue contents with the medium during incubation (ii) one can know how much incubation fluid has adhered to the tissue, and its chemical composition	the tissue swells; there is exchange this problem is only solved subjectively
6. Per g of tissue protein	(i) it is difficult to measure protein satisfactorily (ii) a calibration using a pure protein can be used for different tissue fractions	different methods give different quantities of protein (Table 6) it can not
7. Per µg of DNA	(i) all cells in a particular animal contain the same quantity of DNA	untreated nuclei can vary from 2 to 30 µm diameter in the same animal so, this is unlikely
8. Enzyme activities mg/ml substrate per period of time	(i) the activity is not changed by the isolation, preparation or extraction	the Second Law of Thermodynamics says that it will be (Chapter 3)
9. Ion-sensitive measurements with micropipettes	(i) the membrane's permeability to an ion in very small concentration in a tissue is only determined by the one ion (ii) a calibration with pure solution is relevant to that in the same tissue	extremely unlikely; has not been tested comprehensively one does not know how much of the ion is bound

Table 5. *The essential problem is that the tissues are heterogeneous, so that concentrations per quantity of tissue give no information about concentrations in particular phases. It is difficult to decide the 'correct' parameter in a particular experiment. Please see also Hillman, (1983).*

Different proteins are composed of different sequences of amino-acids and different tissues contain different concentrations of proteolytic enzymes. Proteins may be measured by their peptides, their nitrogen concentration, their fluorescence, their affinity for a resin, and other methods (Table 6; Boyer, 1993). Different

procedures measure different parameters. The proteins extracted are calibrated against particular purified proteins. Unfortunately, different tissues, homogenates, cells and subcellular fractions, contain quite different mixtures of proteins from those used to calibrate the assay systems, so it is necessary to use several purified proteins, and to draw a different calibration for each preparation or fraction.

Measurements of proteins in tissues

Precipitation	Electrophoresis
Extraction	Mass spectrometry
Lowry	Calibration with pure protein
Biuret	Calibration with mixture of proteins
Bradford	Spectrophotometry
Biscinchoninic acid	Recovery measurements
Autoanalysis	Use of radioactive precursors
Chromatography	Spectrophotofluorimetry

Table 6. These are listed in Boyer, (1993)

A body is composed of organs, tissues, cells, organelles and phases (Table 7). However, it is remarkable how many different phases there are in a cell, and each atom, ion and molecule has a different behaviour in each phase; there are also many different states in which ions and molecules can exist (Table 8).

The phases of cells in vivo

1. Extracellular fluid
2. Bound to other extracellular constituents
3. Bound to the cell membrane extracellularly
4. Cell membrane
5. Bound to the cell membrane intracellularly
6. Free in the cytoplasm
7. Bound to other cytoplasmic constituents
8. Bound to the cytoplasmic surface of the nuclear membrane
9. Nuclear membrane
10. Bound to the nucleoplasmic surface of the nuclear membrane
11. Bound to cytoplasmic surface of mitochondria
12. Mitochondrial membrane
13. Bound to the internal surface of the mitochondrial membrane
14. Free in the mitochondrial matrix (the mitochondrioplasm)
15. Bound to other matrix constituents
16. Bound to the unidentified cytoplasmic particles

- | |
|---|
| 17. Cytoplasmic particles (assuming they are solid) |
| 18. Free in nucleoplasm |
| 19. Bound to the other nucleoplasmic constituents |
| 20. Bound to the constituents of the pars amorpha |
| 21. Bound to the external surface of the nucleolonema |
| 22. Bound to the nucleolonema |

Table 7. *This is the minimal number of phases in a cell to which any substance may have any degree of affinity. Thus, in respect of the measurement of substances, the cell or tissue should not be treated as being one phase only. Please see also Hair, (1971).*

States of ions and molecules

Fully ionised, N	Denatured, S
Partially ionised, N	Moving by osmosis, N
Diffusing, N	In dynamic equilibrium, N, S
In bulk phase, N	In static equilibrium, S
Adjacent to interface, N	In open systems, N
In suspension, N, S	In closed or partially closed systems, S
Dissolved, N	Bound by hydrogen bonds, N
Precipitated, N	Bound coordinately, N
Charged, N	Bound covalently, N
Colloid, N	Bound by Van der Waals forces, N
Insoluble, N	Distributed by voltage gradients, N, S
Secreted, N, S	
Crystallloid, N	

Table 8. The variety of states of ions and molecules in tissues. N = natural state, S = conditions in which they are often studied. *Many publications assume that they can exist in only free, partially bound, and fully bound states.*

Attempts are often made to measure the intracellular concentrations of substances by measuring the total tissue concentrations, and the volume of the extracellular compartment of the tissue, and then calculating the intracellular concentrations from the equation: tissue concentration x 100 = extracellular fluid volume x extracellular concentration + unknown intracellular concentration x intracellular volume. The total tissue concentration is measured. The extracellular (and therefore intracellular) volume is measured by using markers, which are believed not to enter cells. These markers have included Na^+ , thiocyanate, sucrose, inulin,

xylose and arabinose. They are *totally* unsatisfactory because (a) no one has shown that they neither enter cells, nor bind to the tissue without entering the cells; (b) one can not recover 100% of them from tissues, indicating that they may be bound, break down spontaneously or are broken down by the tissue; (c) they each give different values for the extracellular space, whereas they should all give similar values, if that is what they are measuring; (d) tissue slices (please Chapter 13) swell as soon as they contact incubation media, and the chemical composition of this swelling is unknown and unknowable; (e) one of the most popular extracellular markers, inulin, is a heterogeneous mixture of the polymers, which varies with the source of the plant from which it is extracted (Bassir, 1956). It is used clinically as an extracellular marker on the basis that it is completely cleared by the kidney in living animals and human beings (Smith, 1951); (f) it is assumed that markers have no effects on the osmotic pressure, chemical properties, or membrane permeability, but the warrantability of these assumptions has never been tested. Obviously, if one can not be satisfied with the measurements of the extracellular space, one can not calculate the intracellular concentrations.

Neither histology nor electron microscopy can be used for measuring extracellular or intracellular volumes (please see Chapters 8 and 9).

Other methods have been used for measuring the areas and volumes of cells, although frequently they have been applied to dehydrated, sectioned or frozen tissue. Caspersson, (1950) scanned cells with ultraviolet light to examine the nucleotides, Ross, (1967) used phase contrast microscopy. Brattgård and Hydén, (1960) used x-ray microscopy. Probably, the most successful method is the use of fluorescence, on cells in incubating media (Allanson, Khatibi, Gustavsson et al, 1999).

Measurements may be referred to the dry weight of tissue. The tissue is heated to 100–105°C for 1–2 hours until it reaches constant weight. This measurement implies: firstly, that only water evaporates, while none of the other substances with lower boiling points are lost. This is obviously untrue, as one can smell volatile substances during the drying. Secondly, that these substances, other than water, do not have significant weights; thirdly, that the heat and dehydration do not change the cell constituents significantly; fourthly, that the crust which forms allows uniform and complete dehydration; fifthly, that none of the tissue is lost by ‘spitting’ during the drying. These all add up to the belief that the overall recovery is not 100%.

One may measure several different parameters of enzyme activity (Table 9).

Furthermore, it is often implicitly assumed that the activity is a linear function of the quantity of enzyme present in the tissue. Since, classically, the relationship between an enzyme activity and the substrate is sigmoid, calculation should not be made as if it were linear. For example, it is obviously wrong to dilute a preparation x or y times, and then to multiply the measured activity by x or y, unless one has shown that the relationship between the enzyme and the substrate is linear within the ranges used. However, in comparing enzyme activities in the *same* tissue under different conditions, it is probably reasonable to assume - although it is much better practice to demonstrate experimentally - that the different experimental conditions affect the enzyme activities in the two different situations equally.

Different activities of an enzyme

- Concentration in a particular phase in vivo
- Activity in intact living cells in natural conditions
- Activity in living cells in tissue culture
- Maximal activity in tissue slices or homogenates
- Activity when the enzyme is partially denatured
- Activity in stimulated muscle, brain or nerves
- Enzyme preparations in conditions in which constituents produce submaximal activity
- Enzyme preparations containing known or unknown inhibitors
- Activity in closed or partially closed systems
- Activity in extracellular fluid
- Pure enzymes in aqueous solution
- Crystallized pure enzyme
- In presence of radioactive isotopes
- Activity in homogenates
- (Spontaneous breakdown of substrate in the absence of enzyme activity)

Table 9. The activity or concentration is normally only measured in homogenates, in which it is assumed that homogenisation itself does not change the activity. However, exo-enzymes, and activities in unicellular organisms can be measured without disruption.

Physical agents affecting tissues

- | |
|---|
| 1. Rise in temperature above the physiological range |
| 2. Light |
| 3. Fall in temperature below the physiological range, including cooling and freezing. |
| 4. Rise in pressure above the physiological range |
| 5. Fall in pressure below the physiological range |
| 6. Dehydration |
| 7. Subjection to ultraviolet radiation |
| 8. Subjection to ionising radiation |
| 9. Microtomy |
| 10. Centrifugation |
| 11. Homogenisation |
| 12. Electrophoresis |
| 13. Heavy metal deposit |
| 14. Gas-liquid chromatography |
| 15. Radio activity |

Table 10. These agents can alter the rates of reaction in tissues or denature proteins.

Biochemists recognise different enzyme activities. However, they have generally not shown much interest in the effects of the physical agents they use on the chemical - particularly enzyme - activities within the tissues. A list of such agents is given (Table 10). The Editor of the 'Biochemical Journal' wrote in 1964 that it was 'revolutionary' to suggest that physical agents could have biochemical effects! The response was that Count Rumford had demonstrated the untruth of this heresy, when he tried to homogenise cannons in 1797.

Ion activators and inhibitors of enzymes

<i>Enzymes</i>	<i>Activating ions</i>	<i>Inhibitory ions</i>
Phosphatidyl acetyltransferase	K ⁺ , NH ₄ ⁺	Na ⁺
Acetyl-CoA synthetase	Mg ²⁺ plus K ⁺ or NH ₄ ⁺	Na ⁺
Pantothenate synthetase	Mg ²⁺ plus K ⁺ or NH ₄ ⁺	Cu ²⁺ , Na ⁺
Pyruvate kinase	Mg ²⁺ plus K ⁺	Ca ²⁺ , Na ⁺
ATPase	Mg ²⁺	Ca ²⁺
5-nucleotidase	Mg ²⁺	Ca ²⁺
Arginosuccinate synthetase	Mg ²⁺	Ca ²⁺
Glutamine synthetase	Mg ²⁺	Ca ²⁺
Riboflavin kinase	Mg ²⁺ , Co ²⁺	Ca ²⁺
Inorganic pyrophosphatases	Mg ²⁺	Ca ²⁺
Phosphopyruvate hydratase	Mg ²⁺	Ca ²⁺

<i>Enzymes</i>	<i>Activating ions</i>	<i>Inhibitory ions</i>
Myosin ATPase	Ca^{2+} , K^+ , NH_4^+	Mg^{2+}
Glycyl-leucine dipeptidase		Ca^{2+}
Alkaline phosphatase	Mg^{2+} , Ca^{2+}	
Creatine kinase	Mg^{2+} , Ca^{2+}	
Malate dehydrogenase	Co^{2+} , Mg^{2+} , plus K^+	
Ketohexokinase	Mg^{2+} plus Na^+ or K^+	
DFPase	Ca^{2+} , Mg^{2+} , Ca^{2+}	
Pyruvate decarboxylase	Mg^{2+} , Co^{2+} , Ca^{2+} , Fe^{2+} , Fe^{3+}	
Oxaloacetate decarboxylase	Co^{2+} , Mg^{2+} , Fe^{2+} , Ca^{2+}	
Trimetaphosphatase	Co^{2+} , Fe^{2+} , Mg^{2+}	
Pantheine kinase	Mg^{2+} , Ca^{2+}	

Table 11. The data for this Table came from Dixon and Webb, (1964), Altman and Dittmer, (1966), and Dixon, (1969). *It can be seen that Mg^{2+} is the most widespread activating ion, and Ca^{2+} is the most popular inhibitor. Any chelating agent added to an experimental mixture will affect the activities of all Mg^{2+} and Ca^{2+} activated enzymes. Edta is widely added in tissue homogenisation.*

In the literature, no attention whatsoever seems to have been given to the possibility - indeed probability - that ions, which activate and inhibit enzymes, can relocate by diffusion during biochemical procedures (Table 11). Since the concentrations of these ions change continuously in cellular compartments, and obviously they often have opposite effects, there is no way of forecasting or calculating the effects of any ions, their concentration or activity in real time, on any particular enzyme in a particular organelle in dead tissue or if any disruptive procedure is used.

Some of the difficulties in the measurement of a chemical component of tissue are illustrated by DNA. It is usually extracted before it is measured, and the extraction procedure is, in itself, quite complicated. Here again, the overall assumption is that no step in the procedure significantly affects the chemistry or biology of the DNA molecule (Table 12). It would require a skilled team of devoted research workers to examine the effect of every reagent and physical agent on these properties of DNA, and, of course, RNA. However, simpler approaches to the problem would be, either to add at the beginning graded quantities of DNA to the

preparation in which one was measuring it, thus using a recovery calibration, *or* to measure the DNA in the sample at every step of the procedure, *or* to take fresh DNA and measure it at every step of the procedure in Table 12.

Extraction of DNA from blood

Blood is drawn into an EDTA tube, mixed and stored at 4°C
Aliquots of 700 µl are frozen to -80°C in centrifuge tubes
Aliquots are thawed, and 800 µl of buffered saline citrate are added
The sample is centrifuged for 1 minute
The supernatant is discarded, and 1 ml of saline - citrate is added; it is centrifuged again for 1 minute, and the supernatant again discarded
To the pellet are added 375 µl of 0.2M sodium citrate, 25 µl of 10% sodium dodecyl sulphate and 5 µl of proteinase K (20 mg/ml of water)
It is vortexed for 1 second and incubated at 36° for 1 hour
120 µl of phenol/isoamyl-chloroform (1:24) are added, and it is vortexed for 30 seconds
The aqueous layer, containing the DNA, is removed and placed in a 1.5ml microcentrifuge tube
It is mixed with 1 ml of cold absolute ethanol
The tube is placed at -20°C, and the DNA precipitates
The tube is centrifuged for 2 minutes to sediment the DNA
The supernatant is decanted off carefully
180 µl of tris-HC1-EDTA are added; it is vortexed briefly
The mixture is incubated at 56°C for 10 minutes
20 µl of 2M sodium acetate are added, and it is mixed by hand
500 µl of cold absolute ethanol is added, and it is again mixed 'gently' to avoid damaging the DNA
It is centrifuged for 1 minute, and the supernatant decanted off
The pellet is rinsed at room temperature with 1 ml of 70% ethanol
It is centrifuged immediately for 1 minute and the supernatant decanted, without disturbing the DNA pellet
The tubes are centrifuged in a warm low-speed centrifuge for 10 minutes to remove excess ethanol
200 µl of tris-HC1-EDTA are added and it is incubated overnight at 56°C to dissolve the DNA
The mixture is vortexed for 10 seconds the next morning. It is ready for spectrophotometry to measure the quantity of DNA

Table 12. Steps in the isolation of DNA from blood samples (from Easteal, McLeod and Reed, 1991, pages 149-151), by kind permission of Harwood Academic Publishers, Elsevier. *This protocol is used by the Federal Bureau of Investigation.*

See also Marmur, 1961; 1963. The same principles are used for extracting DNA from tissue samples, tissue cultures, amniotic fluids, buccal cells, tooth pulp, muscle, bone, liver, kidney, cardiovascular tissue and skin (Kirby, 1990; Krawczak and Schmidtke, 1998). Do any of these reagents or agent affect the biochemical properties of the DNA?

Chapter 6

Control Experiments

It is a truism that an experiment is only as valid as the control experiments carried out in parallel with it. Most research workers assume that their supervisors, or the pioneers of the procedures which they are using, have done all the crucial controls, and they suffer serious intellectual crises, when they have to face the fact, that it is not always true.

There are several different kinds of control experiments. In most cases, controls in living intact animals are better than those carried out in isolated tissues or *in vitro*. The following controls are available.

(a) a *previous* control in a tissue, which can be examined, before and after an agent has been added, sometimes several times. This sort of control is often used by pharmacologists. Thus one is safer doing control and experimental observations on the same tissue sample, or individual animal, rather than on populations;

(b) control observations in *parallel* with the experimental, in which the agent is not added to exactly the same preparation;

(c) control experiments in which *ischaemia, hypoxia or absence of substrate* indicate when one is studying a biosynthetic, rather than a simply chemical reaction;

(d) controls represented by *fixed or boiled preparations*, with no likely biosynthetic properties but similar - if denatured - proteins to those in the living tissue;

(e) *temperature* controls in which a temperature gradient is used to decide whether a reaction is metabolic or chemical;

(f) '*specific*' *inhibitors* are widely used in the belief or hope that they are likely to block only one step in a metabolic pathway or cycle. Actinomycin is believed to block RNA synthesis, cyanide the respiratory cycle, and ouabain some of the Na^+ activated ATPase activity. Unfortunately, inhibitors have not been tested on several, many, or all, of the reactions in metabolic maps. However it would seem *highly* unlikely that such powerful reagents would only inhibit one of so many reactions. As the late Horace Davenport put it, in a personal communication, "The specificity of an inhibitor is inversely proportional to its familiarity." I would stress that I am not suggesting that there are no 'specific' inhibitors, rather that it would require an immense number of control experiments, (many not yet done) to establish

the specificity of an inhibitor unequivocally, and, secondly, that the inhibitors of one particular reaction in vitro may not be so specific in the whole intact animal. One should always bear in mind the multiple interconnections in metabolism (Table 13).

Interconnections of metabolic pathways and cycles

<i>Pathway or Cycle</i>	<i>Common Compound</i>	<i>Pathway or Cycle</i>
Glycolysis	Glucose 6-phosphate	
	Fructose 6-phosphate	Pentose phosphate
	Glyceraldehyde 3-phosphate	
Fatty acid synthesis	Acetyl coenzyme A	Citric acid cycle
Fatty acid synthesis	Acetyl coenzyme A	Glycolysis
Citric acid cycle	Acetyl coenzyme A	Glycolysis
β - oxidation	Acetyl coenzyme A	Citric acid cycle
Respiratory chain	Nicotinamide adenine dinucleotide	Citric acid cycle
Fatty acid synthesis	Acetyl coenzyme A	β - oxidation
Glutamate synthesis	NH ⁺ ₄	Purine nucleotide cycle
Purine nucleotide cycle	Fumarate	Citric acid cycle
Urea cycle	Glutamate	Citric acid cycle
Citric acid cycle	Acetyl coenzyme A	Cholesterol synthesis

Table 13. Some of the important compounds linking major pathways. The data comes from Greenberg, (1967-1975); Dixon, (1969); Salway, (1999). *Obviously a change in any enzyme, substrate, or activator, of one pathway or cycle, would rapidly have effects throughout metabolism.*

(g) when new drugs are tested, the control group usually consists either of patients treated with the *commonest* or *most effective* drugs against their disease to date, or a group of patients treated with a *placebo*. The patients are assigned to each group at random, and neither the patients receiving the drug, nor the research worker analysing it, know whether particular patients have been given the older, the new drug, or the placebo. This is the ‘double blind’ experiment. This prevents the interpretation of the success of the treatment from being influenced by the knowledge of which drug the patient was receiving. It is also possible to do a ‘triple blind’ study, in which neither the doctor administering the drug, the patient receiving it, or the person analysing the results, knows the expected result.

However, in normal biochemical experiments all the parties involved, the

experimenter, the technician and the supervisor, usually know the likely result of an experiment. Why should one believe that their expectations would not influence the result? Sheldrake, (1998, 1999) reviewed literature in the physics, biological and medical sciences, and compared it with that from parapsychology. His survey shows that single or double blind experiments were not reported at all in the physics journals he surveyed, but that they were reported in 0.8% of the biological journals, 24% of the clinical, 5% of the psychological and animal behaviour journals, and 85% of the parapsychology journals. He found that teaching of 'blind' methods of research was extremely rare in the 11 British universities from which he received replies. This rare use of such relatively objective techniques was due to the historic belief that physical, biological and medical, research workers do not allow their expectations to influence the results of their experiments;

(h) when lithium salts were first used in the treatment of manic depression (Schou, Juel-Nielsen, Strömgren and Voldby, 1954), the publication was attacked on the grounds that there were no control patients. The authors were concerned that it would be unethical to withhold what they believed to be an effective drug from patients. Therefore, they devised a new kind of control. All the patients were initially given lithium salts, and then, halfway through the clinical trial, the drug was stopped for a randomly selected half of them. The relapse rates of the two groups were then compared, and it was found that those treated with lithium salts had a much lower rate of relapse (Baastrup, Poulsen, Schou, et al, 1970).

Chapter 7

Subcellular Fractionation

Since the Second World War, this technique has been used by biochemists, pharmacologists and biophysicists, to break up a tissue into its subcellular components, and study each of their properties. The tissues are homogenised and centrifuged in non-biological media to separate layers believed to be enriched with, *inter al:* nuclei, nucleoli, mitochondria, submitochondrial particles, microsomes (cell membranes plus endoplasmic reticulum), lysosomes, synapses, and synaptic vesicles (De Duve et al, 1955; De Duve, 1963; 1965a; Whittaker, 1972; De Duve and Beaufay, 1981). The overall assumption behind the use of these techniques is that the procedure does not alter the properties of the organelles, from their conditions *in vivo* in the intact animal to the subcellular fraction

It is useful to list the steps of the procedure widely and routinely used to produce fractions (Tables 14-17). Unfortunately, when they purport to describe the chemistry of nuclei, mitochondria or cell membranes, they are actually talking about the properties of the nuclear, mitochondrial or microsomal *fractions*. Thus, if there are significant changes, the following beliefs may be untrue: that: oxidative phosphorylation occurs in the mitochondria; DNA is housed mainly in the nuclei, but a little is also in the mitochondria (Cummings et al, 1979); the receptors are mostly located in, or near, the cell membranes; the lysosomes are the seats of acid hydrolysis of proteins; ribosomes are the location of protein synthesis in cells.

Preparation of a mitochondrial fraction

<i>Step</i>	<i>Comment</i>
The animal is killed	there are agonal changes in biochemistry
Liver, brain, heart or muscle is minced in a pre-chilled meat grinder	homogenisation redistributes tissue components
The tissue is diluted with double the volume of ice-cold 0.25 M sucrose + 0.01 M tris-HCl	sucrose inhibits enzymes (Hinton, Burge and Hartman, 1969)
The sucrose is squeezed out	sucrose effects may not be reversible
The mince is then diluted again with double the volume of sucrose tris-HCl	

Step	Comment
The tissue is homogenised for the second time rapidly with a motorised pestle for 10 seconds <i>or</i> in a blender for 15 seconds, and then 5 seconds	the homogenising tube is surrounded by ice in the expectation that this will prevent heating in the tissue during homogenisation. The cold <i>increases</i> the viscosity of the preparation, therefore increases the heat generated. It is hoped that heat will be dissipated more rapidly due to the larger temperature gradient between the homogenate and the cold outside of the homogenising tube
The pH is adjusted to 7.8 with tris buffer The homogenate is centrifuged for 20 minutes at 1,200 g (24,000 g. min), which produces a pellet	The pH must have changed the pellet is believed to be composed of unfragmented cells and nuclei
The supernatant is decanted and filtered through cheese cloth to remove lipids	
The pH is adjusted with 2M tris to 7.8	the pH has changed during homogenisation
The suspension is centrifuged for 15 minutes at 26,000 g (390,000 g.min) producing a light upper layer, and a heavier lower layer	this is believed to consist of 'damaged' mitochondria as judged by appearance by electron microscopy. The lower layer is believed to consist of intact mitochondria. Centrifugation generates friction, pressure and rise in temperature in the homogenate
The upper layer is discarded	one does not know what proportion by weight, volume or enzyme activity, of the original mitochondria is discarded in the fraction of 'damaged' mitochondria
To the heavy fraction, 10 ml of sucrose-tris are added and it is stirred	
The mitochondrial fraction is homogenised in a motorised glass Teflon homogeniser tube for 5 seconds each	the rationale for this step is not clear, and its use implies again that homogenisation does not affect the chemistry of the homogenate
The pH is adjusted to 7.8 with tris which dilutes it to a total volume of 130 mls	The pH must have changed
The suspension is centrifuged for 15 minutes at 26,000 g (390,000 g.min)	this produces the same effect as the previous centrifugation. The rationale for this is presumably to purify the fraction.
The fraction is suspended in 60 ml of sucrose-tris and adjusted to pH 18 with 2M tris	the necessity to adjust the pH shows that the procedure changes the pH
The fraction is stored in ice	to prevent diminution of enzyme activity
Sodium desoxycholate is added to disrupt the mitochondria and release the protein to measure it	this assumes that this reagent has no effect on the protein content or recovery of the fraction
Bovine serum albumen is used for calibration of all fractions	

The protein content is determined by the biuret method

Table 14. The fractionation has many effects which have not been recognised. The total centrifugation is 804,000 g.min. This Table is reproduced from Boyer (1993) by kind permission of Benjamin Cummings Publishing Company.

Preparation of a fraction of submitochondrial particles

<i>Step</i>	<i>Comment</i>
The mitochondrial fraction is first prepared (Table 14)	the effects of mitochondrial preparation must always be added to the preparation of submitochondrial particles
The mitochondrial fraction is diluted to give 10 ml of 15 mg of protein/ml	
It is cooled	
It is sonicated at maximum energy for 6 periods of 5 seconds each, with 30 seconds between each period to cool. The temperature outside the tube is not allowed to rise more than above 10°C during sonication	please see the effects of cooling in Table 14. Cooling <i>increases</i> the heat generated but it is hoped that it prevents temperature rise in the fraction
Ice cold buffer is added.	
It is centrifuged for 10 minutes at 10,000 g (100,000 g.min) with the centrifuge temperature set at 5°	this 5°C is not necessarily the temperature within the homogenate
The supernatant is centrifuged 3 times each for 20 minutes at 100,000g (6,000,000 g min)	again it is assumed that this massive and prolonged centrifugation has no significant effect on the chemistry of the submitochondrial particles. These measurements also imply the assumption that neither the isolation of the mitochondrial fraction nor the submitochondrial particles has any significant effect on the recovery, activity, distribution or naturation of enzymes.
Enzymes, such as malate dehydrogenase and monoamine oxidase are measured	Enzymes are particularly sensitive to temperature rise

Table 15. The fractionation procedure followed is that of Boyer, (1993) by kind permission of Benjamin Cummings Publishing Company. *The subcellular fractionation of mitochondria (Table 14) required 804,000 g.min, and of the submitochondrial particles a further 6,100,000 g.min.*

Preparation of a fraction enriched with nuclei from the liver

Animal is killed

The blood is washed out of the liver with 20 ml of 150 mM NaCl, then 20 ml of 250 mM sucrose

The liver is forced through a press

Sucrose (0.25M, 2M or 2.4M) plus Ca^{2+} (0.5 mM to 10 mM) is added, 10-15 ml/g tissue

The tissue is cooled and homogenised with 3 up and down strokes in a Teflon homogeniser with a clearance of 0.15 mm to 0.25 mm

The cooled homogenate is passed through 8 layers of gauze and a 90-mesh stainless steel wire screen

The homogenate is centrifuged at 40,000 g for 60 minutes

Each g of pellet is suspended in 1 ml of 1M sucrose containing 1 mM Ca^{2+}

The sample is again homogenised in a Teflon homogeniser at 500 r.p.m. with a clearance of 0.25 to 0.30 mm

The homogenate is centrifuged 3,000 g for 5 minutes

The precipitate is the fraction of enriched nuclei

Table 16. The generalised procedure for preparing a fraction enriched with nuclei, in which nuclei can be seen by phase contrast microscopy. The technique was described by Hogeboom, Schneider and Streibich, (1952), and Chaveau, Moule and Rouiller, (1956). The DNA, RNA and protein ratios, per nucleus were 11,3 and 44 respectively (Busch and Smetana, 1970). There are many variations of these procedures, see, for example, Roodyn, (1972), Kay, Frazer and Johnston, (1972), Franke and Scheer, (1974), Franke, (1974), Kasper, (1974) and Rickwood, Messent and Patel, (1997). The purity of the fraction is measured by the numerical proportion of nuclei, relative to other particles identified and counted.

Preparation of nucleolar enriched fraction

A nuclear fraction is prepared

It is sonicated with a sonifier for 15-120 seconds in ice

The sonicate in a volume of 20 ml is layered over 20-25 ml of 0.25 M sucrose

It is centrifuged at 2,000 g for 20 minutes in a swing out bucket

The sediment of nucleoli is resuspended in 0.25 M sucrose, and centrifuged for a few minutes

The fraction is examined by light and electron microscopy

The appearances and ratios of nucleoli to other particles are examined

The DNA, RNA and protein, concentrations are measured

Table 17. This generalised procedure is described by Busch and Smetana, (1970), pages 532-537.

Subcellular fractions, like tissue slices can be studied using manometric methods (Dixon, 1952; Umbreit, Burris and Stauffer, 1972).

A mitochondrial fraction may be any of the following:

(a) a fraction entirely composed of only mitochondria. This means that few mitochondria have been broken, made unrecognisable, diffused away, or have had their chemical properties changed significantly, during the separation procedure (Tables 14-17). There is no way of telling whether or not these assumptions are true, but they apply to any 'enriched' fraction.

(b) the majority of the structures which can be seen in this fraction by light or electron microscopy, are mitochondria. This implies that one can identify the numerical majority of the particles one can see, while other particles do not contribute to the chemical properties of that fraction. It also implies that the 'purification' of the fraction, by increasing the numerical proportion of the mitochondria in it, does not affect the total activity of the enzyme believed to be located there;

(c) the chemical activities of the 'markers' of a particular organelle - say succinic dehydrogenase for, mitochondria - are a measure of the 'purity' of the fraction. However, it is only an *assumption* that the latter enzyme is a marker, because it is found in the fraction at the end. It is a circular argument to say that the purity of a fraction is measured by the activity of the 'marker' and a high marker activity proves that the fraction is pure. So far, it has always been accepted that there are biochemical markers for different organelles, which seems to be unproved and possibly unprovable. However, it is generally agreed that many classical 'lysosomal' enzymes have also been found in other fractions (Tappel, 1969, pages 209-215).

(d) The organelles in the fraction are not incubated in a similar environment to that which surrounds them in intact cells in the living animal, which means that they will behave differently, (Table 18). It is not at all surprising that fractions possessing allegedly different chemical properties - or even originally present in a uniform compartment - when subjected to a different army of agents and reagents, would exhibit different properties.

Different environment and physical conditions of the mitochondria in vivo and in vitro

<i>Cause</i>	<i>Changes occurring in vitro compared with in vivo</i>
Agonal changes	O ₂ down, CO ₂ up, Na ⁺ up, K ⁺ down, Ca ²⁺ up, H ⁺ up
Homogenisation	Chapter 15
Centrifugation	Chapter 15
Incubation and measurement of enzyme activity	in entirely different chemical environments
Washes and re-suspensions	dilution of all soluble compounds in the original cytoplasm
Protein calibrations	different for whole tissue and particular subcellular fraction

Table 18. Similar changes occur during preparation of all subcellular fractions.

An experiment is considered satisfactory if 70% to 130% of the enzyme activity of the crude homogenate is recovered in the total added activities of all the subcellular fractions. This ignores the effects of the killing and the homogenisation on the enzyme activities of the original tissue. More seriously, the fact that the total enzyme activity recovered from all the fractions is 100%, does not mean that the enzymes, the substrates, the activators or inhibitors, have not relocated during the procedure. When recoveries of enzyme activities relative to those of the crude homogenates are low, some biochemists call the total recovered activity of the fractions 100%, and express the activity of a particular fraction as a percentage of that 100%. Nørgaard, (1986) found that only 0.2% to 3.6% of ATPase was recovered.

In subcellular fractions, light and electron microscopy are used to decide which is the predominant organelle in a particular fraction. Nuclei, nucleoli and mitochondria can be identified fairly easily, but ribosomes are largely amorphous in fractions. However, membranes and the reticulum appear trilaminar and fairly straight, while microsomes all appear circular or oval (Figure 1). Although lysosomes are seen to be of similar dimensions to nuclei or mitochondria, they are not seen in unstained cells by light microscopy; they are generally seen in stained tissues next to artificial materials, which they are believed to take up. Although vacuoles appear in unstained aquatic marine protozoa and in tissues in cultures, they are hardly ever seen in unfixed mammalian tissues. Claims have been made that one can make a subcellular fraction of vacuoles, (De Duve and Wattiaux, 1966; Gordon, 1973). These and many biologists regard a vacuole - which appears to be as a clear translucent sphere - to be identical with a lysosome - which appears to be amorphous solid

granule. Liposomes exist outside fat cells only in oil droplets. However, neither vacuoles nor liposomes can be studied by any light or electron microscope technique, which involves dehydration and extraction of lipids. Vacuoles increase in number when a tissue culture is dying.

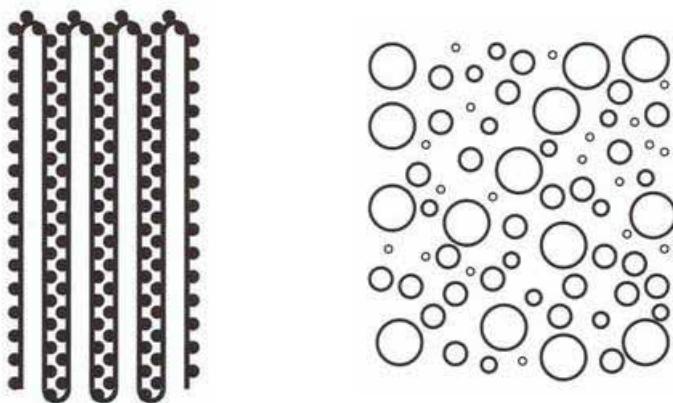


Figure 1. Diagrams of *left*, the endoplasmic reticulum with adherent ribosomes, *right*, the microsomal fraction. It is widely taught that the former appearance represents the same structure as the latter.

The force and duration of the centrifugation, as well as the chemical reagents necessary to separate a particular fraction, are arrived at empirically, as is the recipe for a Black Forest gateau. However, it is reasonable to suppose that the amount of energy dissipated during the centrifugation, is a function of the energy injected into the system to separate the fractions, because, of course, the biochemists go on centrifuging the tissue until they obtain what they regard as a satisfactory separation. One rather crude measure of the energy injected into the fractions is the g force multiplied by the time, t , for which that force is applied. It can be seen that the gt used to separate different fractions varies from 5 - 120,000 thousands (Table 19). Thus, there are enormous differences in the energies used to separate the fractions; such energy may activate enzymes, change equilibria and denature proteins.

The centrifugal force and the time to which some fractions are subjected

<i>Fraction</i>	<i>g x t minutes (thousands)</i>
Nuclear	5 - 6
Mitochondrial	400 - 800
Submitochondrial particles	13,000 - 14,000
Nucleolar (rat liver)	80 - 90
Microsomal	5,000 - 10,000
Peroxisomal	10,000 - 12,000
Lysosomal	12,000 - 13,000
Ribosomal (rat liver)	70,000 - 100,000
80m ribosomes (rat liver)	110,000 - 120,000
Cytosol (rat liver)	4,000 - 5,000
Synaptosomal	6,210 - 7,020
Myelin	6,000 - 7,000

Table 19. These values are calculated from data in Rodriguez de Lores-Arnaiz, Alberici and de Robertis, (1967), Whittaker, (1984) and Graham and Rickwood, (1997).

Unfortunately, the use of *g* forces to designate the conditions of a particular centrifugation obscure an important approximation. The *g* refers to the whole centrifuge tube. However, the *g* force is very different at the top of the tube from that at the bottom of it, because the *g* depends upon the radius of rotation, which, itself, depends upon the *angle* of the tube during centrifugation. In many preparations the *g* force at the top, which is subjected to the minimal force, may be less than half of that at the bottom of the tube, which is much farther from the axis of rotation. Knowing the force applied, the time to maximum speed, the duration of centrifugation, the time to stop, one may be able to calculate the energy *injected* into the centrifuge tube. However, ignorance about: the exact *g* force; the viscosity in different parts of the homogenate, and the heat capacity and conductivity of each of the parts of the homogenate, the plastic homogenising tube and the vacuum around the tube and the rotor, means that *in the real conditions* of the experiment, it is virtually impossible to know the energy dissipated or the temperature rise in the preparation of any particular fraction.

Receptors are believed to be regions which have particular affinities for ligands, transmitters, hormones, etc. The study of affinities or bonds in subcellular

fractions implies that the whole preparation procedure does not alter the affinities of these substances. This likelihood does not seem to have been addressed experimentally.

Some years ago, the assumptions inherent in subcellular fractionation were listed (Hillman, 1972, pages 33-34). These can be examined, bearing in mind that the validity of an experiment - like a chain - depends on the weakest link. If any important assumptions are unwarranted, the validity of the whole experiment is threatened. The assumptions implied by the use of the procedure are as follows (Table 20)

Assumptions inherent in subcellular fractionation

- | | |
|--------|--|
| (i) | the stress of handling and killing the animal has no effect on the results of the experiment (t); |
| (ii) | the agonal changes have no significant effect; |
| (iii) | post-mortem changes have no significant effect (t); |
| (iv) | cooling to room temperature, 0°, -25° or -196°C has no significant irreversible effects (t); |
| (v) | the enzyme activity of a homogenate decreases linearly with dilution (t); |
| (vi) | the medium in which the tissue is homogenised, containing, for example, sucrose, edta, detergent or bile salts, does not alter the chemical activity significantly and irreversibly (t); |
| (vii) | the enzyme activity measured finally is not changed significantly by the incomplete replacement of soluble constituents of the tissue which are lost on gross dilution, homogenisation and centrifugation in a quite different chemical environment (t); |
| (viii) | movement during preparation of known co-factors, such as cations, and co-enzymes, or unknown ones, will not alter substantially the apparent localisation of enzyme activity as measured (pc); |
| (ix) | soluble materials originating from any compartment <i>in vivo</i> will not diffuse into the supernatant or become bound to another fraction during preparation, and thus be supposed to have originated in the location where they are found (pc); |
| (x) | no step in the preparation or lytic enzymes will render substances which were slightly soluble <i>in vivo</i> more soluble, and thus more diffusible, which may change their affinity for the different fractions (t); |
| (xi) | the heat necessarily generated during the homogenisation is so rapidly conducted away that the temperature does not rise sufficiently high to change enzyme activity irreversibly (t); |
| (xii) | refrigerating the centrifuge diminishes temperature rise at the surface of particles being homogenised (pc); |
| (xiii) | enzyme activities are not irreversibly changed by pressure during homogenisation and centrifugation (t); |
| (xiv) | the same amount of work is done on each different part of a centrifuge tube (pc); |
| (xv) | the same amount of heat is generated in different layers of the homogenate in media which have different viscosities (pc); |
| (xvi) | the extractions from each of the final fractions are equal and complete (t); |

- (xvii) a recovery of enzyme activity in all the subcellular fractions, added together of 60% to 130% of that in the initial crude homogenate, implies that the enzyme has not been relocated (pc);
- (xviii) the enzyme activity in the unphysiological substrate mixture is approximately similar to that in the chemical environments *in vivo* (t);
- (xix) the similarity in appearance on electron microscopy of, for example, a mitochondrion in a section to that in a mitochondrial fraction is evidence that its biochemical properties have not changed during fractionation;
- (xx) the microsomal fraction consists mainly of cell membranes and endoplasmic reticulum;
- (xxi) the apparently high but unquantifiable incidence of a particular identifiable organelle in a particular fraction as seen by electron microscopy is evidence that the biochemical properties of that fraction are dominated by that organelle;
- (xxii) when the enzyme activity is referred to the protein content of a subcellular fraction, the protein can be measured satisfactorily (t);
- (xxiii) the calibration method for protein in one subcellular fraction is applicable to other fractions, unless this has been tested for specifically (t);

Table 20. *Most of these assumptions are testable (t). Some of them are contrary to the laws of physical chemistry (pc). Particular ones can only be ignored, if it has been demonstrated that failure to recognise them makes no significant difference to the results of the experiments.*

There is plenty of experimental evidence in the literature that assumptions (i), and (iii) - (vii) are untrue (Hillman, 1972 pages 1 - 33). Assumptions (viii), (ix), (xii), (xiv), (xv), (xvii) are contrary to the laws of physical chemistry. Most of the others could be, can be, and should be, tested experimentally. In view of the fact that it has often been said that there is no way of controlling the procedure, a list is given here of the sort of standard experiments which could be carried out to answer the question, 'How much does the biochemistry of the fraction reflect that in the living cell in the intact animal?' (Table 21). It may well be that the uncertainties are so great that the technique can not be used to study the subcellular localisation of the chemical constituents or activities in cells. However, despite frequent assertions that the relevant control experiments have never been carried (Hillman, 1972, page 110; 1979; 1991) the procedure has been used and the results of experiments have been interpreted for 60 years. This represents an invitation for all biochemists to carry out long overdue controls. An enormous edifice has been built up on what may well be unsure foundations. Should not the builders and the occupants be deeply concerned?

However, if it should turn out that this high energy procedure is unsuitable for localising tissue constituents and chemical activities in the cell, there is a range of other less energetic and less disruptive techniques, which can be used (Table 3).

A new approach to the problem of subcellular localisation was conceived. When one looks at unfixed unstained cells down the microscope, one sees two natural 'fractions'; all the structures one can see are obviously insoluble; the translucent fluids are either solutions, or suspensions of particles. The former was designated 'the insoluble fraction' and the latter 'the soluble (and suspended) fraction', respectively. The following procedure was used to separate them (Ahmed and Hillman, 1982). Ox brain was obtained from an abattoir, soon after the animals has been slaughtered. To each 3 g of brain 10 ml of distilled water was added. The samples were homogenised by hand at room temperature in a Potter - Elvehjem homogeniser. They were then dialysed in a Feinstein dialyser for 12 hours against 20 - 24 l of double distilled water. The dialysed samples were filtered through normal filter paper and then dried at room temperature with a hair dryer; during this time, they occasionally had to be stirred to prevent a crust forming on them, which would prevent the deeper layers drying. The resultant dry powder was designated the 'insoluble fraction'. The affinities of Na^+ , K^+ , Ca^{2+} and Mg^{2+} for the fraction were tested, and the effects of oxygen, glucose and the other ions in the physiological range of concentrations, were also examined. This insoluble fraction was devoid of any water soluble materials, and contained all the cell membranes. It was originally intended to concentrate the soluble material, which had diffused out of the dialysis bag, down to the approximate volume of the cytoplasm, that is from 20l to about 80ml, and study this fraction to represent it. However, it was found that when double distilled water plus dialysate was concentrated so considerably, it attracted dust, fungi and bacteria; furthermore unmeasureably low levels of ions are concentrated to measurable levels. In other words, it was not practicable to prepare a fraction containing only cellular fluids.

It has often been suggested that the reason for which inadequate controls for subcellular fractionation - other than 'recovery' (please see page 34) - have been carried out so far, is that no relevant experiments are obvious. A list of such possible experiments is given (Table 21).

Control experiments for subcellular fractionation

<i>Step</i>	<i>Reason</i>
Measure enzyme activities in tissues during each step of procedure	to find out which steps change the activity most, to devise procedures to limit enzyme changes, or account for these changes
Put the final fraction all the way through the procedure again	to see how the whole procedure has changed the activity of the enzyme
Use a boiled control, shown to have no enzyme activity	to find out how much of the breakdown of substrate is due to the non-enzyme activity; to measure the enzyme activity and its distribution at each stage
Put a pure enzyme system through subcellular fractionation	to measure the enzyme activity and distribution at each stage
Homogenise an enzyme preparation 6 times, and measure the loss of activity and extrapolate back to no homogenisation	to find out how homogenisation affects enzyme activity
Centrifuge an enzyme preparation at different g - forces and measure loss of activity and calculate its effect	to calculate how centrifugation effects enzyme activity
Take an enzyme and substrate in pure solution with the same particles of different size	to see how the activities are distributed to particles of different size
Mix enzymes with milk, or solutions of gelatin or albumin, and measure the enzymes in each fraction	to examine how uniformly distributed enzyme activities change during separation of different fractions
Add substrate to the enzymes, at the beginning and at the end of the fractionation	to carry out recovery experiments for enzyme activity and protein concentration
Assess the effects of each agent and manoeuvre used on the enzymes being studied	to see how such agents affect the enzyme activities
Inject radioactive phosphate, and use autoradiography to find out if it is taken up into mitochondria (seen by confocal microscopy) in living cells	to see if oxidative phosphorylation occurs in mitochondria in living cells

Table 21. *Experiments involving subcellular fractionation should not be considered complete, and should not be interpreted, until comprehensive control experiments have been carried out.*

In view of the possible reasons for which an enzyme born and bred in one organelle might appear in an ‘enriched’ fraction of another, it is useful to summarise some of the possible reasons why an enzyme may be found in a fraction (Table 22).

**Reasons for which an enzyme activity may be found in a subcellular fraction, or
a microscopical site**

- It was located in that organelle originally
- It has moved to that site during preparation
- The substrate or co-factors moved there during preparation
- Inhibitors moved away during preparation
- Chemicals added activated enzymes in the final fraction
- Heat generated during the preparation activated the enzyme, or denatured inhibitors
- Protein may have diffused from the site
- The incubation medium of the fraction or tissue section activated the enzyme
- Dehydration of a particular organelle altered its apparent activity

Table 22. *Each of these possibilities should be explored.*

Subcellular fractionation and histochemical localisation are both carried out in view of the belief in ‘compartmentation’ - that is, that each organelle and phase enjoys the uniqueness of its own role in the biochemical drama.

Chapter 8
Histology and Histochemistry

These microscopical techniques are used to examine the shapes, dimensions and chemistry of cells. They involve fixation and staining, and should be contrasted with methods of study of unfixed unstained cells, (Chapter 11). An important distinction must be made between histology and histopathology, used to diagnose disease, on the one hand, and biological histology, histochemistry, electron microscopy and immunocytochemistry, which are used to attempt to elucidate the properties of living cells, on the other hand (Hillman, 2000). The former is a correlation between the appearance after histological preparation of tissue, from an organ of a healthy animal or patient, with the appearance of the same organ from an animal or patient, which or who has a disease or syndrome, independently diagnosed by a veterinary surgeon or a physician. The latter knows well that in life nuclei are not violet and cytoplasm is not pink, but the pathologist knows the appearance of the tissue after all the artifacts of haematoxylin and eosin staining, both from a healthy organ, and an organ inflicted with disease. However, if one wishes to describe the properties of living cells as accurately as one can, it essential to examine the effects of the staining procedures on the cells. For reviews, see Mann, (1902), Adams, (1965), Pearse, (1968), Bancroft and Stevens, (1982).

For example, one may analyse the effect on the tissue of the most popular procedure in the world, haematoxylin and eosin, bearing in mind that the same steps, fixation, dehydration, mounting, sectioning, etc. are common to most staining procedures (Table 23).

Staining with haematoxylin and eosin

<i>Step</i>	<i>Chemical Effects</i>	<i>Other effects</i>
Animal is killed, biopsy is taken, or patient dies	ischaemia; hypoxia; collapse of homeostasis	blood pressure falls; tissue cools
Tissue is excised	proteins denature; electrochemical gradients diminish	organ loses its blood supply
Tissue is fixed in formalin	metabolism stops; tissue is diluted; tissue exchanges with fixative; enzymes are inhibited	tissue becomes opaque
Dehydration	water leaves tissue; solutes precipitate; osmotic effects; water-soluble and ethanol-soluble substances extracted	solutes crystallize; tissue shrinks
Ethanol is extracted with xylol	further fat extraction; osmotic effects	tissue shrink
Embedding in wax	wax extracts xylol	tissue becomes opaque
Sectioning	heating by microtome knife	tissue is compressed; it is sheared; cells and processes are cut
Section floated on to slide	unfolds section	
Wax extracted with xylol	further lipid extraction	removes air bubbles
Xylol extracted with ethanol	further lipid extraction	
Rehydrated with gradually weaker ethanol	water re-enters tissue; aqueous solutes extracted	
Section stained with haematoxylin	unnatural agents added	colours different parts of tissue
Differentiation with acid-ethanol	acidifies tissue	colour contrast increases
Washing	removes acid-ethanol	
Counter-stain with eosin	unnatural agent added	enables tissue to fluoresce
Dehydration with ethanol	as previous dehydration	as previous dehydration increases section transparency
Clearing with xylol	substitutes xylol for ethanol	section is subjected to pressure
Mounted in DPX		
Examined under the microscope	photochemical effects; section heated; bleaching	photodynamic effects
Photography	as above	

Table 23. Similar analyses could and should be made for any histological procedure.

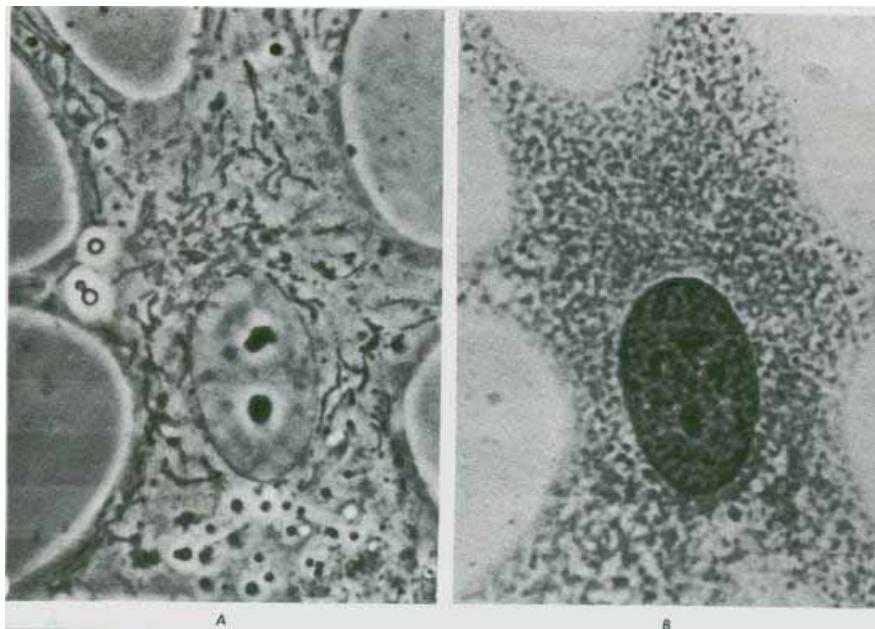


Figure 2. *A.* Dark phase contrast photomicrograph of a living fibroblast from a cell culture. Threadlike mitochondria and spherical lipid droplets are clearly visible in the cytoplasm. The cytoplasm matrix shows little structure and the nucleoplasm is homogeneous except for two prominent nucleoli and a few small karyosomes. *B.* The same cell after fixation in alcohol and staining. The fixation has produced a coarse granular precipitation of the proteins of the cytoplasm and nucleus, and the organelles are no longer visible. This illustration comes from Bloom W and Fawcett DW, (1968), page 8, by kind permission of Elsevier Ltd.

Fixation is intended to stop structural changes, diffusion of solutes and particles, and bacterial invasion of tissue. It itself causes denaturation of proteins, inhibition of enzymes, precipitation of the cytoplasm and nucleoplasm, and cessation of intracellular movements until bacterial invasion liquefies the tissue.

Fixation alone inhibits many enzymes, Seligman, Chauncey and Nachlas, (1951). Janigan, (1964) examined the effects of 4 - 10% concentrations of several aldehydes on alkaline phosphatase, acid phosphatase, esterase, sulphatase, β -glucuronidase, β -galactosidase, β -glucosidase and N-acetyl- β -glucosaminidase enzyme activities. The enzymes activities remaining varied between 27% and 91%. They were also dependant upon the temperature at which the tissues were fixed. Bancroft, (1982) noted that "fixation destroys many of the oxidative enzymes; there

are few exceptions to this." Please see also Arborgh, Ericsson and Helminem, (1971), Stoward, (1973) and Boon and Drijver, (1986).

Barka and Anderson, (1963) and Hopwood, (1969) listed the main factors that inhibit enzyme activities in histochemical procedures, as follows: loss of chemical constituents during storage; fixation; dehydration; embedding; cutting and handling; loss of co-factors; non-optimal substrates; poor solubility of substrates, inhibition of enzymes by diazonium, lead and calcium salts; heating; incubation; insolubility of precipitates; composition of substrates; loss of soluble products to the reagents in any step of the histology or incubation. In addition to the inhibition of enzyme activities, the following factors cause redistribution of water, ions, aminoacids, fatty acids and metabolic intermediaries: washing; filtration; fixation; freezing; dehydration, heating; embedding; addition of non-isotonic reagents, (Pool et al, 1983).

Fixation precipitates proteins in the nucleoplasm and cytoplasm. The latter is well illustrated in a fibroblast in tissue culture, from Bloom and Fawcett, (1968), shown here (Figure 2). Precipitation of both the cytoplasm and the nucleoplasm makes their contents largely unrecognisable. Intracellular movement is stopped. Although precipitation is likely to inhibit intra-cellular movements of particles, it can only stop *diffusion* of soluble substances, if the whole tissue is frozen to absolute zero, or if it becomes solid, or if solid substances attach to insoluble membranes and macromolecules (see also Wollman, 1955). Fixatives sterilize tissue, and prevent bacteria growing. They also produce artifacts (Kuthy and Csapo, 1976).

Although dehydration shrinks tissue (Kushida, 1962) it is usually thought of as removing water only, it also removes substances soluble in water and ethanol, and, possibly fine particles beyond the resolution of the light microscope. It is used twice during the staining procedure. All the cell membranes are believed to consist of 24-79% lipids (Guidotti, 1972; Eisenman, 1975-1978), and nearly all the lipids are extracted during preparation for electron microscopy (Cope and Williams, 1967), yet electron micrographs show the 'unit membrane' which is believed to shows the lipid layers of Davson and Danielli.

It is widely believed that the embedding medium or the mountant restores the volumes of the cell components to their original volumes in the unfixed state, but this is not so. Staining with haematoxylin and eosin, Palmgren's, or osmic acid, shrunk the areas of the neuron cells bodies to 20%, 19% and 14% of their original respective areas, (Chughtai, Hillman and Jarman, 1987) (Figure 45).

The facts that the tissue shrinks and that sections are cut, mean that in a stained section one can not measure the dimensions of any part of cells, and assume that the measurement reflects the dimensions in the whole intact tissue. Serial sections are cut from shrunken tissue, in which all the organelles do not shrink to the same extent. By applying mathematics to stained serial sections, one can work out the range of dimensions in the shrunken tissue, but this does not help one to know them in the intact animal.

One aspect of staining, which has been rather neglected, is that each of the reagents, - in volumes many times larger than those of the sections, draws out and dilutes soluble tissue constituents. Therefore, it seems highly unlikely that the original concentrations of many soluble constituents survive in stained sections. This is not important in clinical histology, but could be of considerable significance in histochemistry, autoradiography and immunocytochemistry, when one is attempting to carry out quantitative studies on cells and their organelles.

There are two kinds of stains, those which colour the cells, for instance, eosin, methyl violet or malachite green, and those which are deposits of silver, osmium, or lead, salts (Baker, 1948a). The former are *not particulate* and stain a whole organelle, while the latter are *deposits* on particular parts of cells or kinds of cells. The salts of heavy metals come out of solution when the sections are dehydrated or bombarded by electrons. These salts are used mainly in electron microscopy, and they deposit on organelles for which they have affinity. Any part of a cell, which has no affinity for the heavy metal salt used will not show up in an electron micrograph. Their poor solubility makes them come out of solution without much provocation, and they may form precipitates, especially in the cytoplasm (Figure 2). They also adhere to insoluble cell membranes.

The intensity of the staining as seen under the microscope, or photographed, depends upon many variables, in addition to the concentration of the substance in the cell or organelle. One has no way of knowing how much of the intensity of staining is due to the concentration or activity of the particle or substance under study (Table 24).

Factors contributing to assessment of histological staining

- Treatment of animal during killing
- Beer-Lambert's Law does not apply
- Chemistry of tissue at the time of death
- Particular tissue
- Age of animal
- How soon the tissue was fixed during life or after death
- Chemistry of fixative
- Chemistry of dehydrating agent
- Affinity of a particular organelle or cell type for the stain
- Degree of differentiation
- Section thickness
- Embedding agent
- Distance of the cell from the surface of the section
- Number and order of reagents, including water
- Duration of application of all reagents
- Chemistry of all the reagents used
- Illumination system under which they are examined
- Selection of field
- Skill of the histologist
- Bias of the histopathologist due to clinical knowledge
- Previous expectation of the histological appearance
- Statistical considerations

Table 24. *Many of these factors are subjective.*

In a normal histological preparation, 5-15 powerful reagents and agents are imposed on a tissue. Little interest has been shown in what they do to the cells, although there is plenty of evidence that they cause denaturation, dehydration, extraction, dilution, freezing, heating, osmosis, diffusion, compression, etc. Although these effects occur both in the control and experimental sections, the possibility exists that the powerful reagents and manipulations obscure the subtle differences in chemistry between a healthy and an unhealthy tissue. Jobbing histologists and electron microscopists see many shapes, which they label as 'granules', 'vesicles', 'bodies', 'apparatuses', 'vesicle-like bodies', 'particles', 'specialisations', 'heterophagic vacuoles', 'ribosomes', etc, whose chemical nature in sections they have no way of knowing. Histologists show profiles of what has been left in the tissue after all the manipulations, not what was there at the beginning. The variety of apparent structures forces the histopathologists and electron microscopists to *describe* the structures they see empirically, and they talk about 'cell necrosis', 'sloughing',

'degeneration', gliosis', 'liquefaction', 'apoptosis', without being able to define their *chemical* natures.

Some cells, such as those in striped muscle, myometrium or kidney tubules, are orientated. Like nuclei and nucleoli, they have relatively simple shapes. If one sees a large number of them in the several orientations, one can know their shapes. However, when one looks at a *single section* of a cell, one can not know its dimensions, shape, presence of an organelle, structure of an organelle, distance between organelles, number of organelles, presence of a vacuole, size of the vacuole, number of processes, length of processes, depth of staining, number of synapses, diameter of synapses, etc. (Hillman, 1986a, page 51). In the Frontispiece of 'Fine Structure of the Nervous System,' Peters, Palay and Webster, (1998) have a diagram illustrating the shapes of neurons, astrocytes and oligodendrocytes. It has not been appreciated that if they constructed solid models of each of these allegedly different kinds of cells, and cut sections of them in other orientations, (analogous to a histological section), the appearance of each of them would be indistinguishable. Staining of cerebral slices showed that whole slices shrunk to a different extent than the neuron cell bodies or nuclei within them (Hillman and Deutsch, 1978). This is, of course, because the slice contains cells in addition to neurons and extracellular fluid, and is altered by cutting.

Both the experiments in which staining reagents were added to single cells, and to cerebral slices, showed that - contrary to what is widely believed, - the shrinkage of cells and organelles during dehydration is not reversed by the embedding medium or the mountant. The cells remain smaller (*ibid*).

Histologists have attempted to prevent shrinkage by embedding tissues in water-soluble carbowaxes, which do not require dehydration. We attempted to make up a single histological reagent, consisting of a fixative and a polychrome stain in carbowax, with the intention that sections could be made of it (unpublished). However, during the course of the experiments, we found that although tissues could be saturated with the carbowax, they would not *harden* until water evaporated from the preparation. Thus, we did not succeed in embedding them without dehydration.

Theoretically, one could measure or calculate the dimensions of a cell or organelle in a histological section, if one knew: (a) the changes in volume and projected area of the parts of interest, as a result of the staining procedure. Unfortunately, membranes are beyond the resolution of the light microscope; metal is

deposited on them, and they shrink under electron microscopic preparation since they contain water, so that their thickness during preparation can not be monitored; (b) the real thickness of the sections after microtomy. They are compressed by the microtome and expand when placed in water; (c) that the objects at which one was looking were approximately symmetrical in three dimensions, as are, for example, nuclei and nucleoli; (d) one had sufficient measurements on a particular organelle to analyse them statistically; (e) that the dimensions of the cells and organelles were not distorted by staining and mounting.

Whereas in most sciences, statistical methods are used to indicate the significance of differences between experiments and controls, this is not the case in histology or electron microscopy. In clinical histology, it is usually assumed that changes seen in the tissue are pathognomonic, because they occur in every sample, but it is the practice in biological histology to *choose* the fields to demonstrate a feature, and dub it 'typical'. If a Martian were to look down a microscope at, for example, a sample of albumen, an area of necrosis, or a piece of male bovine faeces, she would see a wealth of unidentifiable granules, particles, vesicles, fibrils, etc. From this, the histologist deliberately chooses a part of the field, either in which the structure under question is clear, or it illustrates the point he wishes to make, or it is closest to his preconceptions. With this attitude, histologists and electron microscopists stand in danger of trading the merits of their own procedures, for the luxury of making useful generalisations.

Supposing one had a lymphocyte in saline, and one could be sure that its separation from the blood sample had not changed its diameter. It appears under phase contrast microscopy to be 12 μm in diameter, and after staining, say, with Leishman's, it was 10 μm . One could cut 1 μm thick sections on the microtome. If one ignores the possibility that cutting compresses the cells, one could cut 10 sections of which those of maximum diameter would represent the measurable diameter in the stained section, provided that all the cells were lying in a uniform layer in the same horizontal plane. However, if one can measure the diameters of whole cell in fresh preparations by phase contrast microscopy, one has to ask oneself, "Why should one embed and section the cells?"

In every day histology, the organelles and cells one is examining are not spheres, are not visible in a single layer, and one does not know the orientation of the microtome relative to the positions of the organelles and cells. Therefore, it is

virtually impossible to measure their dimensions which are intended to reflect their properties in the earlier intact lives by any histological procedure.

Comparatively few types or cells, such as lymphocytes, ova or granule cells from the cerebellum, are spherical or oval. Many, such as hepatocytes, cubical cells of the intestine, muscle cells and fibroblasts, have symmetrical shapes in histological sections, but one still can not solve the problem of the microtome cutting cells from any direction. In addition, many cells by light or electron microscopy, do not appear to be furnished with cell membranes. Tissues in which such cells appear are called syncytia, and the irregularity of the shapes of the cells exacerbates the problems of cutting sections. The ubiquity of syncitia is not always appreciated (Table 25).

Syncytial tissues

<i>Connective tissues</i>		<i>Alimentary tract</i>
Mesenteries		Dental pulp
Pleura, pericardium, peritoneum		Submucosa of whole tract
Perivascular tissues		Appendix
Pericytes around blood vessel		Longitudinal and circular muscle
Aponeuroses		
<i>Nervous tissue</i>		<i>Urogenital system</i>
Cerebral neuroglia		Bladder mucosa
Cerebellar white matter		Ovarian stroma
Cerebellar neuroglia		Fallopian tube stroma
Granule cells of cerebellum		Uterine mucous layers
Spinal neuroglia		Vaginal corium
Capsule cells of sympathetic ganglia		Graafian follicle stroma
Schwann cells		Urethral glands of Littré
Outer and inner layers of retina		Interstitial tissue of prostate
Muller cells of retina		Interstitial cells of Leydig
Choroid plexus		
Corneal epithelial cell		<i>Lymphatic system</i>
<i>Respiratory system</i>		Medullary sinuses
Nasal corium		Cortex of lymph nodes
Tracheal and bronchial submucosa		Small lymphocytes
Lung parenchyma		Tonsils and adenoids

<i>Endocrine system</i>	<i>Embryonic tissue</i>
Trabecular cells of pineal gland	Trophoblast
Medulla of thymus	Neural tube
Interfollicular tissue of thyroid	Lung
Zona fasciculata of adrenal cortex	Liver
Anterior lobe of pituitary	Blood vessels Ducted glands Pre-cartilaginous cells
<i>Spleen</i>	<i>Malignant cells</i>
Red and white pulp	Carcinomas
Giant cells of spleen	Sarcomas
Small lymphocytes	
<i>Skin and hair</i>	<i>Muscle</i>
Dermis	Developing (Fischman, 1972)
Cutis vera	Perimysium Epimysium Endomysium
<i>Bone marrow</i>	
Erythrocyte precursors	
Leucocyte precursors	

Table 25. These may be seen by examining histological sections or textbooks.

Information which can be derived from a single stained section

The gross anatomy of an organ
The presence of nuclei, nucleoli and membranes
The general histology of tissue
The development of the embryo
The relative incidence of subcellular organelles, if statistics are used
The presence of bacteria and parasites
Malignancy and its spread
The spread of infection and inflammation
The presence of haemorrhage, ischaemia, thrombosis, fatty infiltration, hypertrophy, and atrophy
The presence of necrosis, but, not its chemistry
Rejection of a grafted tissue
Artifacts of preparation
Hyperplasia

Table 26. This Table should be compared with Table 27. Both of them cover histology, histochemistry, immunocytochemistry, and all procedures of electron microscopy, in which sections are cut.

Information which can not be derived from a single stained section

- The dimensions of any part of the cells
- The real and relative presence, dimensions, shape or incidence of organelles, vesicles, vacuoles or spaces in cells
- Whether the cell or organelle has shrunk or swollen
- The movement, or rate of movement, of an organelle or particle in a living cell
- The change in shape of organelles
- The location, e.g. central or peripheral, of an organelle in the cytoplasm or nucleus or whether it is bound to, or near, a membrane
- The presence or location of any particle, or of any chemical component soluble in, or precipitated by, any of the reagents used during preparation
- The distance between two membranes
- The chemical or enzymic activity of any organelle
- The presence of any chemicals or structures, which have no affinity for the stains or other reagents used
- The accuracy of calibration of a micrograph, relative to its dimensions *in vivo*
- The presence or incidence of fibres, fibrils, dendrites, protrusions, neurites or processes from the surface of cell membranes
- Whether pyknosis or clumping has occurred
- Whether an apparent structure in the cell results from reaction with any of reagents used, or was precipitated during preparation of the section
- The particular plane separated by different freezing procedures used in electron microscopy
- Whether cells occur in isolation or in clumps
- The physiology of dying
- Changes during the dying or post mortem of any of the above

Table 27. This information is normally sought using histological procedures.

A section by definition, does not include the whole structure, so serial sections were devised to cover the total volumes of structures (Stilling and Wallach, 1842). However, the reconstruction by adding all the sections does not compensate for the shrinkage of the tissue.

Chapter 9

Electron Microscopy

Most of the comments about histology apply to electron microscopy, but this procedure has additional difficulties. Living cells can not survive low pressures, subjection to electron bombardment and x-irradiation. Therefore, they have to be stained by deposition of heavy metal salts of osmium, lead, tungsten, manganese, etc. (Weakley, 1972; Rash and Hudson, 1979). Electron microscopists can only examine metal deposits on those parts of cells, which have affinity for these salts. They are not looking at a cell membrane, a nuclear membrane, a mitochondrial membrane or a macromolecule. (Please see Scanga, 1964).

For the reasons above, the electron microscope can not be used to look at living cells, but one can examine tissues before and after events, which occurred in life, such as movements, phagocytosis, infection and necrosis. The main advantage of the electron microscope is that, under optimal conditions the resolution of the electron microscope is 1.5-2 nm, compared with the light microscope of 200-250 nm.

There are several different kinds of electron microscopy, including transmission, scanning, freeze etching, freeze substitution and freeze fracture (Moor, 1969; Breathnach, Stolinski and Gross, 1972; Stolinski and Breathnach, 1973). It is often said that each of these different procedures represent independent evidence for the existence of very small structures, but this view is not sustainable, since all the procedures always involve fixation, heavy metal deposits, dehydration, low pressure, electron bombardment and x-irradiation, so that any real or apparent structure visible after any of these steps common to all the procedures, may appear in several of them.

It has been asserted that freezing can be carried out rapidly, without causing dehydration of the cellular fluids (Saubermann and Echlin, 1975; Linner et al, 1986). Unfortunately, this is not the case. When a chemical mixture is frozen to temperatures below the eutectic points of its main soluble constituents, water comes out of solution as ice, leaving all other constituents dehydrated. This principle is used in desalination of sea water. The rapidity of the freezing may stop the solutes moving far, but the appearance of ice is *prima facie* evidence that dehydration of adjacent fluids has occurred. It is also believed that rapid freezing may vitrify solutions and prevent dehydration (Ryan and Macklin, 1968; Franks and Skaer, 1976; Franks, 1977; 1985; Westhoff, 1993). However, the glasses are formed at particular rates of

freezing, temperatures, purity of solutions, etc., (Bohler, 1965) and changes also occur during rewarming before cutting (Echlin, 1977; 1978).

Rapid freezing causes tremendous stresses and strains in tissue. For example, rats rapidly immersed in liquid nitrogen for measuring ATP or phosphocreatine in the brain, can be heard to crack several times. Furthermore, rapid freezing does not allow enough time for dissolved gases to escape, so bubbles of very uniform dimensions are entrapped in the frozen tissue. A similar phenomenon is seen in the bottom of a saucepan, when water is heated to the boil. Jobbing biochemists see that specimens of brains, livers or kidneys shrink visibly, when rapidly frozen in liquid nitrogen or Freon.

Electron microscopists have carried out extensive studies on the following effects of electron beams on metals and on biological systems: the fracture planes; precipitation; ‘ageing’ of specimens; heat dissipation; inelastic scattering; dislocations; plastic deformations; distortions; water content; edge effects; quenching; vaporisation of specimens; crystal growth; artefacts, etc. particularly in relation to frozen tissue (Breese, 1962; Echlin, 1977; 1978; Robards and Sleytr, 1985).

Frey-Wyssling, (1953), Luyet, (1960) Luyet, Tanner and Rapatz, (1962), Luyet, (1966) and Meryman, (1960; 1966) were among the pioneers, who examined dried out Ringer’s solution, saline, alanine, glycine, macromolecules and cells, microscopically. They showed the complexity of two-dimensional patterns, which were displayed when they were frozen (Mackenzie and Luyet, 1960). These included ‘spherulites’, ‘dendrites’, ‘spicules’, etc. The two-dimensionality of the deposits arose from the fact that the solutions had dried out on flat crystallizing dishes. They often formed Liesegang rings, and lines, which could justifiably be described as ‘trilaminar’ Robards, (1974). Sleytr and Robards, (1977; 1982) and Robards and Sleytr, (1985), reviewed many of the artifacts visible by electron microscopy in biological tissues (See also, Pentilla, Kalimo and Trump, 1974).

The literature cited indicates that freezing to low temperatures often causes the appearance of crystals, which are generally agreed to damage cells. In order to prevent this, tissues have either been frozen very rapidly, with the intention of forming glasses and minimising the size of crystals, or ‘cryofixatives’ have been added to them. These are also used in the preservation of sperm, ova, embryos and small pieces of tissue (Smith, 1961; Ciba Foundation, 1979). Cryoprotectants included glycerol and dimethyl sulphoxide, which, however, induce their own range

of chemical changes in the tissue. These have been summarised by Franks (1977) listed here, in Table 28. This Table includes the effects of cryoprotectant freezing and cryofixation. Obviously, most of these changes would be extremely difficult to demonstrate experimentally in conditions similar to those in the cells in the intact animal. The parameters of the changes are not known, new equilibria will be established, and most seriously, it is not known whether any, or which, of these changes is irreversible. This would seem to spell out the case, either for doing all the relevant control experiments comprehensively, or for refusing to accept the results of any of these experiments for which controls have not been done.

Effect of freezing with cryofixatives upon the chemistry of biological systems

A. Physico-chemical

1. Colligative reduction of the equilibrium freezing point of substrates and cytoplasm
2. Reduction in the temperature of homogeneous ice nucleation
3. Reduction in the critical cooling rate, above which freezing is controlled by homogenous nucleation
4. A change in the ice crystal growth rate
5. Changes in the various eutectic temperatures of the substrate
6. Changes in the solubilities of electrolytes (buffer species)
7. Changes in the solution of ions and resulting changes in pH of the solvent, monitored as apparent pH changes
8. Changes in the intermolecular order and the molecular dynamics characteristic of outside the cell
9. Deep etching is difficult or impossible

B. Biochemical

1. Changes in membrane permeability and composition permitting cryofixative to enter the cytoplasm, and its contents to leave the cell
2. Changes in the molecular state of the cytoplasm - especially during extracellular freezing
3. Changes in solubility, solvation and ion binding equilibria of cellular components and in the degree of dissociation and pK of acidic and basic groups
4. Changes in enzymatic activities and mechanisms
5. Specific cryofixatives binding to biopolymer species (?)
6. Interference with some physiological functions
7. Changes in immunological behaviour.

Table 28. These are listed by Franks, (1977) and this Table is reproduced by kind permission of Blackwell Scientific Publishers, Ltd.

A few further points must be made here.

Firstly, although freezing is sometimes used to obviate the necessity of chemical fixation, users usually fix the tissues subsequently during their procedures.

Secondly, the tissue is often warmed up subsequently to facilitate section. At this point, any tissue contents which are liquid, will exchange with any of the reagents subsequently used.

Thirdly, whatever the ambient temperature around the microtome at the time of section is, a considerable amount of heat is generated at the cutting edge of the knife and shearing of the specimen. So the edge may be quite hot (Sleytr and Robards, 1977). As in the centrifuge, the temperature in the specimen is unknown and may be unknowable.

Fourthly, *during* the dehydration caused by the freezing, the osmotic pressure of the tissue solutes must increase. This will relocate other solutes not yet frozen and, later, precipitate them.

When one looks at an electron micrograph of the membrane around the cell, the nucleus or the mitochondria, the two lines of the ‘trilaminar’ or ‘unit’ membrane appear remarkably uniformly distant apart, as did the lines of the frozen solutions seen by Luyet, (1966) and his collaborators (please see above). If one cuts sections of concentric spheres, one can see that the apparent spacing of their shells depends upon how close the sections are cut to the periphery or equator (Figure 3). Since the three kinds of membranes under consideration are randomly orientated in any section, and they do not ‘know’ from which direction the microtome will cut, it follows that, even if the concentric shells had exactly the same thicknesses, they should appear in sections in a variety of thicknesses. These would depend upon, whether the section was cut through, the centre of curvature, a segment of the curve, close to its periphery, between any two shells, or, indeed, tangential to them. The fact that such a range of shapes does not appear in any section viewed on the screen of the electron microscope, or adorning the pages of glossy textbooks, means, *either* that a small area of the micrograph has been selected to illustrate the trilaminar appearance, *or* that the trilaminar appearance is two dimensional and occurs after the section was cut.

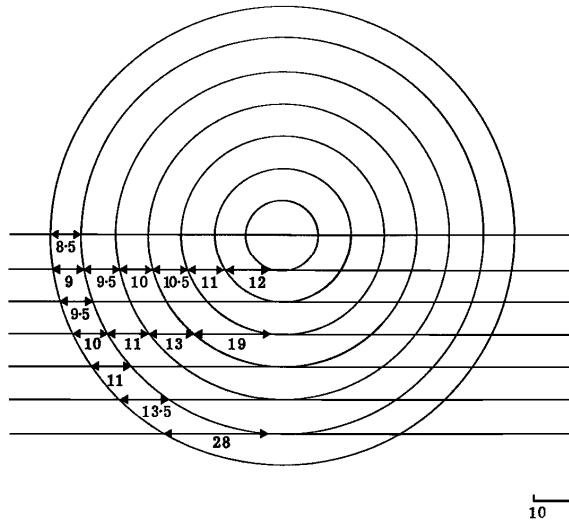


Figure 3. Sections through concentric spheres. *It will be seen that as sections are made nearer the periphery, the distance between adjacent shells appears to increase. The shells only appear equidistant when the section is cut precisely through the centre of the spheres. This geometry applies to all the 'unit' membranes, the endoplasmic reticulum, the mitochondrial cristae, the myelin sheath, the synaptic cleft, and the lamellae of the Golgi apparatus.*

It has been argued that the electron microscopists have selected the illustrations of the trilaminar appearance, because the membranes are clearest when they are cut normally (Horne and Harris, 1981; Michell, Finean and Coleman, 1982). This is not acceptable. When one looks down the electron microscope at many sections, (not only those chosen to demonstrate the crispness of a normal trilaminar membrane, but any others illustrating, for example, mitochondria, nucleoli or vesicles), one fails to see any significant incidence of different apparent thicknesses of the lamellae or the spaces between them. This represents an invitation to any one to publish micrographs not just showing an occasional variation in thickness or some blurring, but in *the same micrograph*, the normal variation in trilaminar spacing, which solid geometry would require. It has also been suggested that the membranes somehow orientate themselves towards the electron beam either as a consequence of section, or as a result of the arrival of the electron beam. However, while the electron beam could not orient a membrane, it is possible that only when it was normal to a section, would enough energy be dissipated in the cell membrane in a groove in the

direction of the beam, that the membrane would explode and blow off the central portion of the metal covering the membrane to reveal a groove, lined by two walls of heavy metal - the 'trilaminar' appearance (Figure 4). The heavy metal, the crystalline trilaminar deposit and the embedding medium, are each grossly different in their physical properties, and they separate when bombarded by the electron beam (Luyet and Rasmussen, 1968).

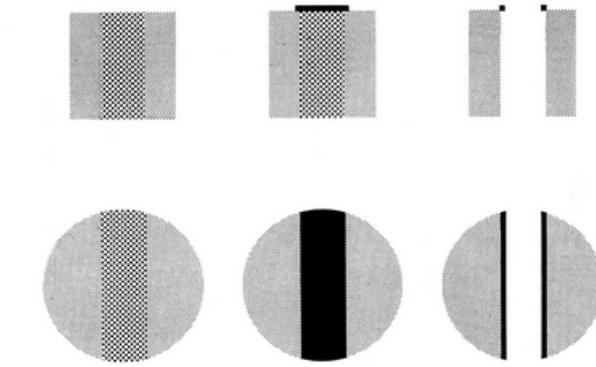


Figure 4. Hypothesis to explain why the membranes always appear double (trilaminar) by electron microscopy. *Left*, stippling shows the side and normal views of an embedded cell membrane; *middle*, black represents the heavy metal deposit on the membrane in the same view, *right*, the electron bombardment, the low pressure in the microscope, the heat dissipated in the heavy metal and the irradiation, cause the membrane itself to volatilise and expel the stippled membrane through the surface of the metal, leaving two lines bordering it.

In the debate at Brunel University in May 1980 on the structure of the cell between Dr A. Robards and Dr K. Roberts on the one side and Mr P Sartory and myself on the other, Robards suggested a reason for which so many membranes appeared normal in electron micrographs. He compared it to a glass door. It could not be seen when the door was closed, but could be seen when it was fully open at the right angles to the house; in this position, there was enough glass in the line of vision to be visible. This explanation is not satisfactory, because it would require that when the membrane was in the plane of the section - as when the door was closed - there should be a large enough empty space not apparently surrounded by a membrane. This is never seen in sections, except of areas of tissue said to be swollen, apparently

containing what are described as vacuoles. This point was not answered at the debate.

The uniformity of the apparent distance between the two lines of the membranes (Sjöstrand 1953 a, b, c) is not only seen in electron micrographs, but also in diagrams drawn to depict these membranes (Figure 5). It would be impossible to make a three-dimensional model of any such structure, which permitted the membrane to appear of uniform thickness. One could not presume that sections pass through the geometric centre plane of every membrane. Curious.

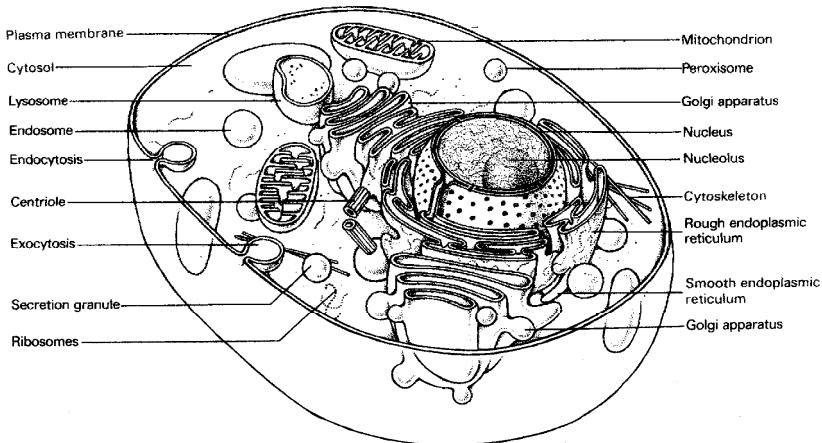


Figure 5. The image of the cell from De Duve, (1984) is reproduced by kind permission of Scientific American, Rockefeller University Press and the De Duve Trust.

It has been suggested that, while a section through an object will only see a two-dimensional plane, the object may be tilted on the stage in the electron microscope to reveal it in other orientations (Gray, 1966; Gray and Willis, 1968; Lange, 1976). This is not acceptable as an explanation for the high incidence of apparently normal sections of membranes in electron microscopy, because: (a) it should not be necessary to tilt the specimens to see a random series of orientations. Light microscopists do not have to do so; (b) the geometrical difficulty is sometimes attributed to the thickness or thinness of sections. This would certainly determine the clarity with which one would see lines on a micrograph, but has no relevance whatsoever to the incidence of cross sections apparently normal to the plane of section in electron micrographs.

The ‘unit’ membrane is believed to be the electron microscopic manifestation of the Davson-Danielli and Singer-Nicolson lipid membrane. Virtually all the cell membranes appear ‘trilaminar’. This is because the heavy metal stains deposit on *both* sides of all membranes, as do linear deposits of precipitated solutions, however thin they are. The stains do not dissolve the membranes, because, if they did, they would not have a membrane upon which to deposit. If one paints a single piece of membrane on both sides of it, cuts a transverse section of it, when laid on its side and viewed by microscopy, it must appear trilaminar. That is, every real thickness - as opposed to a line - must appear trilaminar when stained with a deposit, if viewed with sufficient magnification (cf Figure 4, 5).

Electron microscopists, using freezing techniques, including rapid freezing, scanning, freeze drying, freeze substitution and freeze fracture, state that their planes of cleavage go to one or other side of the membranes or between the laminae (Haggis 1961; Branton, 1966; Breathnach, Stolinski and Gross, 1972; Bullivant, 1977; Plattner, 1981). Their assessments are generally based either on the presence of particles on the faces of the surfaces, (Dalen, Myklehurst and Saetersdal, 1978) which the authors identify quite arbitrarily, or on the convexities or concavities apparent on these surfaces, which can not be identified by any objective criteria. The idea that one could cleave a bi-lipid membrane is based on the belief in respect of both the Davson-Danielli and the Singer-Nicolson hypotheses, that one can *know* the orientation of molecules in the membranes.

When a tissue is being stained for electron microscopy, any particle or precipitate on or near a membrane, will not permit the heavy metal to deposit on that particular region, so that the layer of deposit will appear to take a detour around the particle or deposit, and thus be apparently continuous with the adjacent membrane. Thus the particle or precipitate will appear to be entering or leaving the cell. A similar phenomenon is seen in tablets enclosed in blister packs.

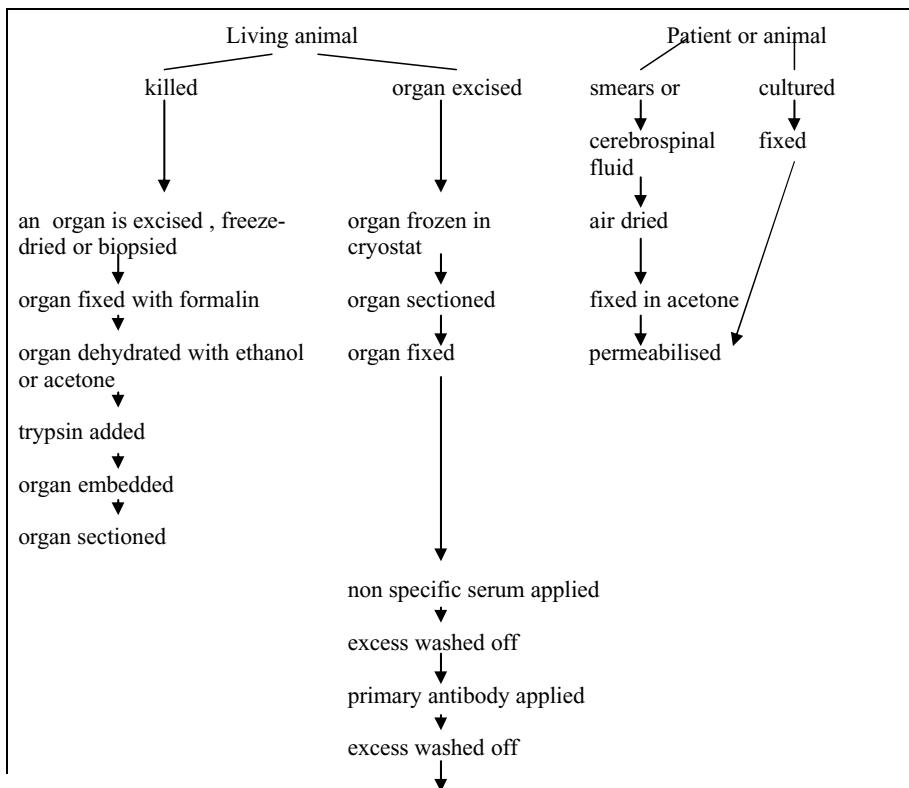
Furthermore, it is difficult to calibrate electron micrographs (Dunn, 1978). The commonest way is to calculate the magnification from the known magnifications of the different lenses of the electron microscope. Another way is to use plastic spheres, crystals or fibres of known dimensions, but there are problems here. Firstly, the heavy metals used to stain the biological tissue, the embedding medium and the calibration specimen have completely different temperature coefficients of expansion, heat conductivity, heat capacity and size of particles. Therefore, each of them will

expand to a different extent when subjected to the electron beam. Secondly, the calibrating objects are measured at room temperature by light microscopy. It is normally assumed that their dimensions do not change on the stage of the electron microscope. In view of the many reported effects of the electron beam on metals, this is not at all likely. See also Barnard and Seveus, (1978).

Chapter 10
Immunocytochemistry

These procedures are based on the belief that different organelles in cells, different kinds of cells and different tissues in the same individual, contain different antigens, proteins, carbohydrates, nucleic acids and lipids. Some of these are fluorescent or can be conjugated with fluorochromes. These are detected by fluorescence or staining, when exposed to specific antibodies (Pearse, 1968; Polak and Van Noorden, 1986; Catty, 1988; 1989; Cuello, 1993; Beasley, 1998). The procedures are complicated, so that one has to have positive controls, consisting of tissue containing known antigen, and negative controls, using other tissue not believed to contain the antigen. Absorbtion controls are used to show that excess of a specific antigen abolishes the immuno-reactivity, but other substances do not. The main steps of the procedures are listed in Table 29.

Immunocytochemical procedures



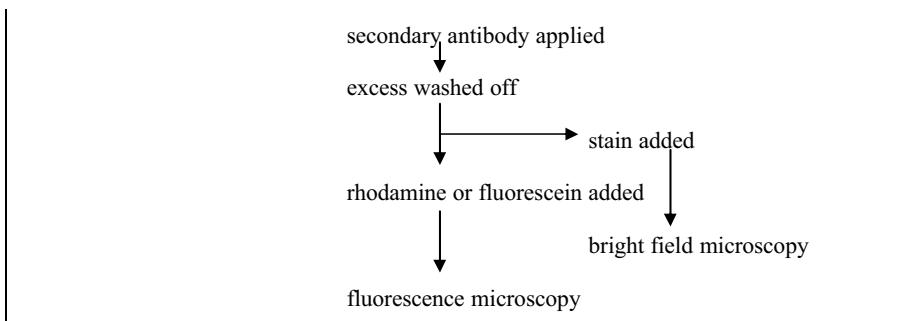


Table 29. *The effects of the chemical reagents used is not always known.*

A few comments are worth making about the procedures.

Firstly, they are largely empirical and require several different reagents, often used in histology. Like all procedures, they imply some important assumptions, which will be listed below. However, it is appropriate to ask why a simple addition of an antibody to a section of tissue, possibly with a fluorochrome, should not produce the observed fluorescence. Why does one need to embed, and add fixatives, ethanol and trypsin? In a simple titration of a diluted serum against a known antigen, such reagents would interfere with the precipitation.

Secondly, tissues are often ‘permeabilised’ with Triton X-100, NP40, formaldehyde or benzoquinone; the former two are described as ‘gentle’, but detergents are powerful surface-active chemical reagents (Helenius et al, 1979). These permeabilisers are believed to render the cell membranes more permeable to the antibodies - as their name implies. However, they are often used on sections, in which fixatives have already increased their permeability, and the cells have been cut open, so that antibodies would have free access to the cytoplasm. This raises the suspicion that the fluorescence may have been enhanced or even caused by the permeabilisers. It is tempting to suggest that if an antibody is applied to an unfixed and un-cryoprotected section, to which a fluorochrome has been added, and it does *not* fluoresce, then the antigen was not present in that tissue.

Thirdly, fresh serum is added to combine with ‘non-specific’ antigens, and then it is washed out. This implies that the serum can distinguish between a ‘specific’ and a ‘non-specific’ antigen; such a distinction seems rather teleological, since the main difference between the two is whether the immunologist is interested in the one or the other.

Fourthly, enzymes such as trypsin can digest some proteins, but not others,

their use underlines the empirical nature of the procedure.

Fifthly, there is a practical problem. Some immunocytochemists photograph fluorescence using automatic exposure - that is, the shutter of the camera remains open until enough photons have been collected to close it. This exaggerates the intensity of pale fluorescence, as well as autofluorescence, which occurs in many tissues, as well as the natural fluorescence of such substances as albumin, γ -globulin, tyrosine, tryptophan and phenylalanine, as well as trypsin (Udenfriend, 1962). The intensity of fluorescence of any region depends upon not only the presence of natural or added fluorochromes and the substances to which they are conjugated, but, also, on the size of the organelle, the thickness of the section, the wave-length of the light, other reagents added, the presence of known and unknown quenchers and enhancers, the strength of binding of added fluorochromes, and the stability of that binding in the presence of tissue and the reagents used.

The following assumptions are inherent in the use of immunocytochemical procedures, but most of them will be dealt with in detail, in consideration of immunology (please see Chapter 51).

- (i) different tissues and organelles contain different antigens;
- (ii) antibodies are specific to the antigens not only in the living intact organism, but also in the precise chemical environments of the prepared sections (Swaab, Pool and Van Lewen, 1977);
- (iii) neither antigens, antibodies or any substances in the tissue which may enhance or quench the fluorescence, move during the procedure;
- (iv) the stability of the conjugation is not affected by the presence of tissue or reagents.

Chapter 11

Light Microscopy of Living and Unfixed Cells

An electron micrograph is composed of deposits of salts of heavy metals. A stained section consists of dead tissues *minus* substances extracted by the reagents, *plus* stains and other substances added during the procedure. By light microscopy of intact living or unfixed cells, in the latter one looks at tissue *minus* substances lost to the medium, in which they are observed. The three different kinds of procedures can be compared (Table 30). It can be seen that observations on unfixed cells are closest to life, since fixation kills cells. Living cells can be seen *in situ*, or they can be separated. Some cells, such as sperm, ova, red and white blood cells, are naturally isolated cells, but, of course, they can not be considered to be 'typical' of somatic and visceral cells. Perhaps, the most important distinction is that between cells *in situ* and solo cells, on the one hand, and those which can be separated even by 'mild' procedures, on the other.

Comparisons of microscopical procedures

<i>Intact animal</i>	<i>Unfixed isolated cells</i>	<i>Stained</i>	<i>Electron microscopy</i>
intracellular movements	intracellular movements	none	none
fully hydrated	may be in incubating medium	dehydrated	dehydrated
cells in normal environment	incubation may be unnatural	tissue loses constituents	tissue loses constituents
natural colour	natural colour	added colour	black and white
normal chemistry	some denaturation	chemistry much changed	heavy metal salts
normal geometry	cells on flat surface	section of cells	section of cells
maximal resolution 200 nm	maximal resolution 200 nm	maximal resolution 200 nm	maximal resolution 10 nm
cells undamaged by dissection	mechanical damage during separation	shearing and cutting	shearing and cutting
poor optical conditions	optical conditions better	better optical conditions	good
animals anaesthetised	animals have been killed	animals have been killed	animals have been killed
measurement possible	measurement possible	measurement impossible due to shrinkage	also impossible for same reason

connections often not visible	connections broken	connections broken	magnifications too large to see
Cheap	Cheap	relatively cheap	expensive

Table 30.

Light microscopical techniques

<i>Technique</i>	<i>Optimal use</i>
Bright field	stained tissues and coloured cells
Dark ground	cell membranes and interfaces
Polarised	birefringence
Oblique light	cell membranes and interfaces
Critical microscopy	centre of field
Rheinberg	coloured structures
Phase contrast or anopteral	unstained cells and colourless cells
Dissecting	manipulation and teasing cells
Inverted	allows easier manipulation of cells from above
Centrifuge	separates organelles reversibly (Harvey, 1950)
Interference	good contrast
Vertical illumination	surface of cells, especially opaque parts
Supravital stains	intracellular movements (Doan and Ralph, 1990; Hung Leo et al, 1993)
Microspectrophotometry	spectra of organelles (Kohen and Hirschberg, 1989)
Modulation contrast	good contrast (Hoffman and Gross, 1970)
Enhanced contrast	uses electronics to clarify images (Allen et al, 1981)
Confocal	focuses on thin plane (Petran et al, 1986)
Real time confocal	used in tissue culture (Jester et al, 1991)
Laser capture	high resolution (Conn, 2002)
Polychromatic light illumination	high resolution (Hewlett, 1983; Oldfield, 1998)
Quantum dot fluorescence	high resolution (Michalet et al, 2003; 2005)

Table 31. Many of these are usable on living or unfixed cells. References to earlier techniques are listed in Hillman and Richards, (1983).

A list of a few of the many different kinds of light microscopy is given (Table 31). Their optimal use is indicated. Phase contrast and anopteral microscopy are perhaps the most important. These translate differences in refractive indices in part of cells, to differences in intensity. The different techniques each require a certain amount of skill for optimal use. Centrifuge microscopy has been used in the past, for

example, on amoebae, to watch the organelles move to one end and return after the end of centrifugation (Chapman-Andresen, 1977), but it has not been used recently. The g forces are in 10's compared with those used in subcellular fractionation, where thousands and tens of thousands of g's are used. Supravital stains, such as Janus green, methylene blue or neutral red, permit mitochondrial movement and respiration to continue in cells, which are two important criteria of the life of cells. Video-enhanced microscopy is a technique whereby noise and background signals may be eliminated from an image by electronic means (Allen et al, 1981; Truss, 1990). It may be regarded as a 'touch-up' technique, in which the operative makes a decision - based on her beliefs - about the relevance of an apparent microscopical feature to the point she wishes to illustrate. This seems a bit dangerous; because it is not always objective. Confocal microscopy permits one to focus on a particular plane in a solid piece of tissue, without sectioning or embedding it (Terasaki and Dailey, 1995). Video-enhanced and confocal microscopy are both relatively expensive.

A list of some of the many microscopical preparations which may be used with little or no disruption is given, to indicate that in cytology there are several serious alternative techniques to subcellular fractionation, histology and electron microscopy (Table 32).

Preparations which may be examined unfixed and alive

<i>Preparation</i>	<i>Comment</i>
Cells growing in culture	not same as in intact animal
Blood vessels, nerves webs, mesenteries and choroid plexuses	can be separated, and maintain properties
Intact pieces of retina	react to light
Naturally isolated blood cells	little or no damage in isolation
Sperm and ova	study fertilisation and cell division
Teased buccal cells, lung biopsies, cervical smears, and urine deposits	examine healthy tissue
Time lapse photography of isolated cells	can record movements of cells and changes in them
Intact skin	by cinemicroscopy
Free hand dissected cell bodies of neurons, hepatocytes, kidney cells or peripheral nerve fibres	minimal energy for isolation
Nuclear transplantation	used in cloning
Injection into cell bodies	under direct vision

Transparent chambers in brain, ears, lungs and skin	needs careful surgery, but tissue intact
Teased muscle fibres	observe contraction
Marking active neurons for subsequent histology	useful in ionophoretic experiments
Any isolated cells	optical tweezers to isolate organelles
Colpoids	to study <i>non-biological</i> properties of biological systems

Table 32. *They may not necessarily involve fixation of tissues or killing animals.*

The advantages of living preparations are that: the cells are approximately isotonic with their normal environments; they do not have to be fixed, dehydrated, embedded, sectioned or stained; they are not subjected to all the necessary reagents; one can measure the dimensions of the cells and their organelles; the effects of changes of the environments of the cells can be examined on them; one can transplant nuclei; one can cut open cells and separate the membranes or nuclei of individual cells; one can observe intracellular movements and how added agents affect them; cells can be micromanipulated and cloned; using fluorochromes or radioactive isotopes, one can observe directly, whether aminoacids, transmitters, hormones, drugs, etc. *do* act on the cell membranes, or must enter the cytoplasm first; one can measure changes in resting and action potential differences, although not necessarily quantitatively (please see Chapter 16).

Of course, light microscopy of living or unfixed cells, also has a significant number of artefacts. These are listed in Table 33. Most of them distort apparent shapes and dimensions, but do not usually create apparently new structures (Table 85).

Artifacts of optical microscopy

Type	Effect	Method of diminishing
Chromatic aberration	colour fringes	compensating eye-pieces with different materials for lenses
Spherical aberration	blurred images, outer zones positive, lenses have shorter focal length than inner zones	stop down lenses may lose resolution; choose positive and negative lenses, and their shapes; achromatic lenses are corrected for one wave length, apochromatic for two

Type	Effect	Method of diminishing
Coma	off axis rays do not come to a focal point, but form comet shaped images, which superimpose	stop down in multi-component systems chose radii of curvature of surfaces
Astigmatism	oblique pencil of rays from off-axis object on passing field through lens, converge to a series of images varying from point to line	negative astigmatism to compensate for curvature of field
Distortion	square appears as barrel, because different parts of lenses have different magnification	can be reduced by putting in stops at suitable points in multipoint lenses, not affected by size of stop
Curvature of field	images of plane surface formed by convex and concave lenses lie on curved lenses	examine only centre of field
Colour staining	differentiates different populations of cells	can be avoided using phase contrast, anoptical video and confocal microscopy
Dehydration	shrinkage	use unfixed, unstained or living tissue
Section	examines only part of tissue	avoid sectioning
Light wave	damage	use minimal energy and longer lengths

Table 33. These are mainly from Needham, (1958) and White, (1966).

A light microscopist may *detect* the membranes, but not measure their dimensions. He may see:

- (a) a difference of refractive index of the cell membrane relative to the extracellular and intracellular fluids;
- (b) an interface between two fluids of different refractive indices;
- (c) deposits of stains or heavy metals, on the membrane, effectively making it thicker;
- (d) an interface between two fluids stained different colours;
- (e) reflection by the membrane;
- (f) fluorescence caused by the addition of fluorochromes;
- (g) a potential difference recorded when a micropipette goes from one phase to another;
- (h) a colour difference between different stained organelles;
- (i) the release of cytoplasm or axoplasm, which has a different refractive index

from incubating fluid, when a membrane is cut open.

It seems to me highly likely that there is no way in which the thickness of the cell membranes in life can be measured, and this reflects sadly on any calculation made in the literature, attempting to find out the following characteristics of the cell membranes: their chemical composition; the orientation of any macromolecules within them; the rates of diffusion across them; the proportion of them in a cell fraction; the voltage gradient across them.

Some of the early developments in micromanipulation of living cells are shown, and the original papers are well worth reading (Table 35).

Pioneers of light microscopic and manipulative techniques for living cells

<i>Techniques</i>	<i>Pioneers</i>
Microdissection	Purkinje, (1838); Hannover, (1844)
Vital stains	Tremblay, (1744); Brandt, (1881)
Microincineration	Alexander and Myerson, (1937)
Micromanipulation	Gray, (1931); Hörstadius, (1950)
Phase contrast microscopy	Zernike, (1934)
Cartesian diver	Lindeström-Lang, (1937)
Nuclear transplantation	Commandon and de Fonbrune, (1939)
Microsurgery	Kopac, (1950)
Microinjection	Knower, (1950); Heilbrunn, (1956)
Microelectrophoresis	Edström, (1953)
Isolation of ganglion cells	Giacobini, (1956)
Confocal microscopy	Minsky, (1961)
Interference microscopy	Beneke, (1966)
Microchemistry	Neuhoff, (1973)
Video enhanced microscopy	Allen et al, (1981)
Polychromatic light illumination	Hewlett, (1983); Oldfield, (1998)
Microspectrophotofluorimetry	Kohen and Hirschberg, (1989)
Optical tweezers	Greulich, (1999)
Wide field microscopy	Allanson et al, (1999)
Quantum dot fluorescence	Michalet et al, (2003)

Table 34. For general reviews, see Carpenter, (1901); Gray, (1931); Heilbrunn, (1952); Chambers and Chambers, (1961); McClung-Jones, (1964); Bourne, (1964; 1967); Ross, (1967); Bracegirdle, (1978); Wilson, (1990); Shotton, (1993); Pawley, (1995); Soll and Wessells, (1995); Bradbury and Evennett, (1996); Greulich, (1999).

Chapter 12

Cell and Tissue Culture

Tissue and cells can survive, grow and multiply in vitro. They maintain many of the same physiological, biochemical and genetic properties, as the original tissues from which they originated. They may be taken from tissue biopsies of human beings and animals, including skin, fibroblasts, epithelial cells, umbilical cords, foreskins and tumours. They may also originate from embryonic tissues and stem cells. They may also come from contamination from other tissue cultures, of which the Hela cell is most well known. There is extensive literature on tissue culture, of which the following are a few references (Willmer, 1965; Fedoroff and Hertz, 1977; Shahar et al, 1989; Freshney, 1994; Cohen and Wilkin, 1995, Spector, Goldman and Leinwand, 1998).

If the tissue is taken from a dead animal or human being, the whole body is hypoxic, but, in all cases the organ becomes hypoxic when it is deprived of its blood supply while being excised. It is transferred from an organ in the body at 37°C to a dish at room temperature and atmospheric pressure. Cells may be disaggregated with trypsin - a powerful proteolytic enzyme. They are then put into a sterile growth medium, containing: salts in similar concentrations to those in serum; embryo extracts; buffer; aminoacids; vitamins; growth factors; fresh calf serum. They are usually oxygenated and there is some carbon dioxide in the atmosphere. They are kept at about 37° or slightly lower, and may be shaken. They are observed using an inverted microscope and photographed at intervals. Time lapse photography of 100-300 times is often used to speed up such dynamic processes as streaming, mitochondrial movement, or cell division. Some tissues just survive in culture, others divide, others last a few generations, and a few are, apparently, immortal.

A large variety of tissues, both non-nervous and nervous can be grown in culture (Tables 35 and 36). These preparations are used to study the properties of the tissues from which they are originated, Rounds and Pomerat, (1959), on the assumption that the whole culture procedure had not changed them significantly. However, there are many variables, which affect the anatomy and physiology of the cultures, and it should be unnecessary to state that the *chemistry* of the media totally determines the chemistry of the cell cultures at the time which they are examined (Table 37). In addition, monoclonal antibodies are produced by cultured cells, and

these are used in diagnosis. They are also used for genetic modification of foods, medicines and agricultural crops, and for making vaccines.

Non-nervous tissues which can be grown in culture

Epidermis	Bone marrow
Breast	Macrophages
Cervix	Thyroid
Skin	Parathyroid cells
Epithelial cells	Liver
Melanocytes	Pancreas
Tooth germs	Salivary glands
Connective tissue	Intestinal tract
Adipose tissue	Cardiomyocytes
Smooth muscle	Bronchial epithelium
Striped muscle	Kidney
Cartilage	Ova
Bone	Adrenal
Endothelial cells	Pineal
Osteoblasts	Hypophysis
Osteoclasts	Endocrine pancreas
Macrophages	Mammalian ova
Lymphocytes	Umbilical cells
Fibroblasts	Stem cells
Foreskins	Hepatomas
Hybridomas	Teratomas
	Tumours

Table 35. These are mainly from Willmer, (1963) and Freshney, (1987). *Tissues are grown in a different variety of culture media.*

Cells from the nervous system which grow in culture

Cerebellar granule cells	Peripheral sensory neurons
Cerebellar Purkinje cells	Enteric ganglion cells
Nigral neurons	Olfactory epithelia
Striatal neurons	Radial neuroglia
Septal neurons	Schwann cells
Hippocampal neurons	Dorsal root ganglion cells
Retina	Autonomic ganglion cells

Retinal glial cells	Neural crest cells
Whole eye	Neuroblastomas
Embryonic motor neurons	Ageing neuroglia
Adult neurons	Glioblastoma cells
Adult astrocytes	Stem cells
Neonatal oligodendrocytes	Primary neurons
Microglia	Brain epithelia

Table 36. Most of these preparations are described in Cohen and Wilkin, (1992), Wood, (1992), Dunnett and Björklund, 1994, Castellano-Lopez and Nieto-Sampedro, (2001).

Variables in the culture conditions for the growth and separation of different kinds of cells

Species of animal	Growth factors added
Age of animal	Whether serum is added
Mechanical dissociation	Freshness of the serum
Enzymic dissociation	Coating of slides
Whether tissue is frozen	Electroporation
Buffers used	Cell density
Whether tissue is centrifuged	Age of culture
Media in which centrifugation and filtration are carried out	Temperature
Composition of culture media	Concentration of carbon dioxide

Table 37. These are decided by research workers who find that they cause particular identifiable cells to grow well for prolonged periods, or to multiply.

Initially, when cells are placed in the growth medium, they become spherical. Some die. Others become flat and their cytoplasm becomes more evident. Many of them - whatever their origin - put out fine processes. These are called 'neurites', when they grow from autonomic ganglia. The processes gradually spread across the media. Some, such as epithelial cells, tend to remain in aggregates. Many cells from specialised tissues, such as brain and epithelia, form flat sheets or monolayers. Over periods of days to weeks, cells change from spherical to flat, to star shaped, and then assume an 'adult' shape. Often, after some generations, they 'de-differentiate' - that is become less and less distinguishable morphologically. Changes in shape are gradual but most cells in culture end up as lozenge-shaped, squareish or with fibres

growing out into the media. The biologists recognise them by their provenance.

When cells die, intracellular movements of all kinds stop. The cells usually shrink, they have blebs on their peripheries, and the organelles become indistinct from one another. Many granules are seen, and finally, they appear to disintegrate. In mixed cultures, in which phagocytes are present, the disintegrated particles are engulfed by phagocytic cells.

Tissue cultures are similar to the tissues from which they come in some ways (Table 38) and very different in other ways (Table 39). It is clear that although there are a few properties in common, there are substantial differences. This is one of the most important questions, in respect of the usefulness of tissue cultures as sources of information about cells in intact animals.

Firstly, a culture is composed of a particular relatively pure population of cells of a particular kind, whereas the body is composed of a large number of different systems, each composed of particular mixtures of cells. Furthermore the cells in culture are significantly different in morphology, biochemistry and environment from the cells from which they originated. The relationship of the tissues in life to the circulation and hormones is different from that of the cells in the culture media.

Secondly, although it is generally assumed that the biochemistry of the cultured cells is the same as that in the original cells, there are rather few chemical experiments comparing them. Furthermore, their staining and immunological properties have not been shown to be similar. From the literature, it is not clear whether such experiments to compare them have been carried out, but it is likely that the experiments have not been published, or they have not been carried out.

Thirdly, the different cells are prepared by different procedures in different chemical environments, and the fact that the chemistry of the environment determines the chemistry of the culture totally, makes it not at all surprising that the cells grown in culture under different conditions have completely different chemical and electrical properties.

Fourthly, it is widely assumed that since intracellular movements occur in cells in tissue culture and in unicellular animals, they probably also occur in cells in intact animals. This assumption is probably warrantable.

Fifthly, most tissue cultures are grown from embryonic, cancerous tissue or stem cells, whose chemistry is different from that of adult tissue (Pollard and Walker, 1997).

Sixthly, many epithelial cells have been grown in culture, including haemopoietic cells, endometrial cells, tracheal cells, mammary cells, corneal cells, skin, thyroid, lymphocytes and macrophages (Table 35). It is hoped that in the future they may be used to manufacture blood, and repair damaged epithelia.

Seventhly, it is usually assumed that the immunological properties of cultures are similar to those in the original tissues, which usually have not been tested. Cells, tissues and organs may change their antigenicity in culture.

Similarities between cells in an intact animal and in tissue culture

Both are derived from the same tissue
They show intracellular movements (assumption)
They contain the same genetic material, which, however, may be modified during frequent passages in culture
They may exhibit the same physiology, although the tissue culture may react at different rates
They share some of the same biochemistry, but the tissue culture may exhibit it with different intensity
They may be excitable if the original cells are excitable
They are claimed to have a wide range of ionic channels

Table 38.

Differences between cells in living intact animals and in culture

<i>Whole animal</i>	<i>Culture</i>
Animal intact	Usually only part of an organ
Homeostatic control	No homeostatic control
Cells normal shape	Cells different shapes
Normal environment of tissues	Grown in abnormal chemical environment
Atmospheric plus tissue pressure	Atmospheric pressure
All tissues bathed by extracellular fluids, such as plasma, lymph or cerebrospinal fluid	Tissues bathed only in particular culture or growth conditions, which are usually artificial
The oxygen and the substrates are derived from capillary circulation	Oxygen diffuses from medium
Excretory products are removed by the capillary circulation	Excretory products diffuse into the medium and may be neutralised there
Energy derived mostly by oxidative phosphorylation	Energy derived by glycolysis
Tissues are normally sterile	Cultures may be contaminated by viruses, fungi, bacteria, mycoplasmas or Hela cells
Long life span	Short life span
Phagocytosis	Phagocytes may not be present

Original staining properties	These have not yet been compared with those of parent tissue
Too thick to examine cells in situ	May be seen by phase contrast, anopteral, enhanced contrast or confocal, microscopy
Cells tend to be three-dimensional	Cells are flattened
Except in the circulations, cells, epithelia and muscle, can not move any distance	Cells move, especially after placing in culture, and growth
The cells are not subjected to the conditions of isolation or culture	Nothing is known about the effects of: death of animal, excision of the tissue, homogenisation, cell separation or the addition of enzymes such as trypsin or hyaluronidase on properties of the cells
Cells grow slowly	Cells grow rapidly

Table 39.

Chapter 13

Tissue Slices

Warburg (1923) measured the oxygen uptake in slices of brain and retina manometrically. He concluded that in view of the rate of diffusion of oxygen in water, slices should not be thicker than 300-500 µm thick in order to ensure that the centres of the slices were fully oxygenated. Under these conditions, the slices of tissue respired linearly, and substances could be applied to alter their rates. Later, it was realised that the same manometry could be applied to measurement of the rates of any reactions, which took up or gave out any gas, including oxygen, carbon dioxide and ammonia (McIlwain, 1951; Dixon, 1952; McIlwain, 1962; McIlwain and Rodnight, 1975; Umbreit, Burris and Stauffer, 1972); this included many enzyme activities. The same procedure was used on homogenates, tissue cultures, single cells and even single nuclei. In addition to slices of the brain, slices could also be made of cerebellum, spinal cord, liver, kidney and muscle. However, most extensive studies have been carried out on *cerebral* slices by Krebs and McIlwain in England, Elliott and Hertz in Canada, Franck in Belgium, Yamamoto in Japan and their collaborators. In view of the large preponderance in the literature of the use of cerebral slices over the other tissues, this account will concentrate mainly on the former.

In consideration of the use of slices of tissue, - as in international treaties -, most of the devils are in the detail, so that it becomes necessary to look carefully at each step of preparation.

An animal is killed, usually without anaesthesia, by neck dislocation, guillotining or neck concussion. Neck concussion increases intracranial pressure in the latter. It is reasonable to believe that neck dislocation is both very painful and stressful to the animal. A more humane method, which should be more widely used, would be to anaesthetise the animal with, say, ether or chloroform, or with intraperitoneal thiopentone, and kill it by exsanguination, while it is anaesthetised. The use of this technique implies that the physiological effects of anaesthesia itself are likely to be less than those of neck dislocation. The guillotine could also be painful and traumatic to the mouse, rat or guinea-pig. Indeed, their eyes often follow the merciless operator, after they are decapitated. This is most easily explained by the fact that the suddenness of the execution leaves a significant quantity of oxygen in the blood and tissues of the head, to permit nervous activity for a few seconds until the

blood pressure falls. Of course, guillotining and neck concussion are not carried out on anaesthetised subjects. Neck concussion - the commonest method used - renders the animals immobile immediately, and it is hoped that they are insenseate. The animals are actually killed by cutting their carotid arteries, and hanging them upside down to drain the blood. It is likely that the blow to the neck causes a sudden severe increase in intracranial pressure, and possibly, tears off diploic vessels, causing instant unconsciousness, as occurs when a boxer knocks out his opponent. The latter condition is often followed by retrograde amnesia. It is really not known which of the methods of killing an animal causes it least pain and stress.

The skull is opened immediately and the brain is cut at the level of the foramen magnum. The whole brain is put on a block, and a guide is applied to it. The guide is thickened at the edges by 200, 250 or 300 μm , which gives the thickness of the sections at the moment when they are cut (Figure 6). Sections are cut with razor blades, but the brain, must be compressed to hold it on to the block, while it is being cut. The pressure applied is not known, but it is enough to make the cortex ischaemic. Compression may damage the cells and capillaries. Certainly, the pressure is much higher than that to which the brain would be subjected in the intact animal.

The guides and the razor blades are often coated with silicone to prevent the tissue slices adhering to them firmly. Some authors wipe off the cerebral sections with a paint brush wetted with saline, others float the sections off on to a Krebs-Ringer saline, perhaps bubbled with 100% oxygen, or 95% oxygen and 5% carbon dioxide, depending on the buffer used. When the slices are freed into saline, they take up some of it, and lose soluble materials into it immediately. The weight of the slice changes at this stage.

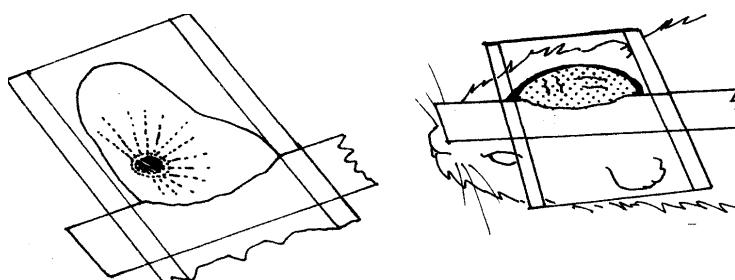


Figure 6. *Left*, cutting a brain slice form an excised brain. *Right*, it is possible to cut a slice directly from the brain of an anaesthetised animal (Hillman, Campbell and McIlwain, 1963).

The slice is then taken from the saline, or off the blade on to a fine hook. It is usually dabbed on a watch glass or ceramic, until the research worker judges that all the adherent medium has been removed. Occasionally, a piece of the tissue is detached. The decision about when all adherent medium has been removed is subjective and unmeasurable, but it is assumed to be approximately constant for a particular research worker. It determines what is considered the weight of the slice of tissue. The slice is then homogenised to make the relevant measurements. Homogenisation is carried out in trichloracetic acid (McIlwain and Rodnight, 1962). If one is measuring, say K^+ or DNA in the tissue, known concentrations of these purified solutions are used to calibrate the flame photometer or spectrophotometer. They are *not* calibrated using a ‘recovery’ procedure, so that results of the experiments must be regarded as qualitative rather than quantitative. The *different* weights of the tissue which may be used are shown (Table 40).

Different weights of tissue slices

<i>Weight of slice of tissue</i>	<i>Comment</i>
Real weight in intact animal at 37°C	true weight, possibly unknowable
Compressed weight at the time of section	some fluid squeezed out by pressure when cutting
Weight after contact with saline on blade or guide at room temperature	some exchange of water and solutes in slices with saline
Weight when removed from blade or guide without contact with saline	small pieces of tissue may remain on blade or guide
Weight immediately after contact with oxygenated incubating medium at 37°C	exchange of solute between slice and incubating medium
Weight at the end of incubation	tissue swells, probably with medium, minus lost solutes
Weight after dabbing off adherent fluid	subjective decision of operative about when to stop dabbing; bits of tissue left on glass while dabbing
Protein in slice at any of the above stages	depends upon method of measuring
Dry weight in slice at any of the above stages	drying causes loss of substances other than water
Non-inulin weight	calculated

Table 40. *Each could be significantly different. Authors do not always specify which they have used.*

It is clear from this table that it may be impossible to know the real weights of tissue slices. In experiments, tissue slices swell by 20-30% of their fresh weights immediately they contact saline. This creates an unanswerable question, 'What is the nature of the swelling?' If it were just water, one could correct the weight by the percentage of swelling. It is rather *unlikely* to be water alone; it is more likely to consist of incubating fluid. However, there is no reason to believe that soluble materials do not leave the slices. Although the late Professor Henry McIlwain, the most distinguished exponent at the use of cerebral slices, believed this to be the case, the research worker or technician, who did the experiments, could clearly see that 50-100 mg of tissue made 2.5 ml of incubating fluid cloudy. As far as I can find in the literature, the chemical composition of the incubating fluid, after the tissue was removed, has not been examined. It would give one some idea of what the tissue had lost. The quantity and composition of the incubating fluid taken up by the slices is unknowable, but it seems much more likely to consist of incubating fluid than water.

Histology of a cerebral slice of 300 μm thickness cut dry showed about the three layers of the cerebral cortex of Brodmann (Hillman and McIlwain, 1961), as one finds in whole brains. After incubation, the same histological procedures show considerable tissue swelling, vacuolation and disintegration at the edges (unpublished).

The following assumptions are implied by the use of tissue slices:

- (a) that the changes during the killing of the animals do not affect the chemistry of the slices irreversibly;
- (b) that the pressure on the organs during cutting do not change these properties;
- (c) that substances leaving the tissue, and entering it after cutting and during incubation, do not affect the biochemistry;
- (d) that one can know the weight of the slices accurately;
- (e) that one can know the volume of the extracellular compartments;
- (f) if the information sought in tissue slices is intended to be relevant to the whole animal, one must assume that the homeostatic mechanisms in the whole body have no significant effects on the chemical properties of the tissue slice. This was tested in respect of the effect of Li^+ salts (0.5-2 mm) on the concentrations of K^+ , Na^+ , Ca^{2+} and Mg^{2+} in cerebral slices (Wraae, Hillman and Round, 1976). Different results were obtained in the intact animal (Wraae, 1980). This crucial experiment is rarely

carried out. Obviously, one must prefer the validity of experiments *in vivo* over those *in vitro*.

The properties of cerebral slices have previously been examined (Hillman and Wraae, 1981). The following aspects were discussed there, where full references are given, (see also Richards, 1981);

- (a) they take up oxygen linearly, and this is increased by high K⁺ and electrical stimulation;
- (b) they carry out glycolysis, also increased by high K⁺ and electrical stimulation;
- (c) they concentrate K⁺ 6-10 times more than the incubating medium;
- (d) they contain some phosphocreatine and ATP after incubation;
- (e) some cells in them have resting potential differences, and discharge spontaneously on penetration;
- (f) if one cuts the slices not tangentially as described here, but radially, slices of piriform cortex show 'synaptic' activity. (It was an important idea to cut slices radially in the direction of the axons, rather than tangentially, as McIlwain and his colleagues (including myself) had done until then (Yamamoto and McIlwain, 1966));
- (g) cells with resting potentials are depolarised by high K⁺ concentrations and electrical stimulation;
- (h) recovery of cells with resting membrane potentials in slices after electrical stimulation was affected by the presence of 0.3 mM phenobarbitone;
- (i) facilitated diffusion of sugars and aminoacids has been demonstrated, but in view of the unknown biochemical status of the slices, the value of these findings is difficult to assess (Joanny, Corriol and Hillman, 1969; Joanny et al, 1971; 1973).

The electrical stimulation that McIlwain and his colleagues used was 5V for 10 minutes, at 100 hertz, square waves, lasting 0.3 msec. This is massive, compared with the stimulation used to induce an action potential in a nerve, for example, one tenth of a volt for 1 millisecond. Calculation of the temperature rise in the cerebral slice, knowing the voltage applied and the equivalent resistance, gives an overall rise of 3-4°C. Furthermore, the heat would be dissipated at the points where the slice is in contact with the grid, so that the temperature could be much higher at these contacts. Indeed, slices were often charred, and one was advised to scrape off the charring, before use of the grid. However, this massive electrical stimulation was regarded as a model for physiological electrical stimulation (McIlwain and Rodnight, 1962).

Perhaps even more alarming was the neglected but important study of Schultze, (1980). He stimulated retina and cerebral slices. However, before doing so, he passed the same currents through incubating fluid in the absence of tissue. The latter caused hydrolysis, acidification, and release of silver ions. This produced the same biochemical changes in the slices as had the electrical stimulation. Therefore, one has to conclude that the effects on cerebral slices were due to the change of chemical composition of the incubating medium, consequent upon the passage of electricity, and *not* the current itself. These two considerations cast a deep shadow over the very large number of experiments in the literature in which massive amounts of electrical energy has been imposed on rather small slices, as being useful models for the biochemical changes during normal electrophysiological activity. Alternatively, one could posit that action potentials normally work by electrical energy, perhaps as heat, being delivered to the tissue.

A further particular problem about cerebral slices was pointed out by McIlwain and Bachelard, (1985, pages 54-60); they compared oxygen uptake, ATP and phosphocreatine in cerebral slices with the same parameters calculated from experiments *in vivo*. They found them to be about 60% in the slices of the parameters in life. They attributed this deficit to the lack of neurological input. However, when they stimulated the slices, although the oxygen uptake rose to average rates *in vivo*, the other parameters fell. Therefore, I think that a better interpretation of the slice was to regard it as 60% ‘undamaged’, and 40% ‘damaged’. There are no objective criteria by which this difference may be settled.

Chapter 14
Isolated Cell Bodies

The commonest and simplest cells, which can be isolated by relatively non-energetic procedures, are buccal epithelial cells, neuron cell bodies, autonomic ganglion cells, dorsal root ganglion cells, rods, cones, hepatocytes and muscle cells.

Cells are isolated as follows: an animal is killed humanely. A small piece of tissue, rich in a particular cell type, or a ganglion is placed in an incubating or growth medium, or in 0.25 M sucrose. Human brains can be obtained at autopsies, but, in Britain, one has to obtain permission from the families. Furthermore, human tissues must be handled in a high grade, sterile laboratory, with sterilised dishes, reagents, microscopes, etc., because of the rare possibility of AIDS.

Ganglion cells are isolated very easily (Figure 7).The ganglia are dissected out and their capsules are torn with mounted needles. The ganglia are shaken in the incubating fluid. Hundreds of spherical or oval cell bodies are liberated (Figure 8).

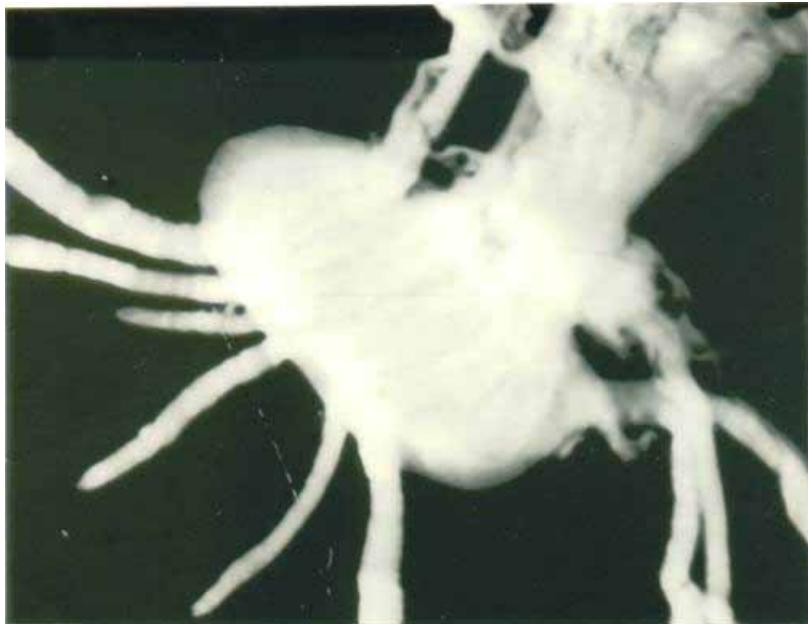


Figure 7. Rat autonomic ganglion with capsule. Its maximum diameter is 3 mm.

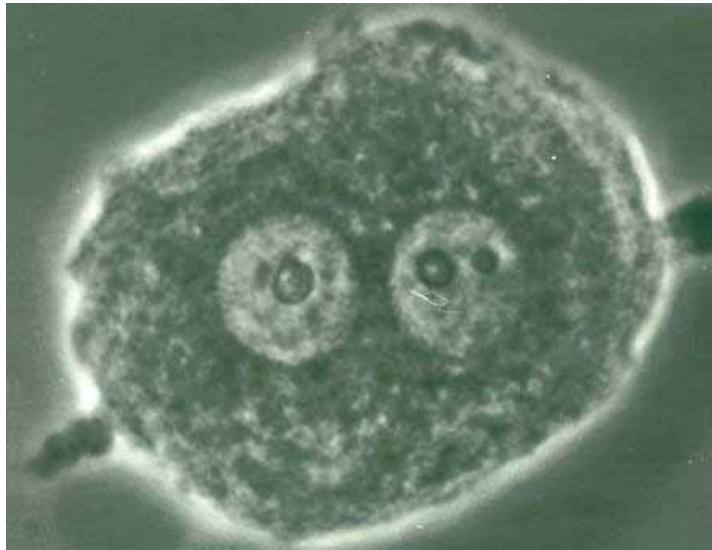


Figure 8. Isolated rat sympathetic ganglion cell, with two nuclei, which is common in this type of cell. Phase contrast. The maximum diameter is 70 μm .

Such cells can be manipulated by hand, using mounted glass rods or stainless steel wires. The wires are of 30, 50 or 70 μm in diameter. One can also use mounted human or pig eyelashes, or eyebrow hairs. Professor Holger Hyden found that all nerve cells rapidly adhered to very clean glass (personal communication). They stuck so well that one could carry out a whole haematoxylin and eosin staining procedure, without dislodging the majority of neuron cell bodies, (Hillman, Hussain and Sartory, 1976). Hyden's technique, (1959) was described in detail later (Hillman, 1986b).

Rods and cones can be easily isolated by cutting out a retina under a dissecting microscope, detaching the sclera, and simply shaking the retina several times in the incubating fluid. Individual rods and cones can be identified by phase contrast microscopy using an objective of $\times 40$, or oil immersion, $\times 100$ (Figure 9). Hepatocytes are easily separated by cutting out pieces of liver 5 - 10 mg, putting them in to saline, and ejecting them through a syringe with a fine needle. Hundreds of isolated hepatocytes can be seen in any field (Figure 10). Muscle cells can be teased apart under a dissecting microscope, using the finest commercially available mounted needles.

Human buccal cells can be isolated as follows. The mouth is washed out with saline three times. The inside of the mouth is then scraped gently with a clean plastic

spoon. The cells appear as an emulsion on the edge of the tip of the spoon. The tip is agitated into a drop of saline on a microscope slide. Using a dissecting microscope, they may be teased under direct vision with mounted needles. (Expensive micromanipulators are not necessary).

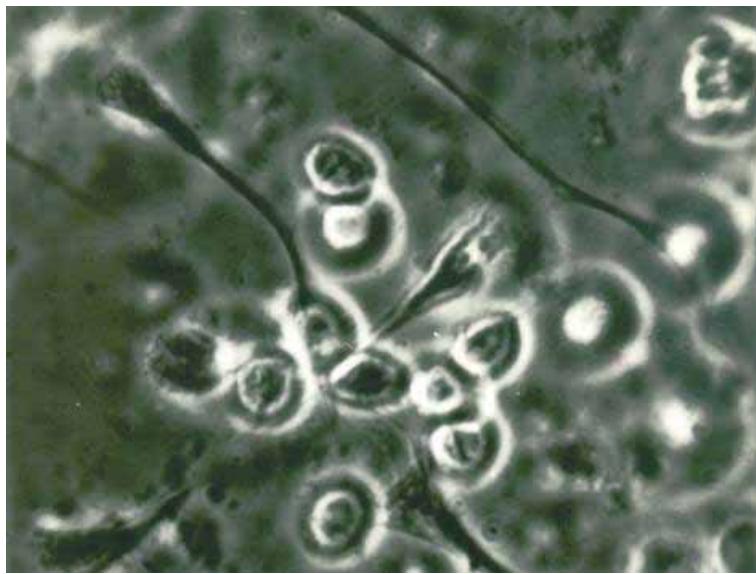


Figure 9. Isolated rabbit retina rods (elongated), cones (spherical). Phase contrast.
The diameters of the cones are 8 - 10 μm .

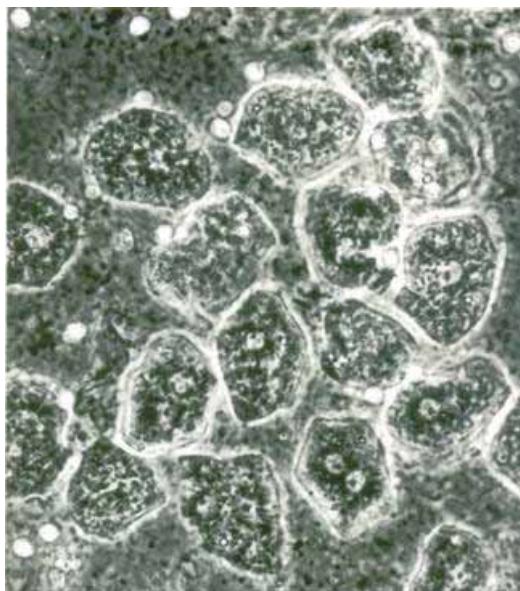


Figure 10. Separated rat
hepatocytes. Phase contrast.
Their diameters are 50 - 60 μm .

In addition to neuron cell bodies, one can isolate neuroglial ‘clumps’ from the areas adjacent to the cell bodies (Hyden and Pigon, 1960). These appear white and fluffy. Hyden used to separate a ‘clump’ which looked about the same size as the cell bodies, although the volume of the clump was unknown. He chose cell bodies of the Deiters’ nucleus of the rabbit, which he judged by eye to be relatively uniform in volume, enabling him to talk about, for example, enzyme activities per cell.

It is also easy technically to pluck out single cells from any living tissue culture, but I am not aware of this having yet been done.

The assumptions inherent in the use of these procedures are as follows;

- (a) their biochemical properties are not changed by the killing of the animals;
- (b) the cooling of the tissue from 37°C to room temperature during transfer does not affect the properties of the cells.
- (c) that reagents, such as sucrose, edta, trypsin or hyaluronidase, used during separation of the cells, do not affect their biochemistry;
- (d) that their being pulled away physically from the adjacent cells and their normal environments does not change their chemistry or physiology significantly;
- (e) that transferring the cells on microwires, detaching them from slides, and putting them into cavity chambers, does not affect their biochemistry.

Isolated cells can be used to measure respiration, enzyme activities, membrane potentials, intracellular movement, and the affects of the addition of other agents to these (preferably in physiological or pharmacological concentrations). They are incubated in plasma, serum, cerebrospinal fluid, Krebs-Ringer, Hanks solution or a growth medium.

Of course, these cells do not have precisely the same properties as they would in the tissues from which they originate, but they are the nearest one can approach to the shapes, dimensions, and intracellular movements, of the living cells in intact animals. It seems that part of the unwillingness to use these preparations is a mistaken belief that the procedures are complicated, or that the operatives need a high degree of skill.

Pathologists examining sputum, bronchial samples, and cervical samples, usually fix them with Papanicolaou’s stain.

Nevertheless, one must bear in mind that procedures of isolating cells or cell bodies, involve pressure, shear, hypoxia, breaking connections and exposure to bright light.

There are many phenomena which could still be studied in unfixed cells, provided they were isolated using minimum reagents and manoeuvres, under clean conditions. They can be examined for up to about 2 - 3 hours after the death of the animal or patient. Post mortem samples from human beings can be used for morphological studies after several days in refrigeration, (Hillman and Jarman, 1991), but not enough investigations have yet been published on their biochemical or microbiological status after these intervals.

Phenomena which could be studied in unfixed cells

Particles entering and leaving cells	Aging
Diapedesis	Allergy
'Active' transport	Anaphylaxis
Pinocytosis	Effects of freezing tissues
Phagocytosis	Cerebral oedema
Location of action of drugs	Regeneration of nerves
Communication between cells	Effects of radiation
Growth	Burns
Fatigue	Necrosis
Muscle contraction	Events during dying
Nature of cytoplasm	Post mortem changes
Nature of axoplasm	Diseased tissues
Effects of hormones	Transmission

Table 41. See also Table 147.

Chapter 15
Homogenisation and Centrifugation

Homogenisation and centrifugation are both widely used in biochemistry in addition to their use in subcellular fractionation (please see Chapter 7), so that it is appropriate to summarise the effects of these manoeuvres.

Whole organs show extremely small enzyme activities, and it is difficult to extract substances for measurement. Substrates may not be able to enter rapidly, and macromolecules can not leave, intact organs are surrounded by capsules, and cells are enveloped by membranes.

When a tissue is homogenised, a solution such as sucrose or Ficoll is added to decrease its viscosity. The effects of the homogenisation in such media are as follows:

- (a) the tissue is diluted by, and exchanges with, the solutions;
- (b) the sucrose or Ficoll have their own chemical effects on the tissues, especially on the enzymes within them;
- (c) the solutions may have osmotic effects;
- (d) if they contain edta, this will chelate Ca^{2+} and Mg^{2+} , resulting in the inhibition or activation of all enzyme activities affected by these ions (Table 11), and any further reactions dependent upon the products of the latter reactions;
- (e) friction and shearing occur between the plunger, the homogenising tube and the tissue, causing unmeasurable rises in temperature around the particles;
- (f) cell membranes and tissue compartments are disrupted, allowing soluble compounds to spread throughout the homogenates;
- (g) any enzyme, activator or substrate may redistribute, so that the enzyme activity required in the homogenate may not indicate the value in the organelles in the living intact cells;
- (h) the affinities of any ligands in the tissues may be changed by the homogenising fluid, the friction, and the temperature rise on particles;
- (i) homogenisation exposes a much larger area of the tissue to reagents;
- (j) some tissue is lost during the homogenisation.

Centrifugation is usually carried out after further reagents have been added. 'Mild' centrifugation may be carried out on a desk-top centrifuge at up to 5,000 r.p.m. for a few minutes, but in subcellular fractionation, 10 - 250,000 g is applied for 10

minutes to 2 hours. This is the centrifugal force defined by the instrument. Most of the energy used is dissipated in the bearings and in compression of the homogenate. Some proportion of it separates the different particles into different layers. Nevertheless, the choices of, centrifuge, temperature, the particular fluid in the centrifuge tube, the centrifugal force, the duration of the centrifugation - including the rates of rise to maximal force and the rotor gradually stopping, - are determined by the operative based on the microscopical appearances of the fractions being separated. The force on any particular particle is determined by the centrifugal force applied to it, its size and density, each of which are very likely to be affected by homogenisation.

Since a particular fraction has been subjected to a particular g force (depending on the radius of rotation), a particular temperature of the centrifuge, a particular duration of the centrifugation, and a particular sucrose concentration, it is unlikely that it would exhibit the same chemical properties, as any other fraction. This would even be true, if one were to subject the *same* homogeneous preparation to all the variables used in practice to separate the different fractions. One would expect each fraction to show different: enzyme activities; ion concentrations; protein saturation; water content; binding properties; density; viscosity; dimensions.

When a tissue is centrifuged, it is subjected to: (a) the reagents in the centrifuge tube; (b) dilution by the latter reagents; (c) increased entropy; (d) compression; (e) friction; (f) shearing; (g) diffusion; (h) heat dissipation.

All the effects of both homogenisation and centrifugation can be diminished by: carrying out procedures for the shortest possible times; using isotonic solutions; using least power; avoiding the use of gradient centrifugation; carrying them out as slowly as possible, so that the heat generated can be dissipated before the temperature rises; using heat conducting tubes for the same reason.

Unfortunately the idea that cooling of the homogenisers with ice, or the centrifuges by refrigeration, *prevents* temperature rise in the preparation, is not necessarily true (please see page 56). The relevant control procedures for testing the effects considered here are listed (Table 21).

Chapter 16

Extracellular Electrodes, Ionophoresis, Intracellular Pipettes and Patch Clamping

Extracellular electrodes are made of tungsten, silver or stainless steel. They have tip diameters of 1-5 μm ; they detect the frequency of action potential ('spikes') of 1-2 mV amplitude in nerve and muscle cells. They are used mainly for detecting neural pathways. Since the electrodes are made of metal, they can not be used for penetration of cell membranes (Bures, Bures and Zachar, 1967; Snodderly, 1973).

Ionophoresis is carried out by driving ions, aminoacids or transmitters to the internal or external surfaces of neurons and muscle cells, with double-barrelled or concentric micropipettes. From one pipette, the substances are driven electrically, while the other inside the cell records the resting and action potentials of the same cell (Coombs, Eccles and Fatt, 1955a,b; Watkins, 1981). This procedure involves certain assumptions: firstly, those arising from the use of liquid filled micropipettes as electrodes (please see below); secondly, that the passage of the current from the extracellular electrodes to drive the substances on to the surface of the cells, propels out only the ion or transmitter of interest to the research worker from the tip of the electrode, ignoring other ions; thirdly, that some of the current from the ionophoretic pipette does not leak, or is not capacitatively coupled, to the recording pipette. The effects of all these potential sources of current are probably very small, because some ions and transmitters depolarise, while others hyperpolarise.

Intracellular pipettes used for measuring membrane potentials, ion concentrations, pHs, and for patch clamping, involve the same general principles. The measurements of a potential difference at the tip of the pipettes are transduced into an electrical signal. Although one speaks generally of a glass micropipette, it is the electrolyte in it which detects the signal, and the glass is the insulator. The micropipettes used for intracellular penetration have the paradoxical properties of having very high resistances and impedances, because their tips are 1 μm or less in diameter, while, on the other hand, the contents of the pipettes are in touch with the fluid media, and the extracellular and intracellular fluids.

The resistances, impedances and capacitances of the micropipettes are so high that the input resistances and impedances of the recording amplifier must be greatly increased by interposing a cathode follower or a field effect transistor, between the

recording pipette and the amplifier. Micropipettes, also referred to as intracellular microelectrodes, are used to record resting and action potentials of nerve cells, muscle cells, hepatocytes, pancreatic and many other cells. There is a large amount of science, skill and artistry in making micropipettes, and this has spawned a plethora of publications (see, for example, Ling and Gerard, 1949; Lavallee, Schanne and Heber, 1969; Koryta, 1975; Thomas, 1978; Corey and Stevens, 1983; and Ogden, 1994). Relatively, little recent literature has been published about the assumptions inherent in their use.

Among the technical problems is the fact that their electrical properties are influenced by how they are filled with the electrolyte. The finer they are, the less they will damage cells, which they are penetrating, but the more they are at risk of being blocked up by tissue. The cathode follower, if necessary, assisted by a Wheatstone bridge, attempts to compensate for the resistance and the capacitance of the micropipettes, but these two properties are continuous and three dimensionally distributed, and are only virtual, in comparison with the geometrically symmetrical and localised resistors and capacitors, used to compensate for them.

The fluid in the micropipette is only connected through the very small hole at the tip to the extracellular fluid or cytoplasm. Nevertheless, one has an unstable equilibrium because the two fluids are intermixing continuously, of course, more rapidly when current is passed through them, during stimulation or patch clamping.

The full recording system is as follows:

- (i) the fluid in the micropipette, which consists of high concentrations of K^+ , Na^+ , Cl^- , or citrate ions;
- (ii) the fluid in the half cells, of similar salts, with 0.5 - 1% agar added to prevent it flowing away;
- (iii) the AgCl deposit of the silver wire, which must not be scratched in any way;
- (iv) the silver wire;
- (v) the cathode follower or field effect transistor, shielded by a metal tube connected to the cathode (if the whole apparatus is not enclosed in a Faraday cage);
- (vi) an a.c., d.c. 'push-pull' amplifier;
- (vii) an oscilloscope, which can record, say 100 μ v to 10 v, at frequencies d.c. up to 1000 hertz.

The latter system may have cameras, tape recorders, computers, analysers,

scanners and printers attached to it. In addition, there will be an earthing electrode, and there may be a parallel system consisting of a half-cell, feeding into a push pull amplifier, to balance off adventitious signals. There are also circuits for measuring the resistance of the micropipettes, and for calibrating the signals they record. The recording system is far more than a voltameter. Stimulators produce small currents, which stimulate single cells extracellularly, or intracellularly via an intracellular micropipette.

The patch clamp is made by stretching a small piece of membrane of up to 40 µm in diameter across the tip of a micropipette, (Kay and Wong, 1986). Small transient currents (pA) of integral values are recorded, and their frequency can be increased or decreased by passing currents across the membranes, or by holding the membrane voltage constant, using a feedback circuit. The transient currents are generally believed to be due to opening and closing of channels in the membranes.

The resistances and impedances of the pipettes used are between megohms and gigohms, and the currents detected are in pA. Current is passed through the system continuously, to keep the membrane voltage clamped. However, the whole circuit contains an orchestra of real and potential small sources of current and voltage (Table 42). Problems have been examined by Lorente de Nò (1971).

Possible sources of current during intracellular recording and with patch clamps

1. <i>Biological</i>	opening and closing of channels; passage of ions through channels; leak current in the membrane; discharge of membrane current; currents generated in other parts of cells; steady state inactivation of voltage-activated currents; biochemical reactions within cells.
2. <i>Preparation</i>	enzymes, such as trypsin and collagenase, to dissociate cells; chelators to lower the Ca^{2+} and Mg^{2+} ; serum gels and other substances added to the incubating media; substances in the pipettes, such as carbachol, glutamate, ATP and GTP, binding receptors in the membranes; detergents to permeabilise the membranes; divalent ions affecting channel charges or selectivity; substances which replace Na^+ , K^+ or Ca^{2+} ; contamination with foreign substances from syringes, tubes, needles and filters;

3. The micropipettes	ions leaking from them into the cells; different activities of the ions in the pipettes and in the extracellular and cytoplasmic fluids; micropipette tip potential resistances and impedances; different osmolarities of pipettes and cells; blockage of the pipettes by materials other than the membranes; bubbles in the micropipettes; Bernoulli effects in pipette tips, (Bingley, 1964); breaking the pipette tip; noise from the high impedance pipette tip; suction of the patches on to the pipettes; connections between pipettes and half cells;
4. Electronic sources	voltages used for clamping patches; electrolysis and heat at the electrode tips; stimulating or ionophoretic voltages; offset of balance of inputs to amplifiers; offset of imbalance of half cells; noise from electronic components; scratches on the chloride coatings of the half cells; temperature variations in the systems; light on the silver salts of the half cells; Johnson noise.

Table 42. Most of these individual sources of current have been recognised (Fenwick, Marty and Neher, 1982; Neher and Sakmann, 1976; Sakmann and Neher, 1983; Madgleby, 1992; Standen, Grey and Whittaker, 1995; Penner, 1995; Aidley and Stanfield, 2000), but their overall contribution to minute currents regarded as originating in the cell membranes has not been determined.

There are some difficulties with the use of micropipettes as electrodes and as patch clamps.

Firstly, it is impossible to measure the small currents and voltages without complicating the matter with all the other sources of current, listed in Table 42. They can not be eliminated, measured or calculated, with any degree of certainty. Sometimes, the values of the individual sources of the currents may exceed those claimed to come from the cells.

Secondly, control experiments have shown that similar bistable transients can be recorded across membranes made of silastic, or even quite artificial membranes. There is now a large literature on this subject (see, for example, Ruknudin, Song and Sachs, 1987; Lev et al, 1993; Sachs and Qin, 1993; Korchev et al, 1997).

Thirdly, the measurements of membrane potentials by intracellular micropipettes or ion selective electrodes depend upon the chemical environments of say, the K^+ , Na^+ , Ca^{2+} or H^+ on both sides of the cell membrane being the same. However, the latter ions are in completely different chemical mixtures in the extracellular fluid or incubation medium, on the one hand, and the cytoplasm, on the other. Thus, the potential differences between the micropipette and the two compartments are likely to be different, even if the concentrations of the particular ions were the same on both sides of the membrane. There seems to be no way to measure or calibrate the voltages at the tips of the micropipettes for this reason. This reservation was first put to me by Dr Brian Freeman of Sydney University in 1995 (personal communication), and does not appear to have been addressed previously.

Fourthly, it is an assumption that these minute currents originate from channels in the cell membranes. The possibilities must be explored that this assumption is unwarranted, not least, if the ionic channels are artifacts. (please see Chapter 21).

Sixthly, the resolution of the recording probably permits one to conclude that a sudden change of potential difference recorded occurs *across* the membrane, but it does not give any information about whether it is between: the extracellular fluid and the intracellular fluid; the extracellular fluid and the extracellular wall of the cell membrane; the extracellular fluid and the intracellular wall of the cell membrane; the intracellular wall of the cell membrane and the bulk cytoplasm. The general belief is that it is the former of these.

Bernoulli effects can be produced in micropipettes (Bingley, 1964).

Chapter 17

Chemical methods

All chemical methods used after extraction of contents of tissue (Table 43) imply the following assumptions:

- (a) that the procedure itself has not changed the quantity or activity of the substance measured, significantly;
- (b) that the extraction procedures either extracts all or the same proportion from each separated fraction completely;
- (c) that the intensity of the signal produced by the product being measured is not affected by the vehicle in which it is measured or the light source used to measure it;
- (d) that the calibrations have been suitable and that there are recovery calibrations, (please see Chapters 4 and 5).

Measurement of concentrations

Chemistry	Flocculation
Titration	Crystallography
Chromatography	Electrophoresis
Absorbtion	Conductivity
Transmission	Voltammetry
Precipitation	Ion-selective electrode
Colorimetry	Radioactive isotope
Spectrometry	Radioimmunoassay
Spectrophotometry	Protein autoanalysis
Photofluorescence	Immunoassay
Calorimetry	Electron spin resonance
Viscometry	Nuclear magnetic resonance
Refractometry	Measurement of redox potential
Densitometry	Rate of diffusion
Osmolarity	Dichroism
Heat conductivity	Birefringence
Heat capacity	Measurement of binding
Ionic mobility	Partition coefficient
Bioassay	Complement fixation

Table 43. These measurements are often carried out on simplified systems or extracted compounds.

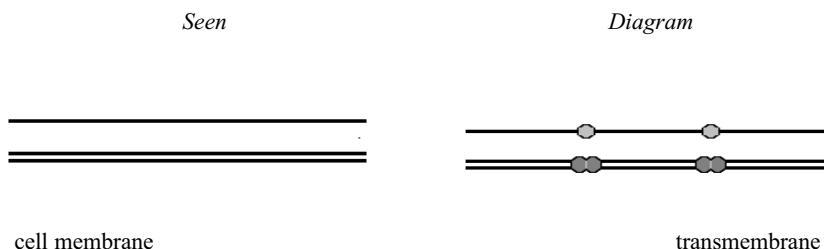
Chapter 18

Diagrams

Several kinds of diagrams are used in cell biology. One kind depicts pathways, such as the citric acid cycle, the electron transport chain, or glycolysis. These show the compounds, the enzymes and the energy changes, involved in the metabolism. They have been shown experimentally to be metabolic pathways, which, rather extraordinarily, are similar in nearly all mammals and many other animals and plants. They are maps of the interactions between the different pathways, but give no information about the rates or locations of the different reactions, *in vivo*.

The second type of diagram is used to show processes, such as transmission, signalling and apoptosis. These represent a depiction of the authors' hypotheses about the order in which a large number of events occur, which make up the particular phenomena. However, many of the alleged events are very rapid, so that they can not be measured while they are occurring. They take place in fractions of seconds, while most measurements take much longer. Many of them are changed by the research procedures. Authors are attempting to put the likely steps within the processes they are studying into a logical, thermodynamically acceptable, order. Nevertheless, one must always bear in mind that the schemes are only hypotheses, and are often neither provable nor disprovable.

Thirdly, there are diagrams representing structures, which can be seen by microscopy. Unfortunately, too often a diagram is significantly different from the structure it is said to depict (Figure 11). Much more populous in the diagrams than in the micrographs are, for example, transmembrane molecules, membrane receptors, nuclear pore apparatus, etc.



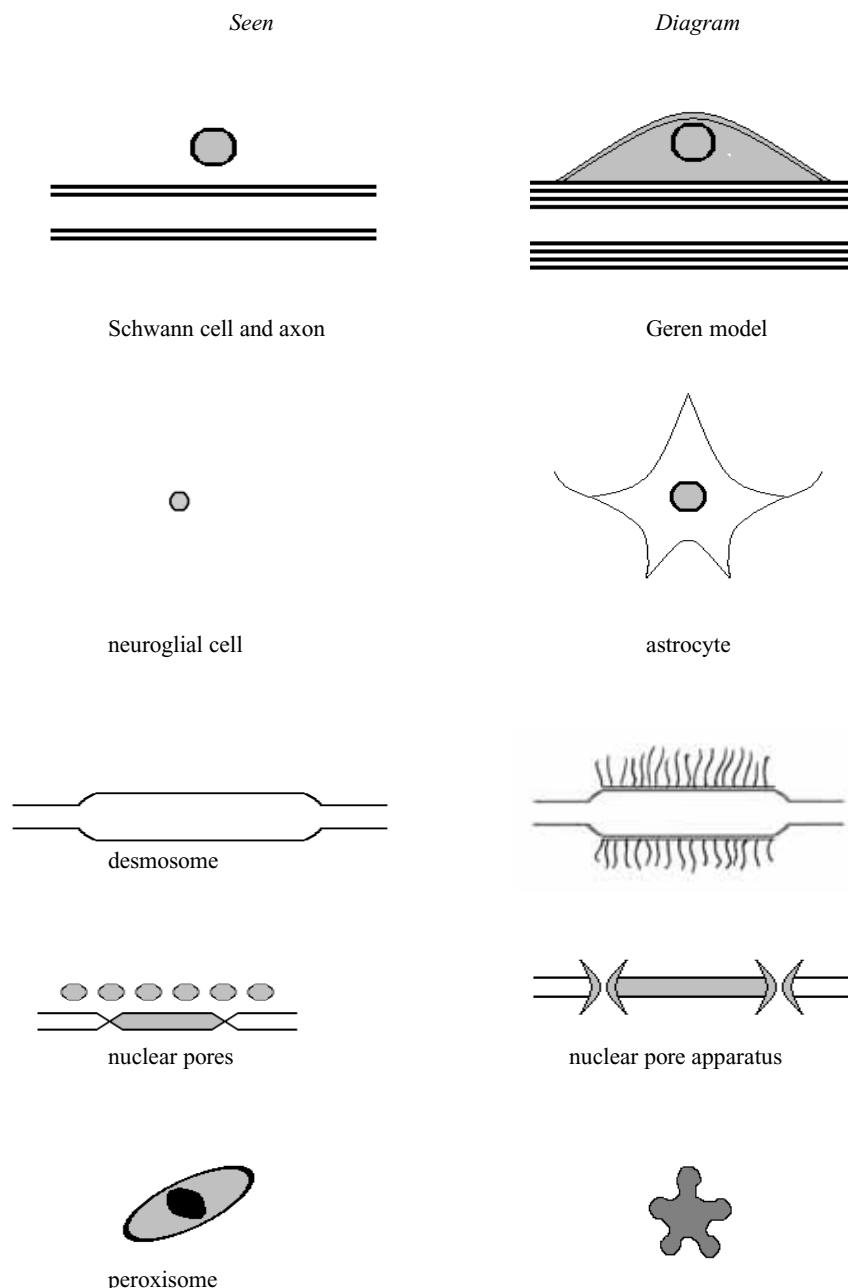


Figure 11. Diagrams of electron micrographs, *left* as seen in micrographs, *right* as seen in text books.

Section B

The Anatomy and Chemical Composition of Cells

Chapter 19
Extracellular Compartment

The extracellular compartment is located between cells, and the extracellular fluids bathe the cells. Traditionally, these fluids are: the serum; the lymph; the cerebrospinal fluid; the aqueous fluid; the vitreous fluid; the pleural fluid; the pericardial fluid; the peritoneal fluid; the synovial fluid and the intercellular fluid (Gamble, 1952). Their chemical compositions are very similar to each other, and generalised values were assigned to the extracellular and intracellular compartments - the latter being indicated for red cells (Figure 12).

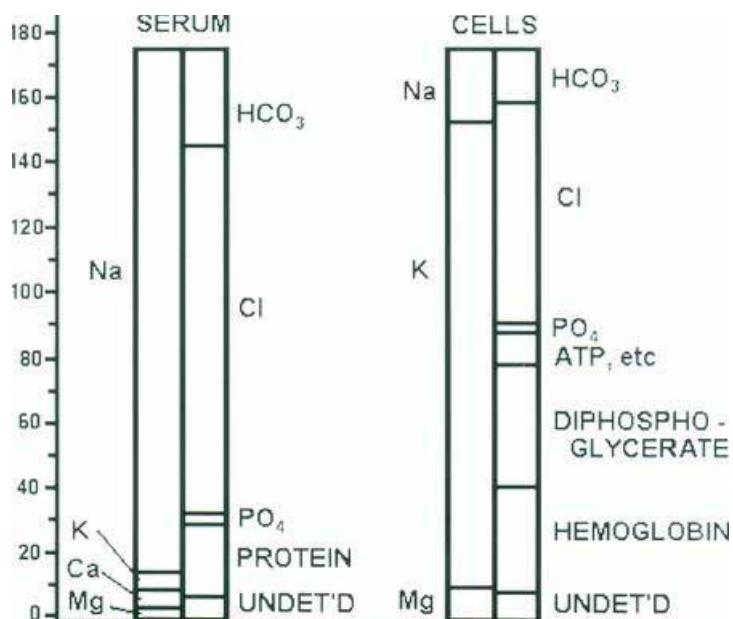


Figure 12. The chemical composition of the extracellular compartment, *left* and the intracellular compartment, *right*, of red blood cells. This is reproduced from Gamble, (1952) by kind permission of Harvard University Press.

The volume of the extracellular compartment is sometimes used clinically to measure that of the intracellular compartment, when dehydration is suspected. It is practically impossible to measure the extracellular compartment around *individual* cells. The following procedures have been tried (a) using a whole tissues or slices and markers, such as inulin, sucrose, thiocyanate, arabinose and Na⁺ (b) measuring the

passage of current across the brain (Van Harreveld 1966), because they extracellular fluid has a much higher conductivity than the cell membrane. These measurements are dependant on the beliefs that there is no significant conduction except along the extracellular pathway between them; (c) measuring the apparent spaces by histology or electron microscopy. The latter techniques are also unsuitable, because they require dehydrating the tissue. The different compartments of the cells contain different proportions of water, so that they each shrink to different extents; (d) one can penetrate a tissue with an intracellular pipette, and calculate what proportion of the distance of penetration is occupied by the pipette recording direct current membrane potentials. The accuracy of this measurement must be questioned, because: firstly, the micropipette tip would push the cells forward or sideways, compressing or avoiding penetrating them, respectively. This can be seen to occur in dramatic pictures of nuclear transplantation; secondly, since most cells, other than egg cells, are not spherical and are not randomly orientated, their dimensions vertically would not necessarily be the same as those at right angles to this plane, so that the proportion of the penetration of cells in the vertical plane is likely to be very different from that at right angles to it: thirdly, it is a very dangerous measurement to make in such tissues as cerebral and hepatic slices, as did Hillman, Campbell and McIlwain, (1963) because they swell during incubation.

It is useful to know the biochemical composition of the fluid around the cells. One may withdraw minute quantities using micropipettes, and use one of the many microchemical techniques, which have been developed to analyse them chemically (Edström, 1953; Giacobini, 1957, 1969; Neuhoff, 1973; Osborne, 1974; Haljamae and Wood, 1971).

Since the cell membrane is semi-permeable, virtually any change in the chemistry of the extracellular fluid must change the chemistry of the cytoplasm, and, subsequently, of the mitochondrioplasm and of the nucleoplasm. Furthermore, the chemoreceptors detect the concentrations of O_2 , CO_2 , H^+ and hormones in the blood, which are continuously changing in the extracellular compartment. A few of the many ways by which changes in the latter may alter the chemistry of the cytoplasm are given (Table 44). It is awe-inspiring how the many feedback mechanisms due to hormones, respiration and excretion, keep the chemistry of the extracellular compartment so constant. The main biochemical parameters in the body fluids found in omnivorous Americans are very close to those found in fish eating islanders and

vegan Jains, despite their vastly different diets. This indicates how effective the extracellular feed back systems are. This constancy of the ‘milieu interieur’ - as Claude Bernard called the extracellular compartment - must keep the cytoplasm, and, in their turn, the nucleoplasm and the mitochondrioplasm even more constant, since they are each furnished about with their own membranes, which must also regulate their contents. Teleologically, one may suppose that the effect of the control of the nucleoplasm within very small limits would result in keeping the environment of the nucleus for cell division remarkably constant, which define the chemical conditions for meiosis and mitosis.

Causes of changes in one phase which may affect another separated by a semi-permeable membrane

Diffusion from one phase to another	Changes of pressure due to muscle contraction and body movement
Mass action	Absorbtion
Substances arriving extracellularly via the circulation	Secretion
Metabolism in the phase	Excretion from another phase
Changes in osmotic pressure	Van der Waal's forces
Changes in extramural pressure from other cells	Phagocytosis
Toxins in the extracellular fluids	Pinocytosis
Membrane leaks	Changes of affinity in the phase
Activation of extracellular enzymes	Changes in transmembrane potential differences
Changes due to feedback systems, hormones and respiration	Changes of permeability of the membranes
Changes in free energy, entropy or temperature in one phase	Electrical or chemical stimulation
Changes in pump pressure in circulations	Drugs in the extracellular fluids

Table 44. See also Claude, (1978).

Chapter 20

The Cell Membrane

Most cells are surrounded by membranes, except for the syncytia, which share communal nuclei and cytoplasm (Table 25). The ubiquity and significance of syncytia are not widely recognised, although there is a considerable literature on ground substances, matrices and connective tissues. There has also been little appreciation of the significance of the syncytial nature of the malignant cells, although the multiplicity and density of their nuclei is well documented.

In this monograph, the term 'cell membrane' is used only to mean the membrane surrounding the whole cell. Other membranes are referred to, specifically, as nuclear, mitochondrial or nucleolar. Other authors use the generic term 'cell membrane' to include all the former, *plus* the mitochondrial cristae, the endoplasmic reticular, Golgi, lysosomal, and myelin lamellar membranes. However, it will be shown that the latter five are artifacts.

The term 'plasma membrane' will not be used, because it implies that the cell membrane is connected to the cytoskeleton or to the cytoplasm - a view widely held among cytologists. The use of the term 'microsome' for the subcellular fraction, believed to consist of cell membranes and endoplasmic reticulum, indicates that one can not distinguish the two in the same fraction, and therefore, many authors talk about the properties of the cell membranes, when they have examined the microsomal fraction. However, a microsome as a body is not seen in electron micrographs.

As pointed out, one can not resolve the cell membrane by light microscopy, or see it by electron microscopy (Chapter 9). Its appearance as having a thickness of 7-10 nm is very unlikely indeed to bear any relationship to its thickness in life. The chemistry of the membrane was postulated by Davson and Danielli, (1936) as being composed of two protein layers sandwiching two lipid layers. This idea came from the experiments of Gorter and Grendel, (1925). They measured the quantity of lipid extracted from red cells, when spread over water, and postulated that the red cell membrane was a bi-lipid layer. This conclusion about red cells was gradually assumed to be true for membranes of all cells, and then the nuclear, mitochondrial, endoplasmic reticulum, and synaptic membranes of all cells.

The thickness of this membrane was found to be approximately the same as the repeating pattern of the lamellae of somewhat dehydrated myelin sheaths by low

angle diffraction (Schmitt, Bear and Clark, 1935; Schmidt, 1936; Finean, 1957, 1960). Later on, Robertson, (1959, 1962) observed that cell membranes appeared 'trilaminar', that is, as two dark lines, enclosing, a lighter region. The whole 'trilaminar' appearance was dubbed a 'unit membrane'. This was regarded as the electron microscopic manifestation of the Davson-Danielli 'bi-lipid membrane'. The latter view was superceded by Singer and Nicolson's, (1972) 'fluid mosaic' membrane. Their contribution was that the bi-lipid layer was punctuated by protruding protein molecules, and that its interior was fluid.

The current consensus nowadays is that: (i) the membranes of the cell, the nucleus, the mitochondria, the endoplasmic reticulum and the myelin lamellae, are represented by the 'fluid-mosaic model'. Incidentally, it is difficult to know why the structure of the membrane should be regarded as a 'model'. If it is believed that the structure is known, it is not a *model*, but a *description*. Perhaps, the use of this term represents an understanding that the current view is only a hypothesis, as Davson and Danielli originally called it. One should go further and say that it is an unproved and possibly unprovable, hypothesis. This is because the chemistry which had been used to define it has all been carried out using destructive and very energetic procedures; (ii) all the above cell membranes appear to have thicknesses of 7 – 10 nm in micrographs; (iii) the insides of the membranes, presumably the lipids, are fluid in life (Does this mean more than that they contain water?); (iv) the cell membranes are punctured by many ionic channels which open during excitation; (v) thousands of different receptors and carriers are in the vicinity of the cell membranes (please see Chapter 23). Some of the channels, other transmembrane structures and macromolecules believed to be spanning the membranes or adherent to them, are shown in Table 45 and 46; (vi) glycoproteins and glycolipids are believed to protrude from the cell membranes in to the extracellular compartments; (vii) the cell membranes are believed to be continuous with channels called 'cisternae' in the endoplasmic reticulum; (viii) all living cells appear to have transmembrane potential differences, which can be depolarised by the hypoxia, lack of substrate, high external K⁺ concentration, or electrical stimulation in excitable cells; (ix) the 'plasma' cell membrane is semipermeable and insoluble, and is impermeable to macromolecules; (x) the myelin sheath is generally considered to be a scroll of cell membranes; (xi) a host of antigens are believed to adhere to them (Table 47).

Classes of transmembrane proteins and peptides

Integral proteins	β -adrenergic receptors
G - protein superfamilies	Vasopressin receptors
Families of 2, 4, 6, 8 oligodimers	Virus ion channels
Connexins	*Extracellular gated channels
Glycophorins	*Intracellular ligand-gated channels
Band 3 dimers	*Inward rectifiers
Families of ion channels for H^+ , Na^+ , K^+ , Ca^{2+} , Cl^-	Intercellular channels
*Voltage dependent ion channels	Cyclic nucleotide – gated channels
17 ATP ion pumps	Rhodopsins carriers
Epidermal growth factors	Water channels

Table 45. Most of these have been identified by the use of ligands on subcellular fractions. *These ion channels have been listed in 2 volumes (Conley, 1996).

Substances believed to be present in or on membranes

A. Proteins	B. Lipids
Integral proteins	Cerebrosides
G -proteins	Cholesterol
88 surface antigens	Phosphatidyl choline
Rhodopsins	Phosphatidyl ethanolamine
Acetyl phosphatase	Phosphatidyl inositol
Acid phosphatase	Phosphatidyl serine
Adenyl cyclase	Sphingomyelin
Alkaline phosphatase	Glycolipids
Several aminopeptidases	C. Carbohydrates
Calcium ion receptor	D-mannose
Cellobiose	N- acetyl - D - galactosamine
Cholesterol esterase	N- acetyl - D - glucosamine
Guanylate cyclase	N- acetylneuraminic acid
Lactase	L - fucose
Maltase	D - galactose
Monoglyceride lipase	
NADH oxidising enzyme	
NAD glycohydrolase	
5' nucleotidase	

Phospholipase A	
Sialidase	
Glycoproteins	
D. Carriers for	E. Channels for
Na ⁺	Na ⁺
K ⁺	K ⁺
Glucose	H ⁺
Glycine	Ca ²⁺
Sphingomyelinase	Cl ⁻
Sucrose	Water
Protein tyrosine receptors	Aminoacids
Connexins	Peptides
Receptors (Table 55)	Toxins
Myelinase	F. Pumps for
	Na ⁺
	K ⁺
	H ⁺
	Ca ²⁺
	Cl ⁻
	Bicarbonate

Table 46. This list is largely from Sheeler and Bianchi, (1987), Playfair, (1992), Lodish, (1988), Lodish et al, (2000).

Most of the substances have been isolated by subcellular fractionation.

Surface antigens

CD			CD		
No.	MW	Cell type	No.	MW	Cell type
1	43-49,000	Thymocyte, APC	41	425,000	platelets
2	50,000	T, NK	42	145,000	platelets
3	16-25,000	T	43	95,000	T, myeloid
4	60,000	T helper, some mac	44	80-95,000	T, myeloid, rbc
5	57,000	some T, B	45	180-220,000	all leucocytes
6	120,000	T	46	180-220,000	T subsets
7	41,000	T, NK	47	56-66,000	all leucocytes
8	32,000	T cytotoxic	48	47-52,000	all leucocytes
9	24,000	pre-B, myeloid	49	41,000	all leucocytes

10	100,000	pre-B,leukaemia	50	(a-f) 120,000-170,000	all leucocytes
11a	180,000	lymphoid, myeloid			
11b	155,000	(adhesion molecules)	51	108-140,000	all leucocytes
11c	150,000				
12	90-120,000	myeloid	52	140,000	platelets
13	150,000	myeloid	53	28,000	leucocytes
14	55,000	mono, mac	54	32-40,000	leucocytes
15	(carbohydrate)	myeloid	55	90,000	act. T.B. mac (ICAM1)
16	50-65,000	myeloid, NK (FCR3)	56	70,000	Leucocytes
17	(lipid)	myeloid, platelet	57	135-220,000	NK, some T
18	95,000	as CD11	58	110,000	NK
19	90,000	B	59	40-65,000	leucocytes, rbc18
20	35,000	B	60	18-20,000	leucocytes, platelets
21	140,000	B (CR2)	61	(glycolipid)	leucocytes, platelets
22	135,000	B	62	115,000	platelets
23	45,000	B, myeloid (FC&R)	63	140,000	platelets
24	42,000	B, granulocyte	64	53,000	platelets
25	55,000	act.T, B, mono(1L-2R)	65	75,000	mono, mac(FCR1)
26	120,000	T	66	(glycolipid)	myeloid
27	110,000	T, some B	67	180-200,000	granulocytes
28	44,000	some T	68	100,000	granulocytes
29	130,000	T, myeloid	69	110,000	mac
30	105,000	act. T, B	70	60,000	act. T, B
31	140,000	mono, granulocyte	71	?	act. T, B
32	40,000	myeloid, B (FCR2)	72	190,000	proliferating cells (transferrin R)
33	67,000	myeloid	73	39/43,000	B
34	115,000	stem cells	74	69,000	B some T
35	160-250,000	myeloids, B (CR1)	75	41/35/33,000	B, some mono
36	90,000	mono, platelet	76	53,000	B, some T
37	40-52,000	B	77	67-85,000	B
38	45,000	act. T, B, thymocyte	78	(glycolipids)	act. B
39	80,000	B	79	67,000	B
40	50,000	B			

Table 47. CD numbers (cluster of differentiation) are used to identify cell surface antigens by monoclonal antibodies. Some of these (e.g. CD3, 4, 8) are also widely

used as markers of particular cell types: act, activated; APC, antigen-presenting cell; CD, cluster of differentiation; CR, complement receptors; FCR, Fc receptor; ICAM, Intercellular adhesion molecule; 1L-2R, 1L-2 receptor; mac, macrophages; mono, monocyte; NK, natural killer. This table is from Playfair, JHL, (1992), by kind permission of the Author and Blackwell Scientific Publishers, Oxford.

Nearly every one of the above expressions of the consensus about membranes is highly unsatisfactory. Each one may be considered separately: (i) The unit membrane is an artifact of electron microscopy (Deutsch, 1962; Deutsch and Mach, 1967). This is because the thickness of the cell membrane, the nuclear membrane, the mitochondrial membrane, the endoplasmic reticulum, the Golgi lamellae, the mitochondrial cristae and the lamellae of the myelin sheath, nearly always appear in micrographs to be cut normal to the plane of section, and the 'laminae' of the membranes appear to be equal distances apart. This defies the laws of solid geometry (Figure 13). The uniformity of distance between the laminae or lamellae has been ascribed to choice of micrographs to illustrate membranes clearly, but when one looks at any section through the electron microscope or in publications, one does not see the cell, nuclear or mitochondrial membranes, except as two clear lines (Chapter 9).

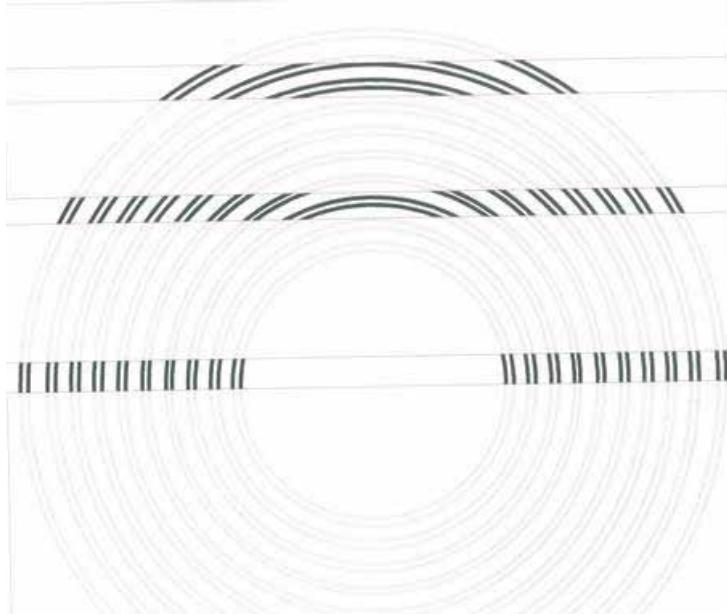


Figure 13. Sections through concentric layers.

The centre of the ‘unit’ membrane, which is assumed to be the lipid in the sandwich, appears pale, not because the lipid does not stain, but because the heavy metal salts *deposit* on both sides of the membrane. A line is a geometrical abstraction, because it has position but no thickness. Every real layer has two surfaces upon which the stain deposits and these can be seen if it is magnified enough. Thus, all one can say about the cell membrane is that it has an unknown finite thickness.

The Davson-Danielli membrane may have a hydrophobic phospholipid core and a protein periphery, but there is absolutely no way of deciding the *orientation* of macromolecules within the membrane, especially bearing in mind that the real chemistry of the membrane is unknown. The knowledge of the latter may be an approximation, but the knowledge of the former must be, and probably will always be only speculation.

Another objection to the ‘unit’ membrane has already been noted. Preparation for electron microscopy involves the use of ethanol, acetone and propylene glycol which extract lipids, so it is difficult to believe that the micrograph shows a lipid-containing membrane.

The Singer-Nicolson membrane (*ibid*) has protein molecules protruding from its surface in all diagrams. Unfortunately, these are not seen in electron micrographs of cell membranes, which appear as smooth as an angel's cheeks.

It should be stressed that the assertion that the ‘unit’ membrane is an artifact does *not* mean that the cell has no outer membrane. It certainly has; intracellular pipettes show sudden changes in phase, when membranes are penetrated.

As early as 1972, Jain recorded sixteen, and Threadgold, (1976) showed eight, different representations of the orientations of the molecules in the cell membrane (Figures 14, 15). The simplest explanation for the number of theories is that there is no way of establishing for certain what the molecular structure of the cell membrane is. Whatever the chemical composition of the membrane of the living cells, and the real orientation of any of its components, (see Robinson, 1975), they will both be altered by subcellular fractionation, and electron microscopy, so that it is extremely unlikely that they are knowable for certain.

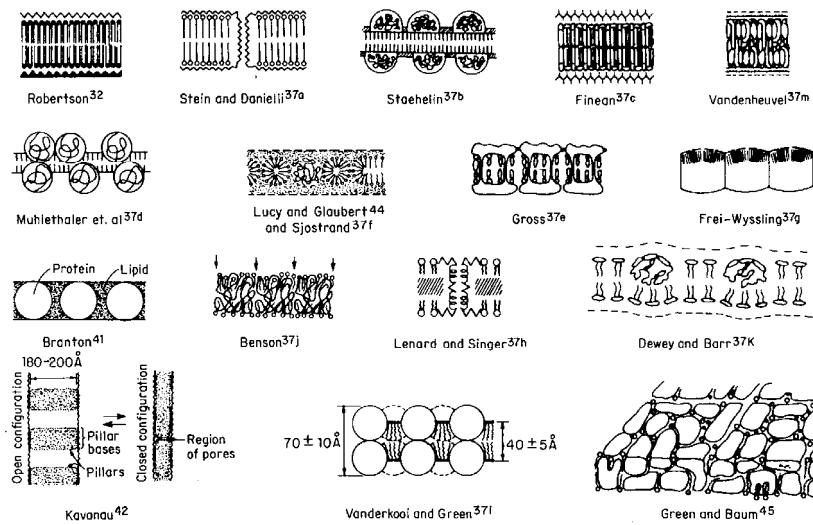


Figure 14. Schematic representation of various models for biomembranes. This is from Jain MK (1972), page 10, by kind permission of Van Nostrand Reinhold, New York. The full references are found in the latter publication. See also Malhotra, (1983).

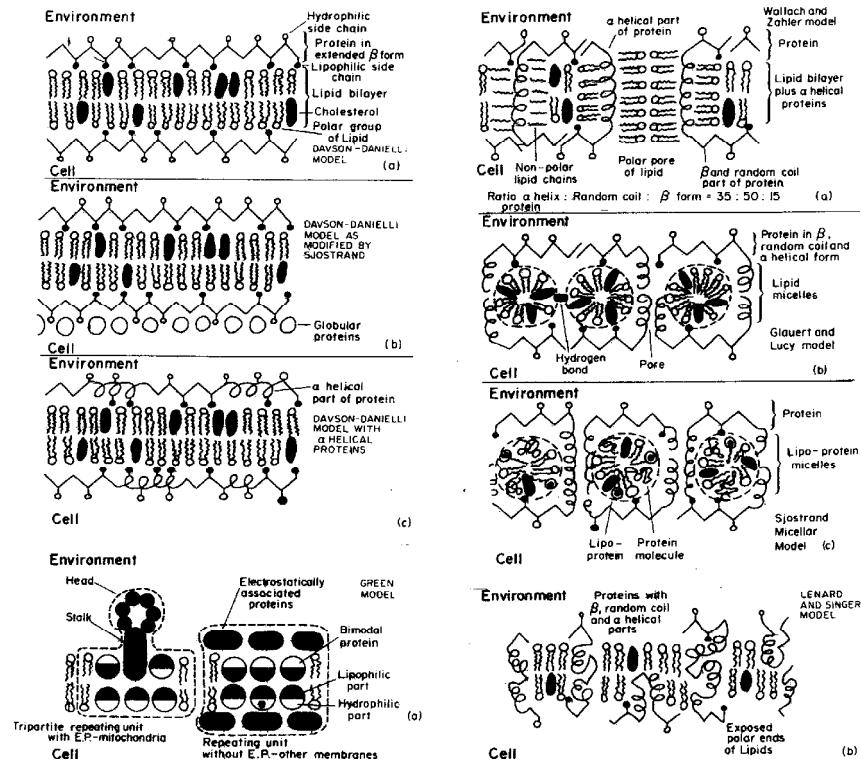


Figure 15. Schematic representations of various proposed chemical compositions of the cell membrane, collected by Threadgold, (1976), 84-88, by kind permission of Elsevier Scientific Publications.

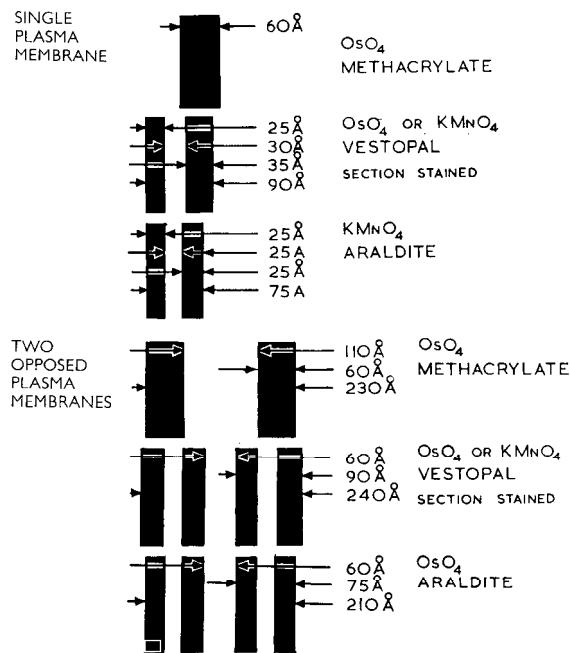


Figure 16. This figure comes from Threadgold, (1967), page 60, by kind permission Elsevier Scientific Publications. The data came from Sjöstrand, FS.

(ii) the thickness of the cell membrane can not be known, not only because it is a metal deposit on a shrunken layer, but also because the apparent thickness depends upon which heavy metal salt and which embedding medium is used (Figure 16). This makes uncertain any measurement made from an electron micrograph, or any calculation using it - as any bespoke tailor would confirm;

(iii) it is impossible to find out by disruptive techniques whether the membranes have a fluid interior or not. Nor, of course, can one find out by using any procedure, which dehydrates the tissue. The experiments in which macromolecules have been shown to move across the *surfaces* of cell membranes cited by (Singer and Nicolson, 1972) do not give information about the *interiors* of the membranes, they show only that particles in fluid move, for example, by diffusion or Brownian movement. This is no more surprising than that one can observe movements of particles in a drop of water on a slide under the microscope,. One is also tempted to suggest that the outer and inner protein molecules would only be held together by

surface tension, or the transmembrane protein molecules. Furthermore, neither the Davson-Danielli nor the Singer-Nicolson hypotheses about the chemistry of the membranes, would leave room for the multiplicity of transmembrane structures and molecules, generally believed to be crowded in to, or adherent to, the membranes (Tables 45, 46 and 47);

(iv) the idea that the channels in the cell membranes open when the cells are stimulated prompts the questions. Are there channels in non-excitable cells? If so, are they always open or closed? If they are always open, diffusion would equalise the concentrations of substances on both sides of the membranes, and would short circuit the resting membrane potentials. If they were always closed, how would ions like H^+ , Na^+ , K^+ , and Cl^- cross the membranes? If they can still cross the membranes with closed channels, would not only a very small proportion of the flux of ions be controlled by the channels? The existence of the channels will be dealt with (please see Chapter 21).

(v) receptors and carriers will be dealt with later (please see Chapters 22, 23), but at this juncture, it only need be noted that the presence of these macromolecules on the membrane surfaces would increase their effective thicknesses significantly;

(vi) all diagrams in the popular text books show glycoproteins and glycolipids hanging on to the outsides of the cell membranes. They are believed to be present there because they are found in microsomal fractions, and when purified, they are long molecules (Montreuil, Vliegenthart and Schachter, 1995). However, they are *never* seen in electron micrographs. Why not?

(vii) the cell membrane is believed to be continuous with the cisternae in the endoplasmic reticulum. This is extremely unlikely for the following reasons. Firstly, this connection would require that the endoplasmic reticulum be four layered, comprising invaginations of two ‘trilaminar’ membranes. It does not appear so. Secondly, the cisternae are very difficult to find in electron micrographs of most cells. Thirdly, one would expect ribosomes to be seen, at least occasionally, within the wall of the cisterna, (as if they were being ‘exported’) and almost always within the cisternae themselves. In other words, ‘exocytotic’ vesicles should be seen normally on the cisternal walls. Are there any micrographs of these of this in the literature?

(viii) all cell membranes appear to have potential differences across them. These would be short-circuited, when any channels were open;

(ix) it appears to be true that cell membranes are semi-permeable;

(x) the myelin sheath has been regarded as a sheaf of cell membranes, so studies of it by electron microscopical and biochemical techniques have been regarded as sources of information about the cell membranes. One reason for this belief is that the repeating period in low angle studies of the myelin sheath corresponds approximately to the width of the cell membrane. However, the former observation was carried out on 25% dehydrated nerve fibres, while the cell membranes were measured from the micrographs of completely dehydrated membranes. The other perhaps more important reason is that the lamellae of the myelin sheaths are artifacts (please see Figure 13).

Brownian movement has been observed in myelin, lipids and in myelinated Schwann sheaths, and Schmidt – Lantermann clefts have been seen to move (Singer and Bryant, 1962; Lee, Ishihara and Jacobson, 1991; Murray and Herman, 1968). These are unlikely to occur, if the myelin sheath were composed of solid lamellae. With all these reservations, one may conclude with reasonable certainty that (a) a semi-permeable membrane is located between the extracellular compartment and the cytoplasm; (b) it has very fine channels through it, allowing small molecules to cross it; (c) a hypothesis for receptor properties, without structural receptors will be given; (d) its chemistry is not known and may be unknowable; (e) the same is true for the orientation of any macromolecules within it; (f) there is a potential difference across the cell membrane; (g) subcellular fractionation and electron microscopy can not be used to extrapolate information back to properties in the living animal.

Many of the proteins in the membranes have been measured in subcellular fractions (Clark, 1984).

Chapter 21

Ion Channels

Narrow channels are believed to occur all over cell membranes and to control the movements of ions across cell membranes. They are believed to be rivet-shaped, with a channel through them, whose opening and closing controls the movements passively (Hille, 1966; 1971; Hille and Schwartz, 1978). Lists of the cells in which they have been reported, (Table 48), and the types of channels (Table 49), are given. It should be noted that channels have been reported, not only in cell membranes, but also in mitochondria, endoplasmic reticulum, sarcoplasmic reticulum, lipid film vesicles, phospholipid bilayers and some non-biological materials. A few are believed to be open at random, but many more open when a nerve or muscle cell is stimulated, and can be seen after clamping. When they are open, by the approach of a particular ion, or by a particular voltage, they allow the passage of the ion, until it is closed. It is believed that the opening of the channel or passage of the ion, triggers a small current (pA), constant for a particular channel, and lasting a fraction of a millisecond, so that one may conclude that the membrane appears to be in a bistable state. When the patch clamp records current from a small enough area of the membranes, this is interpreted as the passage of the current arising from a single channel (Aldrich, Carey and Stevens, 1983; Colquhoun and Hawkes, 1990; Koppenhoefer, 1996; Smith TM et al, 1998).

Tissues with ion channels reported in the literature

Neurons	Keratinocytes
Peripheral nerves (Hille, 1971; Chiu, 1991)	Oocytes
Sensory cells	Cells in brain slices
Glial cells	Mitochondria
Muscle cells including skeletal, smooth and cardiac cells	Nuclei
Endothelial and epithelial cells, including vascular, respiratory and renal cells	Endoplasmic reticulum
Secretory cells, including pancreatic, acinal, lachrymal and juxtamedullary cells	Sarcoplasmic reticulum
Hepatocytes	Liposomes
Pancreatic β cells	

Osteoblasts	Lipid film vesicles
Osteocytes	Vesicle blisters
Cells of the haemopoietic system, including erythrocytes, lymphocytes, macrophages, mast cells, neutrophils, blood platelets	Toxin plus azolectin
Gramicidin (Lee and Jordan, 1984)	Phospholipid bilayers
Silastic	Terephthalates

Table 48. The data for this list is mainly from Cahalan and Neher, (1992) and Saimi et al, (1992).

Types of channels, activators, blockers and inhibitors

Water (Sands, 1998)	Water (Sands, 1998)
Brain specific	TrK1, TrK2
Leak	Muscle specific
Capacitance	Kidney specific
Ion-gated K+, Na ⁺ , Ca ²⁺ , Cl ⁻	K ⁺ channel inactivating peptides
Voltage-gated K ⁺	Antibodies
Ca ² activated K ⁺	Viruses
Ca ²⁺ T, N, L, P, Types	Scorpion, snake, snail and spider, toxins
Voltage-dependant E -type	Gamma-amino-butyric acid
26 Ca ²⁺ channel antagonists and agonists	Glycine
Inward rectification K ⁺	Glutamate
Delayed rectification (closing)	S - hydroxytryptamine
Transmitter - gated	Ryanodine Ca ²⁺
GTP binding proteins	Inositol phosphate Ca ²⁺
Ligand - gated	Endoplasm
Nucleotide - gated	Shaker type
Ca ²⁺ activated non-selective cation	Other activators
Delayed channels	Other blockers

Table 49. These come from Rudy and Iversen, (1992), Conn, (1998), (1999); Waldman et al, (1999); Endo, Kuraichi and Mishina, (2000), Hille, (2001). This list is not comprehensive, and some types overlap. Many of the channels have been seen in tissue culture.

A wide variety of substances is believed to have their own channels; some substances ‘activate’, ‘block’ or ‘inhibit’ them (Table 49). Is there a difference between activation and opening, or between inhibition and blocking. How do

substances, such as gramicidin *induce* pores, other than by boring in to the membranes? Can they cause *synthesis* of the molecules of which the channels are thought to consist.

The ion channels are believed to be controlled by 'gates' (Green and Stokes, 1992). They have been detected, cloned and sequenced, mostly by the use of ligands (Rudy and Iversen, 1992; Conn, 1998; 1999; Ogden et al, 1999; Endo, Kurauchi and Mishina, 2000; Catterall, 2000). Ion channels, which have been isolated, have diameters of, Å, 3.3 x 3.3, 3.1 x 5.1 or 6.5 x 6.5 (Dwyer, Adams and Hille, 1980). The most well known channel, the Na^+ channel, also happens to be the acetylcholine receptor. Its shape was deduced by Kistler et al, (1982) and is illustrated here (Figure 17). The ion channel measured by the electron microscope had a minimum diameter of 9-10 Å, when closed (Unwin and Ennis, 1984; Toyoshima and Unwin, 1988; Unwin, 1995). The Ca^{2+} ATPase detected by antibodies had a diameter five times that of the width of the cell membrane (Green and Stokes, 1992).

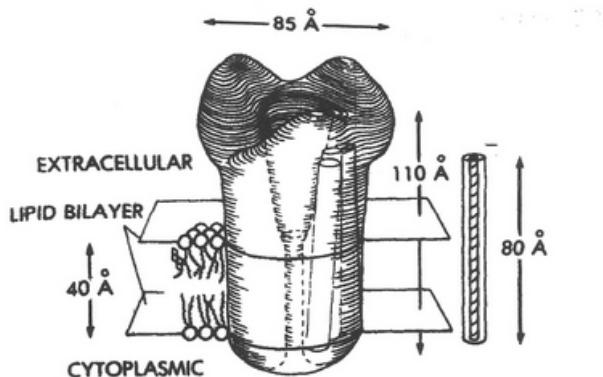


Figure 17. Diagram of the acetylcholine receptor Na^+ channel, modelled by Kistler, Stroud, Klymkowsky, Lalancette and Fairclough, (1982), reproduced by kind permission of Rockefeller University Press. Please note that the diameter of the receptor is 85 Å, and it protrudes from the membrane by about 60 Å.

The patch clamp currents are recorded by impaling and sealing a small piece of membrane or tissue on to the tip of a micropipette. Using electronic feedback, the potential differences across the membrane can be kept at constant voltage (Hodgkin and Huxley, 1952a,b). The current used to 'clamp' the voltage is measured with a micropipette. The impedance of the seals is in gohms, and the recorded noise is 0.2

pA at 1 kHz to 3 pA at 9 kHz (Corey and Stevens, 1983).

The concept of ion channels involves several assumptions:

(i) that there are physical channels in the cell membranes and in many other structures (Tables 48 & 49);

(ii) that the small currents recorded result from the opening of the channels or the passage of ions flowing through them;

(iii) that the ions cross the membrane only at the channels;

(iv) that the opening of the channels is due to the properties of particular ions (Davila, 1999; Goldin, 1999; Nattel, Rodin and Escande, 1999; Coetzee et al, 1999). Unfortunately, each of the assumptions is subject to serious reservations.

(i) there do not seem to be in the literature any electron micrographs as opposed to reconstructions, of *any* of the channels except the Na^+ channel; of Toyoshima and Unwin, (1988). At a packed meeting on the subject of ion channel at University College in 1994, I asked Dr Unwin if he, or any of the hundreds of research workers attending the seminar, had ever seen any other channels in intact tissue - as opposed to subcellular fractions -, and, if not, why not? He answered that he had not, nor was he aware of any literature of any other, and he did not know any explanation for this. No one else at the meeting claimed to have seen any other.

Statistically, only one such observation of the many large number identified in Tables 48 and 49, may not be significant.

It should be noted that these channels for K^+ , Na^+ , Cl^- , and Ca^{2+} are believed to exist in excitable and non-excitable tissues, so they should be seen scattered all over the membranes - like the good seed o'er the land;

(ii) the assumption that the small currents arise after the opening of the ‘gates’ in the channels (Sakmann and Neher, 1983; Aidley and Stanfield, 2000; Hille, 2001) is extremely unlikely, because, (a) it has been shown the ion flow through plastic or polythene terephthalate between a low conducting and a non-conducting phase showed rapid transitions, which looked like patch clamp currents; the latter system showed selective ion flow, and inhibited divalent cations and protons (Sachs and Qin, 1993; Lev et al, 1993); (b) the channels are believed to open when subjected to particular ions, voltages, capacitances, rectifiers, delays, transmitters, etc. This implies that there is a detector in front of the ‘gate’, which instructs it to open. Furthermore, this detector has to ‘decide’ whether to respond to a voltage, a particular ion, an accompanying ion, a capacitance, etc., sometimes to different properties of the

same ion, sometimes after a delay. The detector may also be a discriminator. What mechanism can one envisage or has been published that could react in that way? (c) the diameter of Toyoshima and Unwin's channel was 9-10 Å when closed, but the diameters of the hydrated ions are Å: H⁺ 1.0, K⁺ 5, Na⁺ 7.6, Cl⁻ 4.8; Ca²⁺ and Mg²⁺ are larger, OH⁻ and CH₃COO⁻ are smaller (Boyle and Conway 1941; Jain, 1972, pages 162-163). One must therefore ask how gates so wide could prevent the passage of much smaller ions. One suggestion which has been made is that the channels are charged, and so their walls could exert an electrostatic influence on ions passing through; this is rather a long distance for such an influence to be effective.

In addition, when gates open for the relatively large Ca²⁺ and Na⁺ ions, they can not fail to allow the smaller ions, such as K⁺, to pass through at the same time, and also in the opposite direction.

One of the most dramatic ways of demonstrating the ion channels was to transfer the RNA gene, extracted from various excitable cells, in to Xenopus oocytes, and to produce ion channel currents in them (Gourdon et al, 1971; Methfessel et al, 1986; Stühmer, 1998; Shih et al, 1998). This meant that the RNA from excitable cells created ionic channels in the oocytes. Unfortunately, the RNA is extracted by a complicated procedure involving many reagents, so it is, perhaps an over simplification to describe the injected material as only transfer RNA. Furthermore, perhaps one might expect the whole oocytes to become electrically excitable, as neurons are, but this has not yet been reported.

Another important step in the development of the concept of the channels, is the ability to induce the electrical signals associated with the ionic channels, by adding to black lipid membranes gramicidin, valinomycin, nystatin, etc. (Jain, 1972, page 180; Venkatchalam and Urry, 1983; Lee and Jordan, 1984). Such synporins have even been injected into real and other artificial membranes (Hladky and Haydon, 1984; Montal, Montal and Tomich, 1990), and produce patch currents. Gramicidin is a polypeptide produced by *Bacillus brevis*. The questions are, 'How do these compounds attach and bore into membranes to make channels in them.' 'Do they contain lipolytic and proteolytic enzymes?' 'Do they possess regulatory mechanisms, which choose the ions, their voltage and capacitance, to open?' 'Do they have other mechanisms which close them?'

Most diagrams of patches on micropipettes show the membranes neatly stretched and well fitting across the tips, but micrographs of the same appearance are

fashions to seek. Sakmann and Neher (1983) showed a cone-like filling within the membrane. In order to seal the tip of the pipette, negative pressure must be applied to it. Ruknudin, Song and Sachs, (1987) showed a solid plug of chick skeletal myotube within the tip, containing a collection of vesicles. When they looked at the micropipette tip with dark ground illumination, they saw a column of fluid containing gold liquid occupying the distal 40 μm of the tip of the micropipette, - not like the thin films of cellophane which grandmothers used to stick on their jars of homemade marmalade;

(iii) one is told that the channels occupy such a small proportion of the cell membrane that they can rarely be seen. If the channels were to control the ingress and egress of ions, the rest of the membranes would have to be impermeable. However, all theories and experiments find them to be semipermeable, that is to allow small ions to pass across them without hindrance. Furthermore, since channels are believed to be present in a wide variety of membranes, most of them not excitable, one would have to conclude that they would never open in the latter. Yet experiments with radioactive isotopes show that ions cross all membranes, so one has to ask the following questions. If ions can pass across membranes with closed channels, or no channels at all, how does one know that when the channels *are* present, the ions pass through them?

(iv) the belief that opening is due to particular ions, voltages, capacitances or activating ions, arises mainly from the reproducibility of the effects of any of these agents on patch currents, believed to arise from particular ions, clamped at particular voltages. The overall currents can be calculated and related to the voltages;

(v) ions pass through semi-permeable membranes, such as collodion, black membranes, dialysis tubing. Obviously, they have pores, whose diameters are measured by the sizes of the hydrated ions, which can cross them (Harris EJ, 1972; Jain, 1972). However, such small pores can not be measured or visualised in the hydrated state by electron microscopy.

The minute currents recorded could be from any of the sources quoted (Table 42). In particular, it is worth listing some of the reagents which are added during preparation, and which are assumed not to contribute significantly to the current: trypsin; collagenase; gramicidin; valinomycin; serum; gelatine; carbachol; chelators; glutamate; ATP; GTP; permeabilisers; KCl. In addition, the passage of current causes: localised heating; electrolysis; bubbles; protein denaturation; increased

diffusion; increased free energy. The obvious questions are: How one can discount any of these as being the sources of the minute currents? Do not all biochemical reactions generate currents?

The apparent compatibility of findings from patches (Hille, 2001) with the ionic theory of excitability (Hodgkin and Huxley, 1952 a,b) can not be used as evidence for or against any physical model, since the mathematics of the latter do not depend on any particular physical model (for review, see Trumpler, 1977).

The following control experiments in relation to ionic channels could be considered:

(a) in addition to the preparations cited above, patch clamp pipettes could be blocked with non-membranous starch, lignin, waxes and resins, with high resistances, to find out what currents are generated in the absence of biological membranes;

(b) more experiments could be carried out on collodion membranes, dialysis tubing and black lipid membranes, using patch clamp electronics. All the reagents used in preparing the clamps should be added to the systems;

(c) calculations need to be made on what proportion of the measured flux of ions across a membrane could be accounted for by the channels, and how much by non-channel areas of the membranes;

(d) the properties of the pores in non-excitatory structures need to be examined under the same conditions, as excitable cells;

(e) an attempt should be made to generate testable hypotheses on what ion channels do in non-excitatory cells;

(f) serious explanations need to be proffered for the reasons for which channels are not seen by electron microscopy;

A list of diseases in which ion channels are believed to be affected is given (Table 50).

Ion channel diseases

Atherosclerosis	Long QT syndrome
Central core disease	Malignant hypertension
Cerebral ischaemia	Myocardial infarction
Convulsions	Non-insulin dependant diabetes
Cystic fibrosis	Primary periodic paralyses
Epilepsy	Rickets
Episodic ataxia	X-chromosomes linked Duchenne muscular dystrophy
Hypokalaemic periodic paralysis	

Table 50. These diseases are discussed by Nayler, (1992), Weston and Hamilton, (1992), Wareham, (1995) and Sukata et al, (2000).

Chapter 22

Carriers

Carriers may be defined as membrane proteins, which help substances to cross cell membranes. Originally, it was believed that only ions were carried, but, nowadays, sugars, peptides, aminoacids and toxins are also regarded as being carried (Table 51). Carriers are believed to be present in many different tissues, as well as in, mitochondria, peroxisomes and sarcoplasmic reticulum (Table 52). They are sometimes called ‘transporters’. A list of some of the carriers and inhibitors reported in the literature is given (Table 53). Carriers were characterised by the inhibitors, which blocked them; for example, cytochalasin B in nM concentrations blocked the influx of glucose from human red cells at 20°C, and maltose blocked the inflow (Jung and Rampal 1977; Lacko and Wittke, 1982). They can also be extracted from human erythrocyte membrane fractions, solubilising them in the powerful detergent sodium dodecyl sulphate, and separating the polypeptides on polyacrylamide gel. The carrier was labelled with cytochalasin B (Mueckler et al, 1985; James, Strube and Mueckler, 1989). It can then be incorporated into ‘liposomes’, where it showed ‘carrier’ functions.

Substances believed to be carried

Glucose	Glycerol	Alanine
Fructose	Lipids	Glycine
H ⁺	Purines	Tyrosine
Na ⁺	Urea	Histidine
Ca ²⁺	Peptides	Serotonin
Cl ⁻	Choline	Folic acid
I ⁻	Gaba	Toxins
Galactose	Leucine	Protons
Fatty acids	Valine	Water

Table 51. These have been listed (Stein, 1967; 1988; 1990; Croop, 1998).

Tissues in which carriers have been found

Erythrocytes	Hepatomas
Kidneys	Adipocytes
Intestines	Muscles
Brains	β pancreatic islet cells
Placentae	Platelets
Hearts	Mitochondria
Lungs	Peroxisomes
Livers	Sarcoplasmic reticulum

Table 52. *They are found in subcellular fractions.*

Membrane carriers and inhibitors

<i>Carriers</i>	
Glucose transporter	Carnitine
ATPase Na^+ /glucose co-transporter	Aspartate/glutamate
Anionic transporter	Ornithine
ADP/ATP	P glycoproteins
Fatty acid binding protein	Cystic fibrosis transforming - regulator
Immunogenic antigens	Exchange carriers
Retinoic acid binding protein	Valinomycin
Myelin fatty acid binding protein	
Phosphate	<i>Inhibitors</i>
Uncoupling protein	Cytochalasin B
Dicarboxylate	Phloretin
Ketoglutarate	Maltose
Citrate	Steroids
Pyruvate	

Table 53. Most of these carriers are discussed in Stein, (1986; 1990) Reuss, Russell and Jennings, (1992) and Bernlohr and Banasaki, (1999). *Many have been studied in red cell ghosts or mitochondria. These are believed to be specific for the particular substances they carry, although some of them 'co-transport' and some 'exchange' transport.*

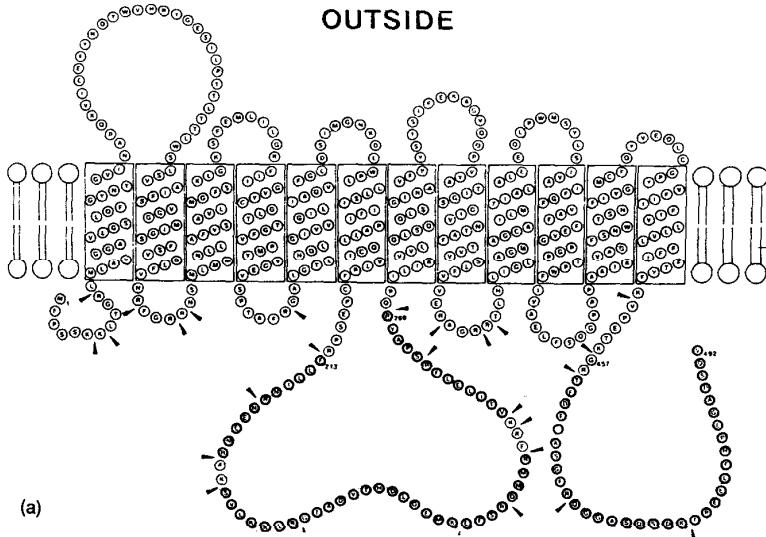


Figure 18. Glucose transporter from Cairns MT, et al, (1987) by kind permission of Elsevier Scientific Ltd. *How could a macromolecule embedded in a cell membrane facilitate the passage of glucose across that membrane?*

Another group of criteria for carriers is the phenomenon of facilitated diffusion; which is attributed to the presence of carriers in the membrane (Bowyer, 1957; Wilbrandt and Rosenberg, 1961; Stein, 1967, pages 127-129). Its properties are as follows: (a) facilitated diffusion does not require free energy, except for the synthesis of the carrier proteins; (b) the rate of penetration is greater than can be accounted for by the number of hydrogen bonding groups believed to be present in the membrane; (c) the penetration of the substance carrier shows saturation kinetics, indicating that it is slowed down when all the sites on the carrier have been bound; (d) there is competition for transport by analogous substances; (e) the rate of penetration may be diminished by inhibitors of enzymes; (f) there are differences between the rate of penetration measured as net transfer of the substance carried, and as unidirectional flux of the isotopically labelled substance; (g) ‘counter-transport’, of structurally analogous molecules may be demonstrated.

Several carriers have been isolated, cloned and sequenced, see, for example, Cairns et al, (1987), Hediger et al, (1987), Thorens et al, (1988) (Figure 18). The large molecule isolated contains several hundred amino acids, which could not possibly fit into the bilipid cell membrane. Therefore, all the diagrams show them to

be folded many times, but, nevertheless, to have extracellular and intracellular domains, protruding significantly into these compartments.

Since no net energy is required for counter transport, it must be concluded either that the original *SC* binding and its dissociation produce or require no energy, or both induce exactly equal and opposite changes of free energy.

Unfortunately, the concept of carriers and carrier transport is threatened by a number of unrecognised shortcomings:

(i) the inhibition of the efflux of glucose from red cells by 1-3 µM cytochalasin B (Jung and Rampal, 1977) is taken to mean that the cytochalasin B 'blocks' carriers, which would have facilitated the egress of the glucose across the cell membrane. However, there are a number of other mechanisms by which the same effect could have been produced, for example: it could have inhibited the synthesis of glucose by the cells, thus lowering the intracellular concentration; it could stimulate glycolysis within the cells; it could decrease the mobility of glucose; it could increase the viscosity of cytoplasm; it could increase the binding of glucose to other cytoplasmic constituents; it could decrease the permeability of membranes; it could increase the binding of the glucose to the cell membrane. The fact that the isolated carrier accords a lipid membrane the characteristics of the carrier, as defined, favours the possibility that the cytochalasin decreases membrane permeability. It is also worth noting that cytochalasin B and phloretin are not naturally occurring compounds;

(ii) it seems rather unlikely that the molecular structure and dimensions of the carriers are not changed by isolation of their membranes, rendering them soluble with detergents, and separating them on columns;

(iii) carriers are sometimes examined in tissue slices, but the value of these as models of living tissues is under considerable doubt (please see Chapter 13);

(iv) the criteria for facilitated transport are somewhat doubtful, because: it is extremely unlikely that either the binding between the substance transported and the carrier, or their dissociation would not be exothermic or endothermic; one can not know the number of hydrogen binding groups available either on the cell membrane, or in the whole preparation; the saturation may not only refer to a carrier, but to any ligand present in the whole system. If a substance binds with any constituent anywhere in the tissue, it will become saturated, as more is added. Competition by analogous substances is common to substrates, enzymes, drugs, transmitters, etc. - indeed, it is one of the foundations of pharmacology. Enzyme inhibitors may have

several sites of action, and may increase or decrease virtually any reaction in a metabolic pathway or cycle. It should not be regarded as surprising that the rate of influx of a substance in to a tissue is different from the rate of efflux. They both start from quite different chemical environments, namely the extracellular compartment and the cytoplasm, and flow to the opposite one. Therefore, one would expect their chemical behaviours to be quite different; ‘counter-transport’ means that as similar substance added to the outside of the cell, stimulates the efflux of a substance carried. Of course, any permeable substance added to the outside of the cell has osmotic effects initially, and, later dilutes and alter the chemistry of the cytoplasm and nucleoplasm, when it enters them. Thus, it is not at all surprising that an aminoacid stimulates the efflux of the same or another aminoacid. Consequently one must ask oneself how cogent each of these criteria should be regarded in relation to the necessity to require carriers to be present in the cell membranes;

(v) the substances carried are presumed to pass through the ion channels and to be controlled by the opening and closing of gates. *Prima facie*, substance + carrier must be larger than the substance itself, and this would hinder passage through a narrow gate. Indeed, it would be so much larger that it would have to fold in on itself several times and protrude from the membrane both extracellularly and intracellularly (Figure 18). It would resemble King Kong carrying a maiden in distress trying to walk through the turnstile at a football stadium. Not only would they have to enter the narrow gate and pass through the channel from the outside to the inside, but would have to hazard the return journey. If the substance and the carrier had a natural chemical affinity for each other in the extracellular compartment, the cytoplasm would have to represent an environment, in which the bond between the two would loosen. Then there would have to be a cytoplasmic mechanism to expel the carriers back through the channels, when the latter opened. Whereas there are believed to be channels for H^+ , Na^+ , Ca^{2+} , and Cl^- , one would have to suppose, either that all the other substances carried (Table 51) would have to share the ionic channels, or that each had their own dedicated channels. The other possibility would be that the substance carried and the bound complex crossed the membrane at points *other than* the channels. This would require mechanisms to allow substances as large as the carriers to cross the membranes at a rapid rate. If such large carriers could cross the membrane, how could the resultant holes prevent the passage of all the small ions, believed to be waiting to pass through the ionic channels?

(vi) small ions and molecules, such as, Na^+ , K^+ , and glucose can cross semipermeable membranes, such as dialysis tubing, ferri-ferrocyanide and collodion. If one places a cation exchange resin, an affinity gel or a fixed anion, on one side, or, if the total chemical activity on one side changes, this would cause temporary movements on both sides of the membrane, which would eventually achieve equilibrium.

Chapter 23

Chemical Receptors

A receptor may be defined as a structure, chemical component or region of a cell, usually believed to be outside or within the membrane, with which a transmitter, hormone, antigen, allergen, anaphylaxin, vitamin, drug, toxin or ligand, binds, and initiates effects. A ligand is a substance which reacts with a receptor. One may designate all the above substances with the letter *L*. Certain other chemical compounds, usually, but not exclusively, similar in structure to an *L*, increase or decrease its activity, and are considered to act on the same ‘sites’. The organs or tissues which react to *L* are called the ‘targets’.

The concept of receptors is used by physiologists, endocrinologists, immunologists, biochemists, toxicologists and pharmacologists. In the late 19th and first half of the 20th centuries, it was regarded as a chemical and a mathematical concept, but more recently, it has also been regarded as an anatomical structure. The idea of receptors grew from the experiments of Langley, (1878; 1905) on the antagonism of atropine and pilocarpine to the production of saliva by cats, following stimulation. This drew attention to the important roles of antagonism and drugs in the development of ideas about receptors. Langley concluded that, “We may, without much rashness, assume that there is some substance or substances in the nerve endings or gland cells with which both atropine or pilocarpine compounds are found, according to some law of which the relative mass and chemical affinity for the substances, are factors.”

Ehrlich, (1877) noted that histologists used the artificial dyes which were being developed in Germany. He thought that illnesses could be cured by using particular chemicals to target the particular cells affected. He developed salvarsan, which contained arsenic, and treated syphilis successfully with it (Ehrlich and Hata 1910). He also coined the phrase ‘*corpora non agunt nisi fixata*’, by which he probably meant that staining could not occur until tissue has been fixed, but is widely interpreted as meaning that substances do not act until they have been bound. His concept of the action of diphtheria toxin also lead him to the idea of receptors. An excellent account of the development of the concept is given by Silverstein, (1989).

Whereas Ehrlich, (1877), Langley, (1878; 1905), Clark, (1933) and Paton, (1955; 1961) envisaged only one receptor for each drug or ligand, the several actions

of adrenalin, acetylcholine and dopamine, led to the idea that each may have several receptors, and now each has ‘families’ and ‘super-families’. These were designated after it was found that several different drugs, not always competitors, enhanced the effects of *L* in vitro. A drug believed to act on a tissue can be defined by its chemistry, that of its agonists and antagonists, the tissue upon which it acts and its physiological effects.

Ahlquist, (1948), noted that the adrenergic activities of adrenalin, noradrenalin and several other homologous substances, acted as if there were more than one receptor, which he named α and β . However, these receptors have since multiplied, and nowadays, pharmacologists speak of α_1 , α_2 , β_1 , β_2 , and β_3 subtypes (Table 54). They are characterised, mainly by drugs which act as agonists or antagonists.

Adrenergic receptors and their effects

<i>Receptor</i>	<i>Tissue and site</i>	<i>Effects</i>
α_1	eye, radial muscle	eye, radial muscle
β_2	eye, ciliary muscle	relaxation for far vision
β_1 , β_2	heart, sinoatrial node atria	increased heart rate, increased contractility and conduction velocity
	atrio-ventricular node	increased automaticity and conduction velocity
	His-Purkinje system	increased automaticity and conduction velocity
	ventricles	increased contractility, conduction velocity and rate of idioventricular pacemakers
	<i>arterioles</i>	
α_1 , α_2 , β_2 ,	coronaries	constriction, but dilatation in <i>vivo</i>
α_1 , α_2	skin and mucosa	constriction
α_1 , β_2	skeletal muscle	constriction or dilatation
α_1	cerebral	slight constriction
α_1 , β_2	pulmonary	constriction or dilatation
α_1 , β_2	abdominal viscera	constriction or dilatation
α_1 , α_2	salivary glands	constriction
α_1 , α_2 , β_1 , β_2	renal	constriction or dilatation
α_1 , α_2 , β_2	<i>systemic veins</i>	constriction and dilatation

<i>Receptor</i>	<i>Tissue and site</i>	<i>Effects</i>
β_2	<i>lung, trachea and bronchial muscle</i>	relaxation
α_1, β_2	<i>bronchial glands</i>	decreased or increased secretion
$\alpha_1\alpha_2, \beta_2$	<i>stomach, motility</i>	decrease
α_1	<i>sphincters</i>	contraction
α_1	<i>secretion</i>	inhibition
$\alpha_1, \alpha_2, \beta_1, \beta_2$	<i>intestine, motility and ton</i>	decreased
β_2	<i>gall bladder and ducts</i>	relaxation
α_1, β_1	<i>kidney</i>	renin secretion
β_2	<i>bladder, detrusor</i>	relaxation
α_1	<i>trigone, sphincters</i>	contraction
α_1	<i>ureter, mobility and tone</i>	increase
α_1, β_2	<i>uterus, pregnant</i>	relaxation (β_2), contraction (α_1) relaxation (β_2)
α_1	<i>male sex organ</i>	ejaculation
α_1	<i>skin, pilomotor muscles</i>	contraction
α_1	<i>sweat glands</i>	secretion
α_1, β_2	<i>spleen capsule</i>	contraction and relaxation
not known	<i>adrenal medulla</i>	
β_2	<i>skeletal muscle</i>	increased contractility, glycogenolysis K^+ uptake
α_1, β_2	<i>liver</i>	glycogenolysis and gluconeogenesis
α	<i>pancreatic, acini</i>	decreased secretion
α_2	<i>islet cells</i>	decreased secretion
β_2	<i>islet cells</i>	increased secretion
α_2, β_1	<i>fat cells</i>	lipolysis
α_1	<i>salivary glands</i>	K^+ and H_2O secretion
β	<i>salivary glands</i>	amylase secretion
α	<i>lacrimal glands</i>	secrete
not known	<i>nasopharyngeal glands</i>	not known
β	<i>pineal gland</i>	melatonin synthesis
β_1	<i>posterior pituitary</i>	antidiuretic hormone secretion

Table 54. These 4 adrenergic receptors are believed to produce all those effects, for example, exhibited by adrenalin, noradrenalin and isoprenalin. The data from this

table comes from Hardman et al, (2003), by kind permission of McGraw Hill. *These receptors are defined by 'specific' blockers. There are also several other α and β receptors.*

The site of action may be anywhere in the cell, but is usually assumed to be on the cell membrane except for the steroids, which are believed to act on nuclei. *L* can act on (a) the extracellular compartment; (b) the outer surface of the cell membranes; c) the cell membrane; (d) the cytoplasm; (e) the mitochondrial membrane; (f) the mitochondrioplasm; (g) the nuclear membrane; (h) the nuclear pores, if they were not artifacts (please see Chapter 31); (i) the nucleoplasm; (j) the nucleolus; (k) any chemical reactions at any of these sites. Obviously, the more rapidly *L acts*, the more likely it is to act on a chemical component or chemical reaction early in the list. There is no reason to believe that *L* only acts on one site, especially if it has many receptors.

The receptor can be either an anatomical structure, the chemical compounds, which it houses, or a chemical reaction. This distinction is not made in the literature. A partial list of chemicals generally considered to have receptors is given (Table 55). Please see Kennakin and Angus, (2000). Others may be added in the future. Of course, the properties of isolated receptors depend crucially on the techniques used to isolate them, and some of these are listed (Table 56).

Classes of chemicals believed to have receptors

<i>Receptors for</i>	<i>Number</i>	<i>Comments</i>
Transmitters	60+	many have 'families'
Hormones	25+	some believed to be nuclear
Ligands	1000's (180 for actin)	for transmitters, hormones and drugs (Sheterline, Clayton and Sparrow 1998)
Antigens to diseases	100+	very specific proteins
G - proteins	16 - 20	present during signalling
Allergens	100's	mainly in foods
Agglutinogens	60+	in blood
Vitamins	25	
Drugs	1000's	may share receptors
Interleukins	15	possibly more to be discovered
Oestrogens	9+	plus some synthetic
Death	5	Ashkenazi and Dixit (1999)

<i>Receptors for</i>	<i>Number</i>	<i>Comments</i>
Toxins	1000's	many chemicals
Autoantigens	26 human diseases (Table 132)	
Cell signals	many (Table 99)	many are protein - linked
Complement	many	in immune and autoimmune diseases
Growth controllers	8+	
Cell division	2	
Viruses (Randall and Phillipson, 1980)		
Differentiation	?	
Recognition	?	
Regulation	?	
Perception	?	speculative
Thought	?	speculative

Table 55. *Most of these are chemical rather than structural receptors.***Methods of identifying receptors**

Assumption when an effect is produced	Use of lectins
Use of ligands, including radioligands	Proteolysis
Subcellular fractionation	Affinity chromatography
Histochemistry	High performance liquid chromatography
Use of agonists and antagonists	Reverse phase chromatography
Fluorescence microscopy	Isoelectric focussing
Monoclonal antibodies	Gel electrophoresis
Autoradiography	Indirect fluorescence
Electron microscopy	Ionophoresis
Isolation and cloning	Positron emission tomography

Table 56. Most of these procedures are described in Venter and Harrison, (1984, vols. 1 - 2), Hulme, (1992).

There is a large literature on receptors, many of which have been identified, isolated, cloned and sequenced (Godin, 1986, Winter and Milstein, 1991; Burgen and Barnard, 1992; Lauffenberger and Linderman, 1993; Ritter and Ladyman, 1995). They appear to be macromolecules with hundreds of aminoacids, molecular weights of thousands and dimensions of 10's of nms. All diagrams in the literature show that their diameters exceed the thickness of the cell membranes in some of which they are

folded, and that they protrude into the extracellular compartments and the cytoplasm. Their dimensions are within the resolution of the electron microscope.

The consensus about the properties of receptors can be summarised (Goldstein, Aronow and Kalman, 1969; Bowman and Rand, 1980; Pratt and Taylor, 1990; Burgen and Barnard, 1992; Hardman et al, 2003; Rang et al, 2003, as follows:

- (i) L acts at low concentrations (Table 57);
- (ii) L or its ligands bind with high affinity for ‘specific’ receptors;
- (iii) the bonds may be electrostatic, covalent, Van der Waals, or hydrogen;

Doses of drugs given to patients

<i>Drug</i>	<i>Use</i>	<i>Single dose (mg)</i>
Aspirin	analgesic	600
Digoxin	heart failure	1.5
Adrenalin	cardiac resuscitation	0.1 - 1
Atropine	premedication	0.3 - 0.6
Thyroxine	hypothyroidism	50 - 100
Pethidine	strong analgesic	25 - 50
Bendrofluazide	diuretic	2.5 - 5
Morphine	strong analgesic	10
Pentobarbitone	rapidly acting anaesthetic	100 - 150
Prednisolone	steroid deficiency	5 - 80
Ergotamine	migraine	2 - 4
Diazepam	premedication	10 - 20
Haloperidol	neuroleptic	3 - 9
Clozapine	neuroleptic	12.5
Salbutamol	asthma	4 - 5
Pyridostigmine	myasthenia gravis	30 - 60

Table 57. Such doses may be distributed in the plasma (3 l) or the total water (40 - 50l) of a 70 kg person. The tissue concentrations of any drugs will be increased by their confinement to particular compartments, and decreased by conjugation and excretion.

(iv) specificity of L for receptors is reciprocal. L binds to certain receptors, and individual receptors ‘recognise’ certain classes of L ;

(v) when bound to receptors, L initiates a number of biochemical events, which are eventually transduced into physiological events, such as secretion, muscle

contraction, digestion, etc. Some ‘non-specific’ chemical binding may occur without producing effects, while some substances, such as rapidly acting anaesthetics, act presumably without pre-formed receptors;

(vi) receptors may be ‘saturated’, that is, a dose larger than that producing a maximal effect, does not produce a further effect;

(vii) ligands are *assumed* to bind to the same sites as the particular transmitters, hormones, vitamins, antigens, drugs and toxins, which they are used to model. (This is on the basis of ‘competition’);

(viii) most agonists, antagonists and other ligands are assumed to have no significant other biochemical, physiological or pharmacological effects in addition to those they are used ‘specifically’ to model;

(ix) L may have a ‘family’ or ‘super-family’ of its own, which will be unique. Does this mean that all the receptors of the family have to bind before it has an effect?

(x) L and its ligands may cause their effects by binding to enzymes, carriers, ionic channels and second messengers. It may also have osmotic, chelating, pH, mass action or ‘non-specific’ effects;

(xi) the degree to which L , when it is believed to be bound to a receptor, causes a biological effect is governed by its ‘efficacy’. Full agonists have maximal efficacy and partial agonists have intermediate efficacy. The term is used because some drugs and agonists do not have the expected effect, but its biological basis is not known;

(xii) the ‘side effects’ of drugs are those which the physician considers undesirable, and the pharmacologist does not like. The fact that the chemistry of a drug may be modified slightly to increase its potency or decrease its side effects, means that a single action of a drug on one tissue (measured by a bio-assay) is not necessarily relevant to its activity in other tissues;

(xiii) ionophoretic application of ions, aminoacids or transmitters, to the outside of neurons in the brain and spinal cord, causes them to respond in milliseconds. G-protein receptors are believed to react in the same time frame. Drugs such as adrenalin, benzodiazepines, pentothal and aminophylline, act within seconds when they arrive by an intravenous route at a site;

(xiv) drugs rarely occur naturally in animal tissues, but cells are believed to have receptors for drugs, antigens, anaphylaxins, toxins and death, to which they have never previously been exposed.

- (xv) some tissues become ‘hypersensitized’ or ‘desensitized’ to drugs;
- (xvi) chemical and anatomical receptors are assumed to be identical;
- (xvii) the ‘non-specific’ uptake of L may be as much as 50% of its total uptake, but it seems a little doubtful that the tissue can ‘recognise’ the difference between ‘specific’ and ‘non-specific’;
- (xviii) receptors can be cloned;
- (xix) it is believed that understanding of the mechanisms of action of any L is increased, when the chemical structure of the isolated, cloned receptor has been elucidated.

One can now analyse in greater detail the concept of a receptor.

Firstly it is believed that a ligand fits a receptor, like a key fits a lock. For example, chlorpromazine is believed to act on D₁, D₂, α -adrenergic, histaminergic I, cholinergic and serotonin, receptors. Since each of these receptors has a prolific family, it would seem that the master key would have to fit rather a large number of locks. Is it suggested that this drug acts on ‘specific’ sites of each of the receptors, or a crucial selection of all of them? The structure of chlorpromazine is CH₃CH₂CH₂N.CH₃; this chemistry indicates that it has 13 potentially reactive groups.

An acetylcholine receptor is regarded as the chemically active site which binds acetylcholine, muscarine or nicotine. Adrenalin and its allies initiates a series of biochemical events which produce ‘adrenergic’ effects (Table 54). Acetylcholine has a different assortment of effects. All of the drugs in Table 58 and 59 are believed to act on the acetylcholine receptors, and therefore to be ligands.

Drugs believed to act on acetylcholine receptors

<i>Drug</i>	<i>Comments</i>
Muscarine	family of receptors; act on autonomic ganglia
Nicotine	family of receptors; act on autonomic ganglia
Cholinesterases	natural occurring; break down acetylcholine
Anticholinesterases	inhibit latter; drugs; include organophosphates
Butylcholinesterases	inhibit cholinesterases
Curare	‘competes’ at neuromuscular junctions
Atropine	‘blocks’ nicotinic effects
Decamethonium	depolarises neuromuscular junctions
Diallylbisnortoxiferine	‘competes’ at neuromuscular junctions
β - erythroidine	‘competes’ at neuromuscular junctions

<i>Drug</i>	<i>Comments</i>
Gallamine	'competes' at neuromuscular junctions
Succinylcholine	depolarises neuromuscular junctions
Benzoquinonium	depolarises neuromuscular junctions
Methacholine	muscarinic effects
Bethanecol	muscarinic effects
Carbachol	muscarinic effects
Lobeline	ganglion stimulator
Tetramethylammonium	ganglion stimulator
Dimethylphenylpiperazinium	ganglion stimulator

Table 58. Most of these come from Hardman et al, (2003). *Similar lists can be made for most transmitters.*

Agonists and antagonists of muscarine

<i>Agonists</i>	<i>Antagonists</i>
Acetylcholine	Atropine
Carbachol	Homatropine
Methacholine	Atropine methonitrate
Bethanecol	Hyoscine
Muscarine	Propantheline
Pilocarpine	Lachesine
Oxytremorine	Cyclopentolate
	Pirenzepine

Table 59. The top 6 agonists and antagonists are homologous. The others have very different structures, but are believed to bind to the same receptors.

In the 1940's and 1950's, acetylcholine had only two actions, 'muscarinic' and 'nicotinic', and, therefore, only two receptors. Some of its actions could be 'mimicked' by each of these drugs. Pharmacologists subsequently described a large number of agonists and antagonists, in addition to 'blockers' to each of these. They believed that elucidating the chemical structures of all the ligands to each of these drugs would give them a clear idea of the chemical conformation of each ligand, and, therefore, of its receptors, (Goldstein et al, 1969). For the sake of brevity only muscarinic agonists and antagonists are given here (Table 59), but there is also a very respectable list of nicotinic agonists and antagonists.

Nevertheless, the *concept* that both muscarine and nicotine together show all

the properties of acetylcholine becomes very questionable, when one realises how completely different are the structures of acetylcholine, on one hand, and muscarine and nicotine, on the other. It is difficult to see which reactive groups the three molecules have in common. Furthermore, muscarine and nicotine do not occur naturally in mammals, and both are extremely toxic. So why is acetylcholine not toxic? Are their actions in ‘mimicking’ some of the effects of acetylcholine - although remarkable - not somewhat fortuitous? Is it stating more than the fact that not only can homologous chemicals have similar effects, but so can others of completely different chemical structures? If one were to take any important biochemical pathway or cycle, would not many active chemical species activate or inhibit it, and would not others agonise or antagonise the latter?

The relationships between the receptors as anatomical structures and the chemical reactions between *L* and the receptors, which trigger the events producing effects, need examination, as it is widely assumed that they are identical. As has been pointed out, there are believed to be a profligate number of receptors and their families, and sequencing has them shown that the receptors are within the resolution of the electron microscope. Their structures have been seen in graphic reconstructions rather than in micrographs (Figure 17). The electron microscopist see *structures*. The *structures* have different dimensions from the macromolecular *chemicals*. They can only be considered to be of the same dimensions, if the structural receptors have the chemical properties of the cloned receptors. However, the anatomical receptors are prepared by the procedures for electron microscopy, while the cloning and preparation of the chemical receptors are subjected to a completely different battery of chemicals, so that one would expect them to have been changed chemically from their native states.

The reasons for which the electron microscopists do not see the receptors (other than that they are not attached to the cell membranes) could be as follows: they have moved during preparation; they do not stain (except occasionally in subcellular fractions); the cloned receptors are not the sites of the *L* - receptor bond; the ligands bind not only to the receptors, but to other molecules as well. Thus, it is possible that the anatomical receptor and the *L* - receptor are different, and the chemistry of the anatomical structure is *not* that of the *L* - receptor complex.

The use of ligands to detect any type of *L* seems questionable. If one wants to study the properties of acetylcholine, why use muscarine or nicotine? For dopamine

why study haloperidol? Why use an agonist, antagonist, competitor or inhibitor? The usual answers given are that acetylcholine, adrenaline or dopamine, would be broken down by local enzymes, or it would diffuse away from the receptor. Or, its concentration in the tissue is too low to be detected. Or, possibly, the bond between *L* and the receptor was too short-lived or unstable. Unfortunately, all these explanations represent clear evidence that *Ls* are different chemically from the substances which they are employed to imitate. One also has to bear in mind that many transmitters are believed to generate autoreceptors.

If the cells are exposed *for the first time* to a drug, which is not a natural constituent of the body, or if the drug is injected on to the surface of a neuron by ionophoresis, it might produce an immediate effect. If it acts on receptors, one would have to suppose either that: *L* acted on other receptors already present; or, receptor proteins could be synthesised within milliseconds, or it modified parts of the cells themselves to combine with *L*, and this *L*-tissue compound initiated the effects.

L and other ligands initiate effects. So do: high K⁺; low Ca²⁺; hypoxia; electrical stimulation; high lactate; high CO₂; high or low pH; heat; cold; pressure; stretch; pain. All these agents produce rapid effects, often without chemical receptors. Or, perhaps, they are 'signals'?

Both acetylcholine and adrenaline stimulate their respective systems at 'low' concentrations, and inhibit them at 'high' concentrations. If they do, in fact, bind with receptors, one would *not* expect that 'supramaximal' concentrations *inhibit* the same systems. They would just have no greater effects.

The ideas that neuroleptic drugs act on receptors, and that schizophrenia is a disease of receptors, because neuroleptics are effective against it, are both thrown into doubt, when one considers the battery of drugs and their reported wide range of structures and receptors. Seeman et al, (1976) listed 17 such drugs. Hardman et al, (2003) and Rang et al, (2003) listed 17 and 15, respectively - some of the drugs in their lists overlap. Examination of any pharmacopoeia shows that neuroleptics include phenothiazines, thioxanthenes, butyrophenone, dibenzapine and benzamide, as well as 'classical', 'atypical', 'modern', 'those acting on negative symptoms', and 'those with side effects'. The multiplicity of chemical structures of neuroleptics and their receptors, makes it very difficult to envisage the structure of such receptors, or even to believe that 'specific' receptors are involved. Of course, in plotting the effectiveness of the different drugs against their uptake in the caudate nucleus,

Seeman et al (1976) were assuming that they did act on the same receptors. The difficulty of having such a variety of chemical substances effective against schizophrenia had led to the suggestion that the term 'schizophrenia' encompasses a number of different syndromes, and that the different receptors, are involved in the different syndromes. Nevertheless, despite extensive studies of the brain chemistry, the blood chemistry and the neuropathology, the genesis of schizophrenia is still not known.

One is now in a position to harvest the crop of possibilities. The following may be regard as certain facts.

(i) small quantities of different transmitters, hormones, antibodies, allergens, anaphylaxins, ligands, drugs and toxins enhance, diminish or produce effects all over the body (Table 57);

(ii) different ones produce effects mainly in the same cohorts of 'target' tissues each time;

(iii) therefore, they must react with the tissues, even if only for a short time, and initiate a series of biochemical changes resulting in the effect;

(iv) small quantities of other, often homologous, chemicals enhance, diminish, or have no effects. Slight changes in the chemistry of L may change its ability to produce desired and 'side' effects;

(v) agonists or antagonists may act on L directly, its binding to tissue, or any of the compounds or reactions connecting the binding or the reaction, to the effects. One may call this the L -effector-transducer chain.

On the basis of these considerations, I would like to propose a more general theory of the relationship between the initiating compound L and the transducer - effector chain.

1. Different chemical pathways and cycles in different tissues are sensitive to different individual types of L . These sensitive reactions are not necessarily *structures*, and may be located in several tissues. Nowadays, they may be thought of as *chemical receptors* for the different types of L .

2. L does not have to *bind* with the sensitive chemical pathways and cycles, it only has to react with them and effect their rates or equilibria.

3. Therefore the problem for biochemists, physiologists, electron microscopists and pharmacologists, is not to seek *structures* affected by L , but to try to locate the particular chemical *pathways* or *cycles*, which may be sensitive to L in

the concentrations, in which the drugs act in the intact animal. A number of possible approaches to this problem, is suggested. The identification of the L – transducer - effector chain represents a further challenge.

The following approach may be envisaged:

(a) the real substances L , rather than its ligands, should be used to try to locate the sites or chemical reactions. If they are unsuitable, for example, because they are not stable enough, they should be injected as closely as possible to the site at which they are believed to act, preferably in the intact animal;

(b) if L can not be examined in the whole animal, it could be examined in the isolated, perfused, organs, in which it is known to act;

(c) high energy and disruptive procedures should be avoided in the search for receptors, since, they can affect the reactions between L and the tissue and they can relocate the reactions, the ligands, the substrates and any co-factors;

(d) one can examine the effect of L on all major pathways and cycles believed to exist in the body. This does not mean examining every single enzyme reaction. If L at physiological or pharmacological concentrations had no effect, for example, on glycolysis or the urea cycle, one would not have to carry out experiments on every single step in these pathways. One would only have to do this, if one found that it *did* have an effect on the whole pathway. Optimally such experiments should be carried out in the healthy intact animal. Slices, cultures and homogenates should be avoided, if possible.

(e) it would be useful to examine the chemical reactions only between L and its antagonists, to exhaust how many of the properties of their reaction in the presence of tissue are actually due to the reactions between each other;

(f) one can inject L outside, or into, isolated axons, neurones, hepatocytes, etc. under direct microscopic vision, if they can be labelled with supravitral fluorochromes to see where they act. Quantum fluorescent microdots might be very useful in this connection;

(g) one can add L to a microdiver containing single cells, and see if it has an effect on the oxygen uptake, or any of the enzymes measurable by this technique;

(h) it is traditional to administer radioactive drugs to animals, and then extract them from the organs, or subject the organs to autoradiography, processes which take hours, and allow all diffusible chemicals to move. The use of positron emission tomography (PET) in living animals is an important advance, because it tells one the

location of the drugs *in life*.

The receptor concept has problems. It is extremely difficult to see the structures in intact animals; there are very wide differences between the chemical structures of ligands; the justification for the employment of ligands instead of transmitters, hormones, drugs, etc; the difficulty of examining ligand-receptor reactions using disruptive techniques; the impossibility of knowing whether a particular ligand acts on the particular system one is studying, but not others; the difficulty of knowing why a ligand affects one particular organ or reaction, but not others in the body (that it is 'specific'); the belief that because one substance activates or inhibits a reaction, in which one is interested, but 'ignores' all others; the fact that many activators or inhibitors are powerful chemicals not naturally found, in the body; the fact that disruptive procedures alter the equilibria of reactions and the locations of the chemicals involved in them.

It seems to me likely that the anatomical receptor does not exist. Particular local chemicals, reactions or metabolic pathways react to a vast range of substances, water, ions, aminoacids, fatty acids, steroids, transmitters, hormones, drugs, allergens, toxins, etc, in low concentrations. Any reaction going on in intact cells in whole healthy animals may be called a receptor. It does not have to be a structure on the cell membrane or in the nucleus. It only needs to be an active chemical, involved in metabolism (Table 13).

Chapter 24

Cytoplasm

The cytoplasm in life is a fluid between the cell membrane and the nuclear membrane. Filiform mitochondria and unidentifiable particles can be seen moving around. The cytoplasm is of low viscosity as measured by centrifugation, injecting small particles, electron spin resonance, and withdrawal of small samples for chemical analysis. The following movements of particles may be seen by high power light microscopy: Brownian movement; streaming; diffusion; convection; secretion; pinocytosis; vacuolation; nuclear rotation. All the above types of movement are seen in tissue cultures, in protista and in plants cells, and it is assumed that they also occur in cells of living intact animals. Indeed, the intracellular movements are regarded as proof that cells in culture are alive.

The main structures, filaments and chemicals, believed to be present in the cytoplasm are given (Table 60, 61). The mitochondria were called Altmann's, (1890) granules for a long time. Golgi saw his apparatus in barn owl neurons (1898). All the other structures were seen by electron or fluorescence microscopy. The mitochondria, Golgi body, endoplasmic reticulum, ribosomes, lysosomes and the cytoskeleton will be dealt with separately.

Cytoskeletal elements

	<i>Number</i>	<i>Authors</i>	<i>Several in class</i>
Actin and associated proteins	47	77	13
Tubulin and associated proteins	20	33	6
Intermediate filaments	15	19	4
Motor proteins	14	20	3
Cytoskeletal anchor proteins	18	25	4
Organelle membrane associated structural proteins	8	10	8
Other proteins	2	2	2
Total	124	186	40

Table 60. The large number of cytoskeletal elements believed to exist in the cell is summarised by Kreis and Vale, (1993) by kind permission of the Authors and Oxford University Press. *In many cases, there are several proteins in one class. Not all*

mammalian cells contain all the proteins. The elements are generally identified by immunocytochemistry. Nevertheless, in the vast majority of illustrations, for example in Dustin, (1984), Björklund, Hökfelt and Tohoyama, (1983-1992), Ockleford, (1988), Roberts and Hyams, (1994), most elements of each kind are nearly always seen in longitudinal view and straight, and the mitochondria are not seen in the cytoplasm at the same time.

Structures and filaments believed to present in the cytoplasm

<i>Structure</i>	<i>Filament systems</i>
Mitochondria	Thick filaments
Golgi body	Intermediate filaments
Endoplasmic reticulum	Thin filaments
Ribosomes	Microtrabeculae
Cytoskeleton	
Lysosomes	
Phagosomes	<i>Chemical constituent</i>
Peroxisomes	Water
Cisterns	K ⁺
Polysomes	Na ⁺
Unidentifiable granules	Fixed anions
<i>Filament systems</i>	<i>HCO₃</i>
Microtubules	Cl ⁻
Stress fibres	ATP, ADP, AMP, and phosphate
Tubulin	Ca ²⁺
Actin	4 microtubule associated proteins
Myosin	Kynesin
Dynesin	Tau
Spectrin	STOPS
Vimentin	Chartins
Filamin	Ankyrins
Desmin	Cytokeratins
Vinculin	
Acropaxin	
Paxillin	

Table 61. *Most of the structures are only seen in stained cells, by fluorescence, or by electron microscopy. There are believed to be many contractile proteins (Pollard, 1981).*

The mitochondria are well seen in tissue culture, but in isolated cells, for example, hepatocytes and neuron cell bodies, their visibility can be enhanced by Janus green or neutral red. Usually, these reagents are alcoholic, which, of course, shrinks cells, but the mitochondria still show up, if only aqueous reagents are used (Figure 20).

The concentrations of diffusible ions and molecules in the cytoplasm tend to be underestimated, because penetrating the cells, for example, with micropipettes, or to take out minute samples, damages the cell membrane, and allows the substances to leak. The use of radio-active isotopes is less damaging. Disruptive procedures should be avoided, when trying to measure cytoplasmic concentrations. Exceptions to this rule occur in the intact animal when one examines tissues with very small extracellular compartments, such as muscle or epithelia. Here, the cytoplasmic concentration of any substance will approximate to that of the whole tissue. Syncytia (Table 25) represent another range of tissues, where there are no visible intercellular membranes. Whenever one homogenises a tissue, the nuclei and the nucleoli are also subjected to pressure, so that the approximation of the total concentration of the cytoplasm to that of a homogenised tissue becomes less valid if the tissues have large nuclei. The relative contributions of the nuclei can be assessed by measuring them under microscopic vision, in tissue culture or pieces of unfixed, unstained tissue.

In addition to taking samples of cytoplasm, one can inject indicators, substrates and particles, directly into the cytoplasm, or one can incubate the cells in different media under direct microscopic vision.

The viscosity of the cytoplasm is low in living and unfixed cells - less than that of 10% glycerol (Hillman and Sartory, 1980, page 57). Intracellular movements may be seen best by time-lapse photography. The refractive index of the cytoplasm, is higher than that of the nucleus. When cells are fixed, their cytoplasm changes from a clear translucent fluid to one containing granules (Figure 2). Of course, the greatest proportion of any tissue consists of cytoplasm.

The view is expressed here - that the living cytoplasm is a homogeneous fluid, containing mitochondria and unidentifiable particles only - is completely different from the consensus view that it also contains Golgi bodies, endoplasmic reticulum, ribosomes, lysosomes and a cytoskeleton; all of these will be shown to be artifacts.

Chapter 25
The Mitochondria

In living cells, these filaments are several microns long, and are sometimes folded somewhat on themselves. They can be seen in every orientation, from transverse to longitudinal, and in-between (Figures 19, 20). The higher resolution of the electron microscope shows extra features: the membranes around the mitochondria appear double; double layered cristae may be seen across the mitochondria (Figure 21), each of the double layers being remarkably uniformly distant apart from its partner. Unfortunately, the two beautiful lines of the cristae always appear in exactly the same orientation whether the mitochondria are in transverse or diagonal sections. This shows that they are two-dimensional, and must appear after the sections have been cut. The simplest explanation for this is that the contents of the mitochondria, (sometimes called the ‘matrix’, and here called the ‘mitochondrioplasm,’) are liquid in life, and dry up during the preparation for electron microscopy, to give the two-dimensional deposit. The mitochondria are regarded as one of the locations of DNA (Anderson et al, 1981).

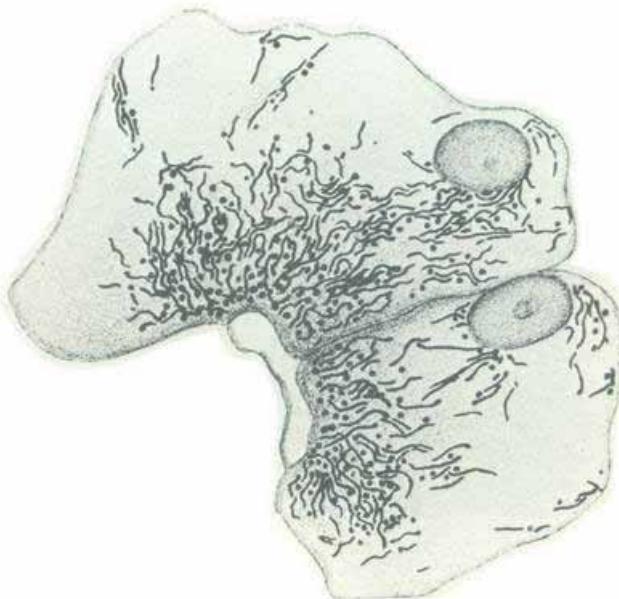


Figure 19. Frog liver cell stained with picric acid, showing mitochondria, from Altmann, (1890).

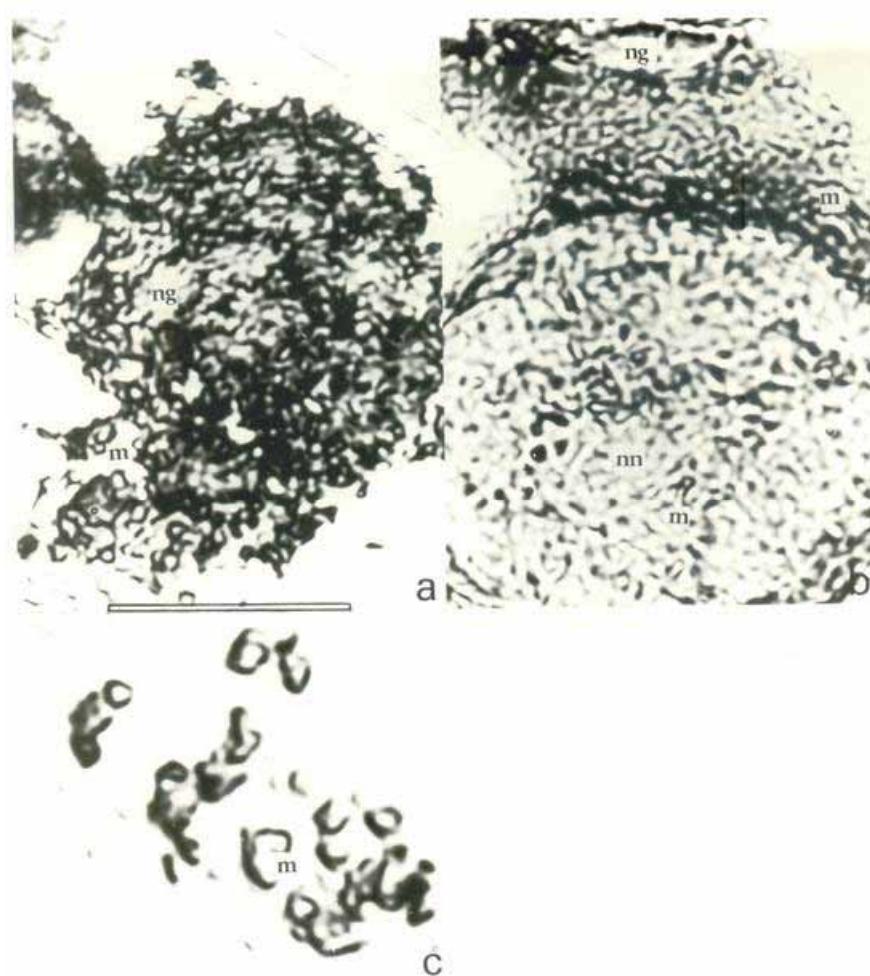


Figure 20. Mitochondria, *m*, in a rabbit frontal cortex, *a*, a piece of neuroglia, *ng*; *b*, upper, neuroglia, lower, neuron cell body, *nn*; *c*, separated mitochondria. Unfixed. Phase contrast. Oil immersion. The bar is 20 μm . The micrograph is from Hillman, Deutsch, Allen and Sartory, (1977), by kind permission of the Quekett Microscopical Club.



Figure 21. Electron micrograph of a bat mitochondrion, showing the two layers, ('trilaminar') of the mitochondrial membrane, and the transverse cristae. Note the uniformity of distance between the two layers. This figure was kindly given to me by the late Professor K. R. Porter.

A mitochondrial fraction was separated (Bendall and De Duve, 1960; Chayen, Bitensky and Wells, 1966; Whittaker and Danks, 1978). For history, please see Slater, (1981). It was further subdivided into fractions believed to be those of: the outer mitochondrial membrane; the intermembrane compartment; the inner membrane and the matrix (Table 15), (Ernster and Kuylenstierna, 1970; Zalman, Nikaido and Kagawa, 1980; Ragan et al, 1987). Using these procedures, it was found that the 'inner' and 'outer membrane' fractions were 'enriched' in cytochrome O oxidase, the outer fraction in monoamine oxidase, and the inner membrane and matrix, in most of the malate dehydrogenase. It would be useful to subject all the latter enzyme systems to all of the reagents and manoeuvres by which the fractions are separated (Tables 14, 15), to find out how they would be affected.

Since the relevant control experiments for subcellular fractionation have not yet been carried out (please see Table 21), one can not yet derive any conclusions from them. Nevertheless, there is no doubt that the fractions isolated by these empirical procedures, *do* demonstrate these enzyme activities. Therefore, it may be

argued that they have managed to ‘hold on’ to the enzymes and their co-factors, despite the huge amount of energy injected into them. This is usually regarded as an indication that the cycles and pathways were in the organelles from which they started (see, for example, Pette, 1966; Tedeschi, 1976).

Although the cristae are artifacts, they do appear in all electron micrographs of cells, and can be used as anatomical markers for mitochondria. The fact that both by light and electron microscopy, the whole mitochondria can be seen in every orientation, whereas the cristae can not, proves that the electron microscope *can* show three-dimensional objects, that is, there is a control for the instrument,. On the other hand, the two-dimensional appearance of the cristae - whether in transverse, oblique or longitudinal section - proves them to be a deposit after section. Liesegang lines have similar appearances. The same reasoning will be used in connection with the endoplasmic reticulum. ATPase molecules are believed to have been seen by electron microscopy (Attardi et al, 1981; Amzel et al, 1982).

A large number of ‘mitochondrial diseases’ have been described. These include those which mitochondria are believed to *cause* the diseases, and those in which the chemistry of the mitochondria has been affected (Table 62).

Mitochondrial diseases

Acetyl - CoA dehydrogenase efficiency	Huntington’s disease
Ageing	Ischaemic reperfusion injury
Alzheimers disease	Kearns-Sayre syndrome
Cancers	Leber hereditary optic neuropathy
Cardiac ischaemia	Myoclonus epilepsy
Cardiomyopathy	Myoglobinuria
Carnitine acetyl transferase deficiency	Myopathies
Deafness	Occipital strokes
Diabetes	Chronic progressive external ophthalmoplegia
Ophalmoplegia	Parkinson’s disease
Encephalopathies	Retinitis pigmentosa
Glutamic acid type 11 deficiency	Sepsis Stroke

Table 62. Mitochondrial diseases include disorders of mitochondrial DNA, respiratory enzymes and other enzymes, believed to be located in mitochondria. The data comes from Harkness, Pollitt and Addison, (1987), Ernster, Luft and Orrenius,

(1995), Beal, Howell and Bodis-Wollner, (1997), Brown, Nicholls and Cooper, (1999), Murphy, (2000) and Dahl and Thorburn, (2001).

Please see also ‘The Chemiosmotic Hypothesis’ (Chapter 47).

Chapter 26

The Golgi Apparatus or Bodies

The Golgi apparatus was first described in spinal neurons of the brown owl by Golgi, using his newly developed silver stain (Figure 22, Golgi, 1898). The tissue was fixed in 2% dichromate and stained with 1% silver chloride and osmic acid. The apparatus or body showed up as a black network within the cytoplasm. Early editions of Schäfer's textbook of histology called it, 'Golgi's deep network', as distinct from a superficial network on the cell surface. Subsequently the structure was found by bright field, dark ground and phase contrast microscopy in most tissues (Figure 23, Table 63; Von Dröscher, 1998 a). Whereas it started out as a network, the name has also been given to: random fibrils in the cytoplasm; a shell of granules around the nucleus; a paranuclear skein of about the same diameter as the nucleus; and a few discrete bodies in the cytoplasm (Figure 24).

The electron microscopists see elliptical 'stacks' of cisternae, at the edges of which are 'vesicles'. They called the concave aspect of the body the 'forming face', and 'secretion granules' and vacuoles are seen in this region. The vacuoles are at the edges of flattened 'stacks' or 'dictyosomes', and the distal membranes are called 'mature' membranes. On the outside of the stacks are pieces of endoplasmic reticulum, and there is much discussion in the literature about the 'storage vesicles' on the concave face 'trafficking' materials across the 'stacks' to the endoplasmic reticulum (Figure 25). (Please see Beams and Kessel, 1968; Rambour, Clermont and Maraud, 1974; Whaley, 1975; Fawcett, 1986; Carr and Toner, 1982; Peters, Palay and Webster, 1991, pages 39).

There was an animated controversy about whether the Golgi apparatus was an artefact of staining or not (Walker and Allen, 1927; Baker, 1955; 1957; 1963). Baker was originally extremely doubtful about its existence in life, but, subsequently changed his mind, when the same structure was claimed to be seen by electron microscopy. He believed that the two kinds of microscopists were looking at the identical structure.

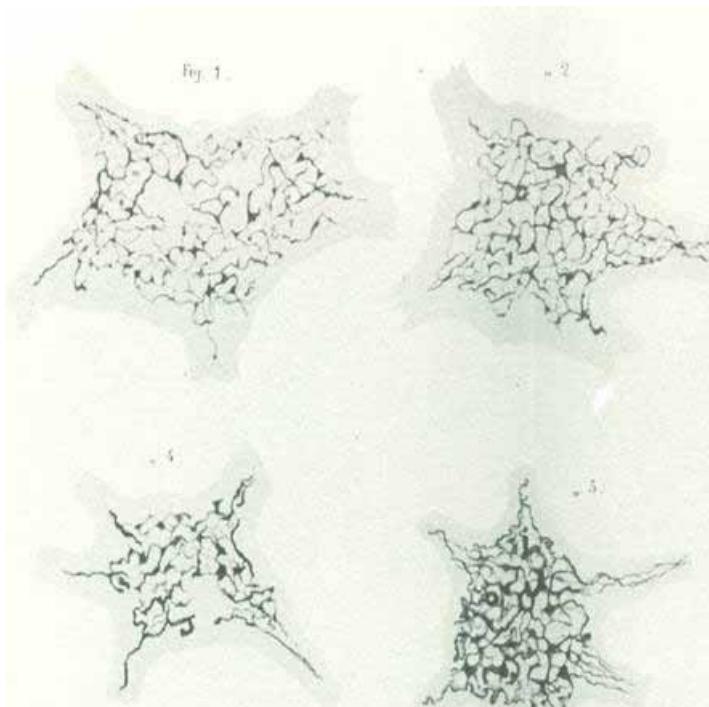


Figure 22. Spinal neurons from the barn owl (Golgi, 1898). *Note that Golgi saw a network.*

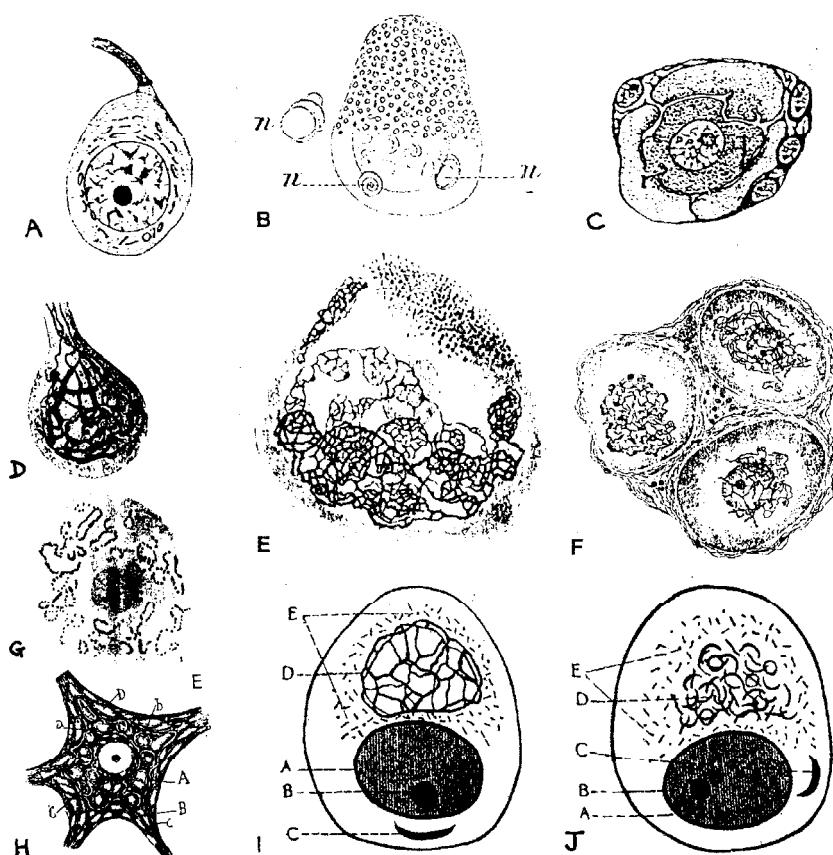


Figure 23. Different appearances recognised by histologists as 'Golgi' apparatuses, collected by Von Dröscher (1998 a, b), with references to them in the latter publications. A. Rings in the auditory ganglion of salmon embryo (Fürst, 1902). B. 'Nebenkern' in a fresh preparation of a pancreas cell of a salamander (Nussbaum, 1882). C. 'Trophospongium' of a spinal ganglion cell of guinea pig (Holmgren, 1902); D. 'Apparato reticolare interno' in a Purkinje cell of a white owl, fixed in potassium dichromate and osmic acid impregnated with silver nitrate (Golgi, 1879). E. 'Apparato reticolare interno' in a spinal ganglion cell of a horse, fixed with potassium bichromate and osmic acid, and impregnated with silver nitrate (Golgi, 1898). F. 'Endocellulärer Netzapparat' of sympathetic ganglion cells of dog, fixed in potassium bichromate, platinum chloride and osmic acid, and impregnated with silver nitrate (Veratti, 1899). G. Neutral red granules in the spinal ganglion of a young

rabbit, in a Grübler's neutral red solution, preserved by Gardner's method (Covell and Scott, 1928). H. 'Golgi's reticulum and Holmgrens ducts' of neuron cell bodies (Ramon Y Cajal, 1909-1911). I. 'Dictyosomes' in living spermatocytes of *Paludina vivipara* (Perroncito, 1911). J. As H, D here is Golgi's internal net (Perroncito, 1911). *Note the wide variety of appearance all called 'Golgi apparatuses'. Perhaps, Camillo Golgi can be considered the best authority of what constitutes his apparatus. This figure is reproduced by kind permission of Dr Ariane Von Dröscher* (from Von Dröscher, 1998b).

Some authors who have seen the Golgi apparatus in living cells and with phase contrast

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Table 63. This list was kindly compiled by Dr Ariane Von Dröscher of the A. Dohrn Zoological Station, Naples. *It is a very useful exercise to examine the original publications, with a view to deciding (a) whether one can see the network clearly (b) whether it is a deposit of cytoplasmic solutes.* Further references are given in Von Dröscher (1996; 1998 a,b).

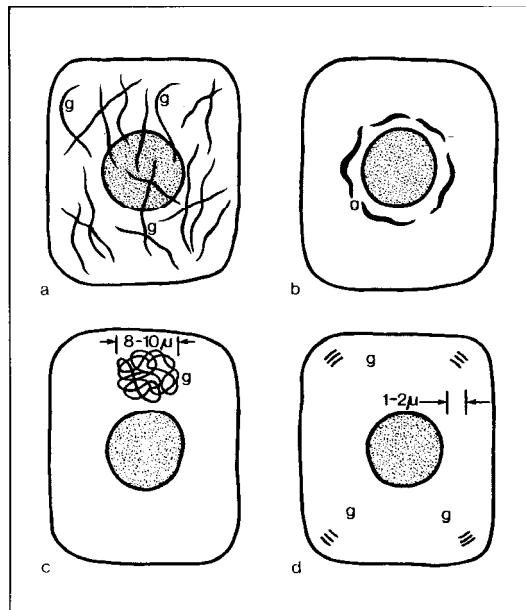


Figure 24. Diagrams of different views of the Golgi apparatus, *a*, Golgi's network; *b*, Holmgren's; *c*, a paranuclear skein; *d*, the modern electron microscope. *Are they all representations of the same structure?*

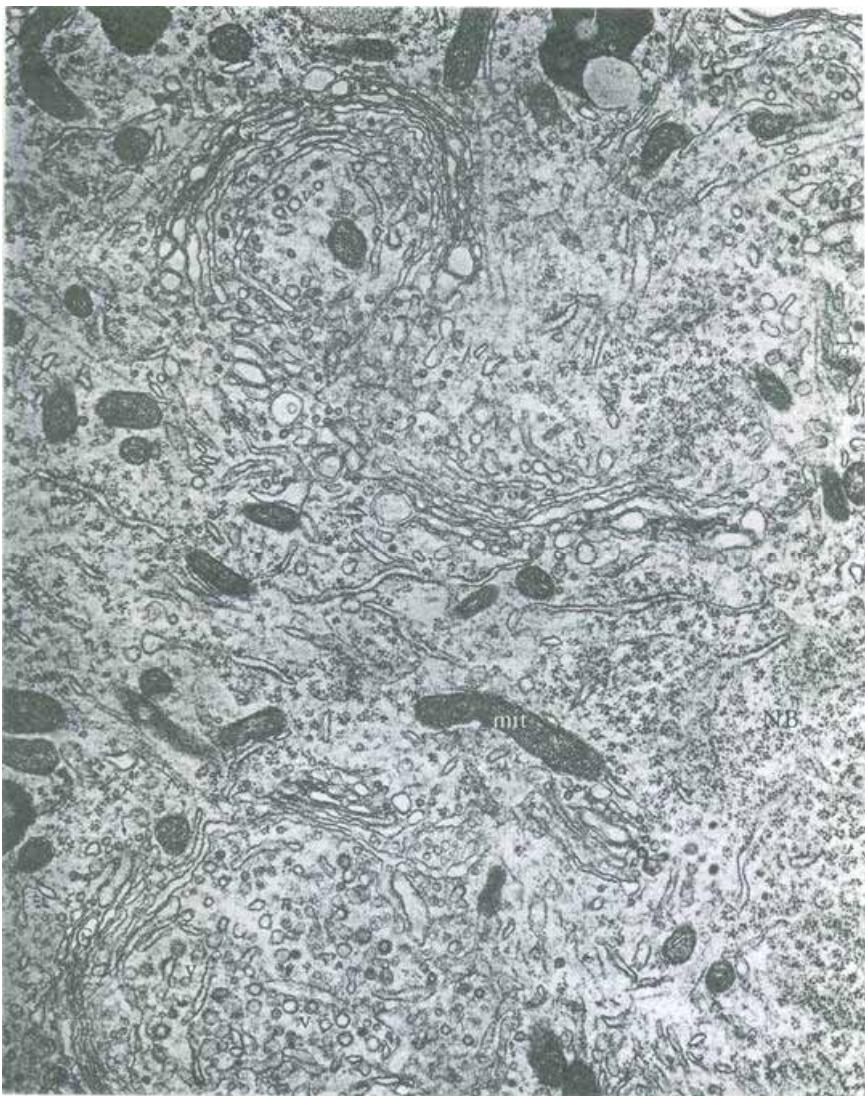


Figure 25. The Golgi apparatus of a Purkinje cell of the cerebellum of a Rhesus monkey. The apparatus can be seen in the upper left, middle, and lower left. Electron micrograph x 20,000. This is from Peters, Palay and Webster, (1998) page 39, and is reproduced by kind permission of the Authors and Oxford University Press, New York.

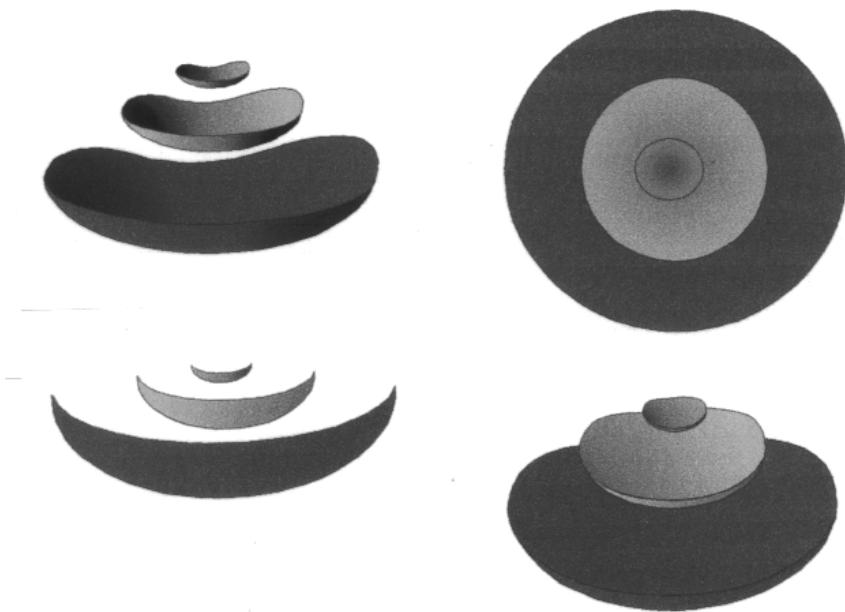


Figure 26. Computer graphics of a model of the Golgi apparatus. All these orientations should be seen by electron microscopy. This graphic was kindly prepared by Dr Nick McKay of Surrey University.

It is clear that the name ‘Golgi’ has been attributed to several quite different structures seen by light microscopy (Figures 22-25). It seems likely that any cytoplasmic particle, granule, piece of network or filament, which was not regarded as a mitochondrion was called a Golgi body, (see Novikoff et al, 1953) although many histologists who identified structures as Golgi bodies, did not show mitochondria in the same cells (Table 63).

Evolution of the description of structures believed to be present in the cytoplasm, is as follows. Firstly, they were seen by light microscopy almost invariably in preparations stained with salts of silver, osmium, lead or tungsten. They were seen to have a variety of appearances depending on the tissue and the staining procedure. Then they were seen by electron microscopy (Dalton, 1951), not without argument as to whether they might be artifacts of dehydration or staining (Baker, 1955-1963). They were then examined by autoradiography, histochemistry, immunocytochemistry and subcellular fractionation, and the organelle fraction was found to be rich in complex carbohydrates (Neutra and Leblond, 1966; Rambourg,

Hernandez and Leblond, 1969; Novikoff and Novikoff, 1977; Morré et al, 1979; Louvard, Reggio and Warren, 1982). A complex picture was then drawn about how protein is ‘transported’ or ‘trafficked’ across the Golgi apparatus to the endoplasmic reticulum, (Farquhar and Palade, 1981; Rothman, 1994). These experiments were reviewed in a special issue of *Biochimica Biophysica Acta* (Storrie, Duden and Allan, 1998), in which the possibility that the Golgi apparatus might be an artifact was not considered. Nor had it been by Berger, (1997), or Berger and Roth, (1997).

The procedures used to identify the body or apparatus engender a number of uncertainties:

- (i) some of them, alleged to detect the body in ‘living’ cells, involve staining or freezing (Table 63);
- (ii) when tissues are stained, it is virtually impossible by histological or electron microscopic procedures to find out the *chemistry* of any subcellular organelle, in view of the large number of manoeuvres and chemical reagents to which the tissue has been subjected (please see Chapters 8 and 9);
- (iii) soon after the publications of La Vallete St. George, (1867) and Golgi, (1898), most cytoplasmic particles, granules and bodies were dubbed Altmann’s granules, and, occasionally, Golgi apparatuses. Since then, lysosomes, endoplasmic reticulum, ribosomes, phagosomes and peroxisomes have been described (q.v.). Unfortunately by light microscopy, and especially in sections, these structures have such a variety of appearances that it is difficult to differentiate them. Electron micrographs themselves show such a bewildering variety of granules, membranes, fibres, vesicles, debris, etc. Furthermore, the elegant figures used to illustrate a particular apparent organelle are chosen subjectively as clear examples. Indeed, in the cytoplasm of animals, the cristae of the mitochondria seen by electron microscopy are the only clear features identifying this organelle. Viewing the others, one can only quote Terence, “that there are as many people as there are opinions”. The nature of electron microscopy is that so much information is available, that the observer has to choose micrographs to illustrate a particular appearance subjectively; because they are clear;
- (iv) subcellular fractionation may change the organelles so much that their chemistry at the end of the procedure, does not reflect that in the original living tissue, and electron microscopy has its own share of artifacts (please see Chapter 7);
- (v) there is much talk about the ‘trafficking’ of proteins. Of course, electron

microscopy requires killing the tissue, and one could only observe dynamic changes if one could photograph the same object several times. For the same reason, one can not know about membrane ‘recycling’, ‘reuptake’ of granules ‘trafficking’, or the direction of movement of particles. Similarly, the belief that a particle ‘stores’ a chemical ingredient is also questionable. Storage implies that the chemical enters, stays and leaves the particle, under some sort of control - another hypothesis that can not be tested by electron microscopy. One can only say that the particle is *present*;

(vi) the idea that the Golgi membranes ‘mature’ as they become peripheral in a stack is difficult to understand. How does one define ‘maturity’, and what are its chemical and histological characteristics?

(vii) in any electron micrograph of dried out salt solutions, urine deposits, bronchial washings, buccal smears, or tissues, there are always a large number of unidentifiable particles, granules, vesicles and filaments. These are usually: ignored; given names; described as particles or granules; regarded as ‘non-specific’; believed to be one of the named cytoplasmic constituents (Table 60, 61); identified as bacteria or viruses; regarded as ‘storage’ vesicles; thought to be stain; regarded as artifacts. Often, the decision about which name is to be given to an apparent inclusion in the cytoplasm rests mainly on the assertion of the author, especially if he is a Nobel laureate.

With these uncertainties in mind, one may now proceed to examine evidence that the Golgi body is almost certainly an artifact:

(a) Golgi’s original body (Figure 22) was a network which is very different in structure from the other forms seen by light microscopy (Figures 23, 24).

(b) the discrepancies between all the appearances by light microscopy, and those by electron microscopy (Figure 25) are even greater. The former have diameters of 3-10 μm , while the latter are generally less than 1 μm . The former are generally open networks, while the latter are concentric segments. Since the latter are so close together that the distances between the cisternae could not be resolved by the light microscope, it should appear only as a black blob, as opposed to an open skein, by light microscopy. It is wise to compare all the light and electron microscopic images closely, and then to ask oneself the simple question, “Can these appearances really all represent the same structure in the living cell?” I believe that they can not;

(c) intracellular movements of light microscopically visible structures such as mitochondrial and Brownian movements, diffusion, streaming, vacuolation, meiosis

and mitosis, can be seen in living cells, such as cells in tissue culture and protista. These would not be possible, either if there were a Golgi network, or a cytoskeleton (q.v.). When powders of carbon, pollens, or iron are injected into cytoplasm, they appear to move around freely, with no evidence of obstruction (please see Chapter 28);

(d) the Golgi body is seen in virtually every electron micrograph, (Figure 25), as stacks of equally spaced cisternae, (Rambourg and Clermont, 1997) which implies that the section cuts the Golgi body near its equator so that it looks like a section of an onion. Of course, solid geometry would require that it should appear face-on, like a circular shield at approximately the same frequency. It should appear obliquely far more frequently than the other two orientations. The face-on view is rarely seen, and the oblique view never. This problem is represented in the graphic of Figure 26. Electron microscopists are invited to send in any micrographs - as opposed to diagrams - showing the full range of expected orientations;

(e) the flattened cisternae are believed to enclose granules, and, therefore should be composed of two 'unit' membranes, that is, 4 lines, whereas only two lines appear on electron micrographs;

(f) the idea that the Golgi apparatus can 'facilitate' the passage of materials to the endoplasmic reticulum is somewhat puzzling. How can not just *one* membrane, but a large stack of them, *facilitate* the passage of particles? Membranes are normally considered to be barriers which slow or hinder movements across them;

(g) mitochondria do not seem to be visible by light microscopy, when the Golgi bodies are seen (Figure 23).

The Golgi apparatus appears when cells are stained with salts of silver and osmium. The salts are rather insoluble, and very near their solubility products. Both of these substances have high affinities for lipids and proteins. When tissues are prepared for histology or electron microscopy, they are dehydrated. About 60-90% of soft tissues are water, so that dehydration must result in the precipitation of all solutes, upon which salts of heavy metals would deposit. This seems to be the most likely explanation for all apparent cytoplasmic structures, other than the mitochondria, which can be seen in unstained cells. This makes it likely that any apparent Golgi apparatuses seen in living and unfixed cells probably consist of mitochondria. Nevertheless, it must be stressed again that it is extremely difficult to elucidate the chemical properties of cellular inclusions.

Chapter 27

Endoplasmic Reticulum, Microsomes and Ribosomes

The endoplasmic reticulum was first seen by electron microscopy as a cytoplasmic network (Porter, Claude and Fullam, 1945; Porter, 1953; Palade and Porter, 1954; Palay and Palade, 1955). It appeared trilaminar when stained with heavy metal salts (Figure 27). See also Terasaki et al, (1984; 1994). The enzymes, nucleoside diphosphatase and peroxidase, were regarded as markers (Novikoff and Heuss, 1963; Novikoff, Novikoff and Quintana, 1971; Novikoff, 1976). When the reticulum is lined by dark granules, it is called 'rough' endoplasmic reticulum, and when they are absent, it is named 'smooth'. The reticulum does not seem to be present in reticulocytes, embryonic cells and tumour cells. In somatic cells, it is regarded either as a network of tubules, or of flattened lamellae. The dark granules of the rough endoplasmic reticulum are believed to have a high content of RNA and are called ribosomes. They are thought to be the sites of protein synthesis.

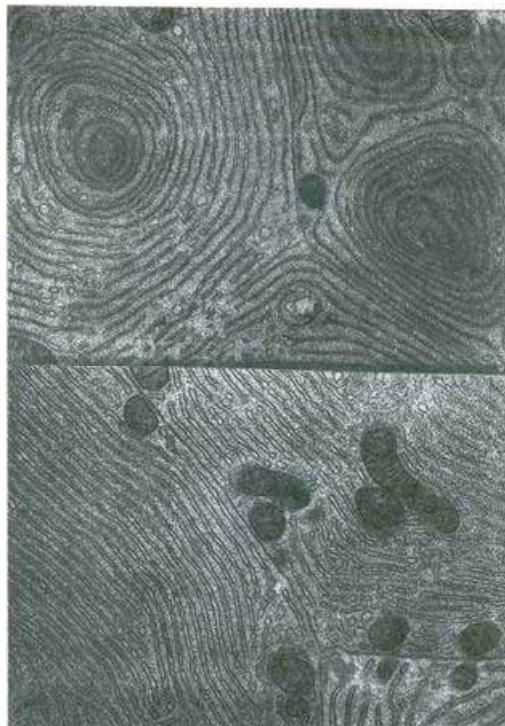


Figure 27. The endoplasmic reticulum in bat pancreatic acinar cells. These micrographs are from Ito, (1962), by kind permission of Elsevier Science Ltd.

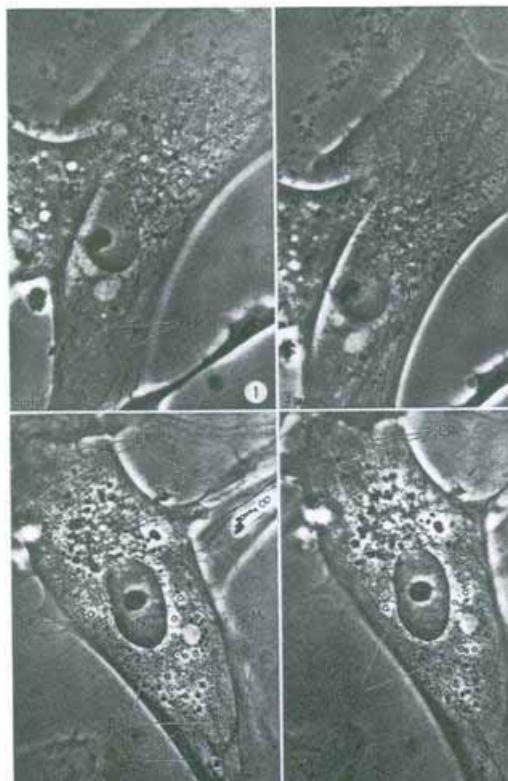


Figure 28. Light microscopy of endoplasmic reticulum in living tissue culture, from Rose and Pomerat, (1960) by kind permission of Rockefeller University Press.

The endoplasmic reticulum has also been seen by light microscopy. Fawcett and Ito (1958) have seen it by phase contrast in testicular cells, but about 40 minutes elapsed before it appeared clearly. Rose and Pomerat, (1960) saw it in tissue cultures of human muscle cells, and, more recently, Stephens and Allan, (2003) showed it up in Vero cells by video-enhanced differential interference contrast microscopy. The clarity of the latter images is extremely poor, as can only be judged by examining the illustrations with attention.

Claude, (1946, 1950, 1969) ultracentrifuged liver, and defined a fraction, which he called ‘microsomes’. This contained what was subsequently called endoplasmic reticulum, together with a significant concentration of RNA. Whereas the endoplasmic reticulum appeared lamellar, the microsomal fraction often looked like an accumulation of fairly empty ovals or spheres (Palade and Siekevitz, 1956; a,b Siekevitz, 1981) (Figure 1).

This procedure to prepare the microsomal fraction requires prolonged high energy centrifugation (Table 64). Like all complex biochemical procedures, the justifications for each manoeuvre and its duration, and each reagent and its concentration, are solely based on the apparent identity by electron microscopy of structures in the whole tissues with those in the fraction.

Preparation of rat liver microsomes

Albino rats <250 g were fasted for 24 hr to lower glycogen

They were killed by concussion and exsanguination

Pieces of liver were excised

Small pieces were fixed in 1% osmium tetroxide for 2 hr

The rest of the tissue was homogenised in an equal volume of 1.46 M sucrose

The latter was diluted to 1 to 10 (w/v) with 0.88 M sucrose

The homogenate was centrifuged at 20,000 g for 30 min to sediment larger particles, including the 'fluffy' layer believed to contain some microsomal material

The supernatant of the latter was centrifuged at 105,000 for 60 min (which should sediment particles > 35 µm within 1 hr)

The volume of each fraction was raised to that of the original homogenate with 0.88 M sucrose

(The pellets in each tube represented microsomes originally present in 1 g of liver)

The supernatant was pipetted off

The pellet was homogenised with a motor-driven glass pestle, and the suspension made up to 10 ml with 0.88 M sucrose

The microsomal fraction was incubated with an enzyme mixture

The fraction was centrifuged at 105,000 g for 2 hr

Trichloracetic acid was added to both the supernatant and the sediment to a final concentration of 5%

Chemical measurements were made in each fraction

Table 64. Preparation of the liver microsomal fraction from Palade and Siekevitz, (1956a). Pancreatic microsomes were prepared similarly by Palade and Siekevitz, (1956b). See also Castle, Jamieson and Palade, (1975).

Electron microscopy of rat liver microsomes

Liver in situ

The animal was killed
 The liver was excised
 It was fixed in 1% osmium tetroxide
 It was dehydrated with graded ethanols
 It was embedded in n-butyl methacrylate at 47°C
 Sections were cut out at 20-50 µm on a Porter-Blum microtome
 Micrographs were taken at instrumental magnifications of 6,000 to 14,000
 The micrographs were enlarged photographically

Liver homogenates

Samples of 2-3 ml were fixed in an equal volume of 2% osmium tetroxide in 0.88 M sucrose for 1-2 hr
 The homogenate was centrifuged to form a pellet (no data about g or time were given)
 The pellet was cut into small pieces
 It was dehydrated and embedded as above

Microsomes

The microsomal fraction was sedimented
 The supernatant was discarded
 The plastic tubes were cut open
 The pellet was cut into small pieces
 It was fixed for 2 - 20 hr
 It was dehydrated and embedded, as above

Experimentally treated microsomal fraction

Some pellets were washed
 They were treated with versene and ribonuclease
 Other pellets were fixed by 10% formaldehyde, 100% ethanol, saturated HgCl₂, or 3% dichromate

Final supernatant

A small aliquot was mixed with an equal volume of 2% osmium tetroxide in 0.88 M sucrose for 20 hr
 The mixture was dehydrated, embedded and sectioned, as above
 It was treated in the same way as the microsomes

Table 65. These steps were also described by Palade and Siekevitz, (1956a,b).

Palade and Siekevitz were pioneers in the identification of the microsomal fraction and its examination by electron microscopy (Table 65). They used osmium tetroxide for fixation, and ethanol for dehydration. When Beaufay, Amar-Costepec and de Duve, (1981) measured the enzymes activities in what they called the microsomal fraction, it contained enzymes, which they regarded as coming from the outer mitochondrial membrane, the plasma membrane, the Golgi membrane, and the

endoplasmic reticulum - cytoplasmic, luminal and rough (Table 66, 67). This led to two difficulties. Firstly, the microsomal fraction could not be considered to consist only of endoplasmic reticulum. Secondly, this procedure could not be used to separate cell membranes from endoplasmic reticulum. The latter has engendered the widespread beliefs that: (a) the latter two are continuous; (b) they are similar chemically; (c) they are both 'unit membranes' in the sense of Robertson (1959).

Microsomal fraction

<i>Putative Location</i>	<i>Enzyme or Constituent</i>
Outer mitochondrial membrane	Monoamine oxidase
	Minor part of NADH cytochrome <i>c</i> reductase
	Minor part of cytochrome <i>b</i> ₅
Plasma membrane	5'-Nucleotidase
	Alkaline phosphatase
	Alkaline phosphodiesterase
	Na ⁺ K ⁺ -ATPase
	NAD-glycohydrolase
	Adenylate cyclase
	Y-glutamyl transferase
	Bulk of sialoglycoprotein
	Nucleoside diphosphatase (ADP)
	Bulk of cholesterol
Golgi complex membrane	Galactosyl transferase
	N-acetylglucosaminyl transferase
	Sialyltransferase
	Some sialoglycoprotein
	Some cholesterol
ER cytoplasmic surface	Most of NADH cytochrome <i>c</i> reductase
	Most of cytochrome <i>b</i> ₅
	Stearoyl-CoA desaturase
	NADPH cytochrome P-450 C- reductase
	Cytochrome P-450
	Hydroxymethylglutaryl-CoA reductase
	Cholesterol 7α hydrolase
	Lysophosphatidic acid acyltransferase
	Fatty acid CoA ligase

	sn-glycerol-3-phosphate acyltransferase
	Phosphatidic acid phosphatase
	Diacylglycerol acyltransferase
	Diacylglycerol cholinephosphotransferase
	Diacylglycerol ethanolaminephosphotransferase
	Phosphatidylethanolamine SAMmethyltransferase
	Cholic acid CoA ligase
	Haem oxygenase
	Part of protein disulfide isomerase
ER luminal surface or content	Glucose-6-phosphatase
	Glucuronosialyltransferase
	Esterase
	Nucleoside diphosphatase
	Signal peptidase
Part of disulfide isomerase	Ribosomes
Rough Er cytoplasmic surface	Ribophorins
	Acyl-CoA cholesterol acyltransferase

Table 66. This list is from Beaufay, Amar-Costepec and de Duve, (1981).

Microsomal enzymes

<i>Enzyme or enzyme system</i>	<i>% in microsomal fraction</i>
Oxidoreductases	
L-amino-acid oxidase (1.4.3.2)	
Aryl-4-hydroxylase (1.14.1.1)	100%
Ascorbate-forming system (1.1.1.19)	
Azo-dye reductase (1.6.6.7)	
Catalase (1.11.1.6)	14%
Cystine reductase (1.6.4.1)	
Desmosterol reductase (1.3.1)	35%
Diaphorases-NAD ₂ NADH ₂ (1.6.99)	
Glucuronolactone reductase (1.1.1.20)	
β-Hydroxy-β-methylglutaryl-CoA reductase (1.1.1.34)	
Lipid-peroxidation system (ADP-activated; coupled to NADPH ₂ oxidase)	
Malate dehydrogenase (1.1.1.37)	
NADH ₂ -cytochrome <i>b</i> ₅ reductase (1.6.2.2)	

NADH ₂ -cytochrome <i>c</i> reductase (1.6.99.3)	62%
NADPH ₂ -cytochrome <i>c</i> reductase	64%
NADPH ₂ peroxidase (NADPH ₂ oxidase) (1.11.1.2)	
Enzymes or enzymeStearate-dehydrogenating system (->oleate)	
Steroid reductase ->5 <i>a</i> isomer)	
Sulphite oxidase (1.8.3.1)	75%
<i>Transferases</i>	
3-Acylglycerophosphorycholine acyltransferase (from acyl-CoA) (2.3.1)	
deCDPcholine: 1,2-diglyceride cholinephosphotransferase (2.7.8)	50%
CDPcholine:1,2-diglyceride cholinephosphotransferase (2.7.8.2)	97%
Diglyceride acyltransferase (2.3.1.20)	84%
Glycerolphosphate acyltransferase (2.3.1.15)	
Phenylphosphate: cytidine phosphotransferase	
Phosphatidylinositol kinasc (2.7.1)	
Pyrophosphate: glucose phosphotransferase	
Ribonuclease ('alkaline') (2.7.7)	low
Transmethylases, to phosphatides or other acceptors (2.1.1)	
UDPGlucose: glycogen glucosyltransferase (2.4.1.11)	
UDPGlucose: glycogen glucosyltransferase (2.4.1.11)	
UDP glucuronyltransferase (2.4.1.17)	high
<i>Hydrolases</i>	
Alkaline phosphatase (3.1.3.1)	42%
Amylase (3.2.1.1)	52%
Arginase (3.5.3.1)	41%
Arylsulphotase C (3.1.6.1)	62%
ATPase (ATP pyrophosphohydrolase; Mg ²⁺ ions in assay) (3.6.1.4)	low
<i>N</i> -Deacylase ("esterase") (3.5.1)	46%
Acetylcholinesterase ("true cholinesterase") (3.1.1.7)	62%
Arylesterase (3.1.1.2)	58%, 67% or 85%
Benzoylcholinesterase (3.1.1.9)	46%
Esterase Carboxylesterase (3.1.1.1)	
Cholesterol esterase (3.1.1.13)	112%
Vitamin A esterase (3.1.1.12)	
Glucose-6-phosphatase (3.1.3.9)	74%
β -Glucuronidase (3.2.1.31)	40% or 37%
Inorganic pyrophosphatase (3.6.1.1)	80%

Lipoprotein lipase (3.1.1)	40%
Lysophospholipase (lysolecithinase) (3.1.1.5)	61%
NAD nucleosidase (3.2.2.5)	93%
NAD pyrophosphatase (3.6.1.9)	63%
NADP pyrophosphatase (3.6.1)	52%
Nicotinamide deamidase (3.5.1)	
ADPase (=CDPhase ?) (3.6.1)	
Nucleoside diphosphatases	>50%
GDP/IDP/UDPase (3.6.1.6)	71%-IDP
5'-Nucleotidase (nucleoside-5'-monophosphatase) (3.1.3.5)	35-40% or 47%
Nucleoside triphosphatase (3.6.1.4)	23%ITP
Phosphodiesterase I (3.1.4.1)	29%
Thiamine pyrophosphatase (3.6.1)	50%
Uronolactonase (3.1.1.19)	94%
<i>Other enzymes or enzyme systems</i>	
Acyl-CoA synthetase (6.2.1.3)	
Cholyl-CoA synthetase (6.2.1.7)	
Demethylase (azo-dye)	
Epoxysteroid lyase	
Ether (aromatic)-cleaving system	
Fumarase (fumarate hydratase) (4.2.1.2)	28%
Glutamine synthetase (6.3.1.2)	47%
Prothrombin-forming system	

Table 67. These activities are listed by Reid, (1967) who gives the references. The Author notes that "some fairly high activities in the literature have been excluded because, for example, the enzyme is really lysosomal". See also Ernster, Siekevitz and Palade, (1962). *In this Table, a low proportion of enzyme in the microsomal fraction must indicate that some of the enzyme must be lost.* The table is reproduced by kind permission of Elsevier Science Ltd.

The microsomes are seen as spherical bodies, with black granules on them, and the latter have been separated into a fraction. The granules are called 'ribosomes' because the fraction was found to contain RNA and protein, and individual granules are called polysomes. They can be separated into 'subunits'. (Table 68).

Preparation of ribosomal fractions and subunits*Ribosomes*

The rats were starved for 1-2 days
They were decapitated
The organ was excised
A piece was cut out
It was diluted with buffer
It was minced and homogenised 5-6 times
It was centrifuged at 13,000 g for 30 min
The supernatant was put aside
An equal volume of buffer was added to the pellet
It was homogenised 3 times
It was centrifuged at 13,000 g for 30 min
This supernatant was pooled with the earlier one
It was filtered through cheese cloth
The filtrate was centrifuged at 140,000 g for 2 hr
The pellet was resuspended in buffer
2.5 M KCl and 10 mM MgCl₂ were added
10% sodium desoxycholate was added
The solution was mixed
The layer was suspended over 0.3 buffered sucrose
It was centrifuged at 176,000 g for 90 min
The ribosomal pellet was rinsed
It was resuspended
It was centrifuged at 20,000 g for 10 min
Dithioerythritol was added to 1 mM for storage
KCl was added to make the suspension 0.5 M
Puromycin was added to 0.5 mM
It was incubated at 37° for 15 min to eliminate peptidyl tRNA and soluble protein synthesis factors
This 80s ribosome fraction was centrifuged at 105,000 g for 4 hr through a 0.5 M sucrose cushion
It was stored at -80°C
It was examined by electron microscopy

Subunits

2.5 M KCl and 1 M Mg Cl₂ were added to raise the concentrations to 1 M and 10 mM respectively
0.1 M mercaptoethanol was added to 20 mM
Mixture dissociated the ribosomes into subunits
The latter suspension was layered on to a 15 - 30% buffered sucrose gradient
It was centrifuged at 95,000 g for 5 hr
The gradients were fractionated
40s and 60s subunits were pooled
The subunits were recovered by centrifugation at 130,000 g for 12 hr

The pellets in buffer were centrifuged at 20,000 for 10 min
 They were stored at -80°C
 The 60s subunits were dialysed against buffer overnight
 The dialysed suspension was layered on to 15 - 30% sucrose gradient
 It was centrifuged at 238,000 g for 4½ hr
 The gradients were fractionated and the 60s subunits were collected
 It was centrifuged at 80,000 for 12 hr
 The 60s subunit was resuspended
 It was centrifuged at 20,000 g for 10 min
 It was stored under liquid N₂ or at -80°C
 It was analysed for purity by loading on to a sucrose gradient
 It was centrifuged at 240,000 g for 50 min

Table 68. This process was described by Spedding G, (1990), pages 1-29. This Table is reproduced by kind permission of the Author and IRL Press. See also Bommer et al, (1996).

Ribosomes are seen by electron microscopy to have diameters of 15 - 25 nm (Boublik et al, 1988). Chemical analysis shows them to contain 3 parts of RNA and 2 parts of protein. They are not seen on 'smooth' endoplasmic reticulum. They are believed to be the site of protein synthesis (Siekevitz and Zamecnik, 1982), although presumably, most cells synthesise proteins. RNA is believed to be present not only in the ribosomes on the endoplasmic reticulum, but also in nuclei, nucleoli and mitochondria (Caspersson and Schultz, 1940; Claude, 1943). It is generally supposed that in tissues in which the reticulum is not rough, or ribosomes can not be seen, they are, in fact, present 'free' in the cytoplasm. Of course, the other possibilities are that they are not present, or that they are not the sites of protein synthesis in the intact animal, although there is no doubt that protein can be synthesised by ribosomal fractions (*ibid*).

The endoplasmic reticulum is seen in transverse section in nearly all micrographs. It has been suggested that this is due to the choice of micrographs to illustrate the structure well, or that it would not be seen, except normal to the electron beam. These explanations are not satisfactory. If the reticulum is composed of flattened lamellae, which are not visible unless they are normal to the electron beam, then one should see a space, when the orientation of the lamellae was in the plane of the section; in oblique orientations intermediate to the latter two, one should see a region of blurring. Unfortunately, these orientations are not seen in any electron micrograph. This geometrical point is illustrated in the photographs of models of a

network and flattened lamellae (Figure 29). A related problem is that there do not appear to be any sections going through oblique chips of diagrams 3 and 7.

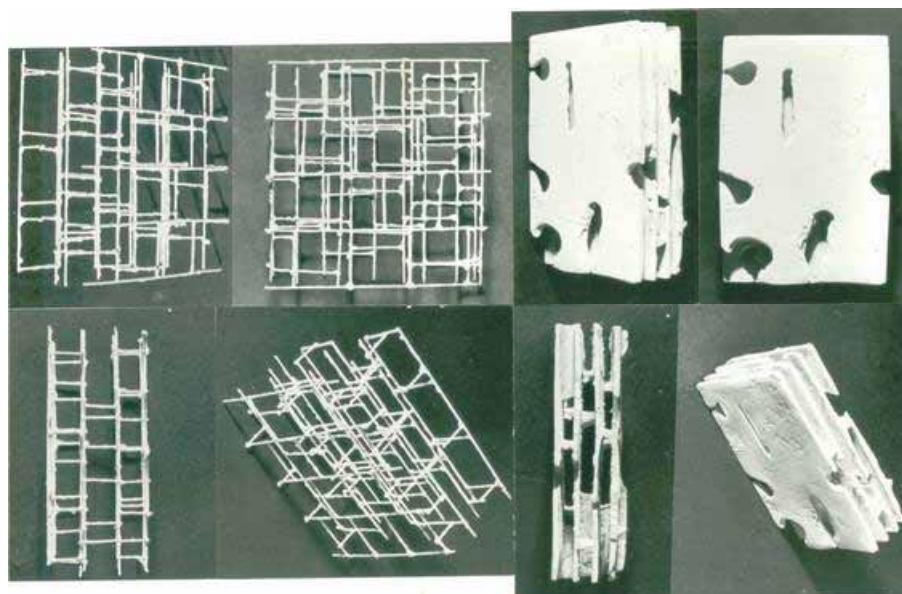


Figure 29. Models of the endoplasmic reticulum, *left*, as a network, *right*, as flattened lamellae in three dimensions.

If the endoplasmic reticulum has the same chemistry as the cell membrane, and if it were also a unit membrane, one would expect that it could be seen routinely by light microscopy for the same reasons as the cell membrane can be seen.

A further anomaly about the reticulum is that it is thought to be partitioned by 'cisternae', along which proteins, thought to be synthesised in the ribosomes, are 'exported' to the extracellular space. Most authors also believe that the reticulum is attached to the nuclear membrane (Hillman and Sartory, 1980, pages 102-103). In this case, the reticulum should appear 4 - layered, consisting of 2 'unit' membranes joining together before they arrive at the cell membrane, as the Robertson model indicates. Incidentally, the junction would prevent nuclear rotation, or would cause the reticulum to appear stretched sometimes. For the same reason, the junction of the cisternae with the nuclear membrane, should make the latter appear 4 - layered. The 'export' of proteins along the cisternae should lead to the expectation that some

granules should be seen in these spaces, but they are not. Cisternae, and their junctions with the cell membrane and nuclear membrane, are extremely difficult to find under the electron or light microscope, or in the micrographs, but are common in diagrams.

The existence of the endoplasmic reticulum is incompatible with intracellular movements of light microscopically visible particles observable in living cells, as is the existence of the Golgi apparatus and the cytoskeleton (please see Chapter 26).

Another way of looking at the intracellular movement problem is to measure the viscosity of the cytoplasm. This has been done by several different ways, for example, centrifuging protista, injecting plant cells, subjecting cells to magnetic fields, injecting air bubbles, and electron spin resonance. Most of the early studies were plant cells, protista and invertebrate eggs, but they also included axoplasm and muscle cells of frog. The authors used Einstein's equation for Brownian movement and calculated the viscosity from Poiseuille's equation (Reiser, 1949; Heilbrunn, 1958). Other techniques have included injection of iron filings, photography of rates of movement of particles and observations on nuclei. All measurements, except those on the viscosity of human embryonic lung tissue, (Keith and Snipes, 1974; Keith, 1979) show that the viscosity of the cytoplasm is approximately that of 10% glycerol.

The equivalent structure to the endoplasmic reticulum in muscle is the sarcoplasmic reticulum. (This will be dealt with under 'Muscle Structure', Chapter 36). Cells probably consist of 60-80% water. When they are dehydrated, the solutes come out of solution and are subsequently stained. One may regard it as axiomatic that dried cytoplasmic solutes would be more likely to precipitate with heavy metal salts, such as those of osmium, silver or lead, which are close to their solubility products, than with the living aqueous cytoplasm. This is similar to oil in a slick binding to the sand on a beach, rather than to the water itself.

This may give a clue as to the nature of the endoplasmic reticulum. Like some other alleged cytoplasmic structures, except the mitochondria, it is a deposit of the salt of a heavy metal on the cytoplasm extracted and dehydrated during the histological or electron microscopical procedure.

The fact that the microsomal fraction is so heterogeneous means that any of its properties may arise from the cell membrane, the Golgi apparatus, the endoplasmic reticulum, the cisternae or the ribosomes. This makes it hardly a localising procedure. The marker enzymes for each of the fractions are, of course, believed to mark the

original organelles. When an enzyme activity is found in a fraction identified by electron microscopy, where it is not believed to be, the procedure is extended until it is no longer present in the fraction, or it is dubbed a 'contaminant'.

There is no doubt that proteins can be synthesised when ribosomal fractions are incubated with the right substrate mixtures, but, for reason indicated, this does not necessarily mean that they are located in the same sites in the original living intact cells. It is also proper to point out that protein is synthesised by all cells, even reticulocytes, embryonic and tumour cells, in which ribosomes are not seen.

Chapter 28

The Cytoskeletons

In this chapter, the cytoskeleton is dealt with separately from the Golgi apparatus, the endoplasmic reticulum and the microsomes. From the literature, it is not clear whether the consensus is that the cytoskeleton is considered to include the endoplasmic reticulum or not. The component elements have been listed in Tables 60 and 61. They include all the fibrillar systems seen by electron microscopy, including the microtrabeculae, seen by Wolosewick and Porter, (1976; 1979), and several other fibres seen by immunofluorescence (see, for example, Björklund, Hökfelt and Kumar, 1984; Niewenhuyss, 1985; Lloyd, Hyams and Warn, 1986; Ockleford, 1988; Bray, 1992; Amos and Amos, 1995; Bloom, Björklund and Hökfelt, 1997; Bershadsky and Vassiliev, 1998). Nowadays, there are believed to be so many cytoplasmic fibre systems, that manufacturers of biological products send out bulky catalogues of antibodies available for the ‘specific’ detection of each of the antigens. There are serious doubts about the alleged specificity of the antibodies for antigens, believed to be present in the cytoplasm. A typical cytoskeleton element in the Vero fibroblasts in tissue culture is shown on the cover of ‘*Guidance to the Cytoskeletal and Motor Proteins*’, edited by Kreis T and Vale R, (1999) (Figure 30). The neuronal cytoskeleton has also been reviewed (Burgoyne, 1991).

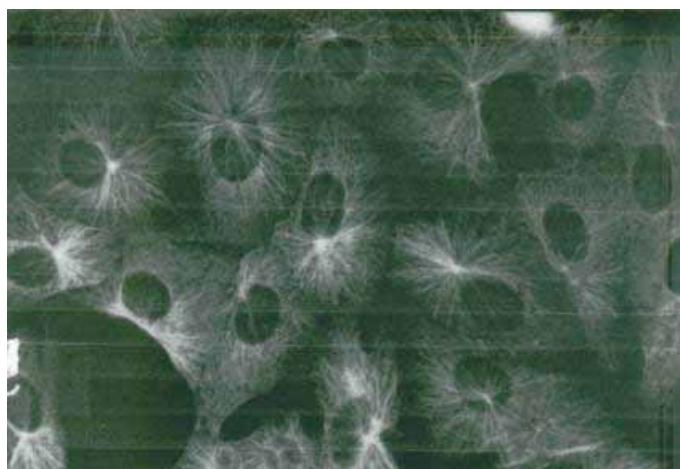


Figure 30. The cytoskeleton of Vero fibroblasts labelled with tubulin antibody from the cover of ‘*Guidebook to Cytoskeletal and Motor Proteins*’, edited by Kreis, and Vale, (1993) by kind permission of the Editors and Oxford University Press.

The reasons for which each of the elements of the cytoskeleton are artifacts are the same as those in respect of the endoplasmic reticulum (please see Chapter 27), but since fluorescence is so widely used to detect cytoplasmic fibre systems and proteins, a few comments are needed about this procedure. Its accuracy in the location of these structures or chemicals depends upon: (a) the specificity of the antibody used to detect the antigen being sought; (b) the belief that different organelles and the extracellular fluid are antigenically different. Although this is the basis of very much immunocytochemistry, it is *extremely* unlikely. All cells in the same animal or human body contain the same DNA, which programmes the proteins in the cells. There is little literature on examination of the presence of antigens, believed to be 'specific' for brain cells, such as GFAP (Eng et al, 1971) or MAP2, (Kay and Wong, 1986; Matteoli et al, 1993) in hepatic, renal or pancreatic cells. More important, perhaps, is the possibility that if different antigens were indeed present in different cells, the body would produce large numbers of antibodies to *its own cells*. This would produce not just occasional international symposia on auto-immune diseases (q.v), but a world wide epidemic in all human beings and animals of auto-immune diseases; (c) the procedures of immunofluorescence are largely empirical (for example, Table 29). If one mixes a serum containing a low concentration of antibody with a known antigen, one sees an immediate clouding of the solution due to precipitation. However, the immunocytochemists used fixatives, 'permeabilisers,' detergents, 'non-specific sera', fluorochromes and protein conjugates all of which are likely to increase or decrease the apparent precipitation. A true antigen-antibody reaction should require an antigen and an antibody alone, not several other chemicals as well. If they do not affect the fluorescence, why use them? If they do influence the intensity of the fluorescence, the apparent localisation of the antibody could be affected; (d) often the proteins are only weakly fluorescent. Therefore the fluorescence as detected microscopically, depends upon: the strength of binding of the conjugate to the antibody; the stability of the bond; and the location in which the conjugate is found - not necessarily where the antigen started. The conjugate may have a higher affinity for a cell organelle than the antibody has, so it will end up at a different site; (e) the likely movement of chemical constituents in the cytoplasm during the procedure has already been mentioned.

The crowding of the cytoplasm by all the cytoskeletal elements will be discussed as a barrier to intracellular movement (Chapter 43). Various explanations

have been suggested, which would allow light microscopically visible particles to move in the presence of the cytoskeleton: (i) they can move between the ‘weave’ of the filaments. They are 10-100 times too large to do so. Whereas the presence of *one* cytoplasmic fibrillar system might permit particles to move, the plethora of such systems (Tables 60, 61) could not permit it; (ii) the cytoskeleton dissolves in front of them, and reforms after they have passed. If this were so, one would expect to see the endoplasmic reticulum and the cytoskeleton compressed at the fore end of the moving particle and, perhaps temporarily absent at its aft end. As far as I can see in the electron microscope, or find in the literature, such appearances have not been observed. Furthermore, if moving particles were to dissolve the reticulum or cytoskeleton, they would have to contain some very powerful proteolytic and lipolytic enzymes, which could dissolve the membranes in real time. Laboratory experiments and the physiology of digestion show that cellular organelles are very stable and difficult to break down rapidly. An even more awkward consideration is that, while it is just conceivable that a mitochondrion would contain the necessary enzymes, injected iron filings or powdered glass - which dance with terpsichorean elegance - simply do not contain them. Even a mitochondrion in Brownian movement would have to contain a control system within it to ‘instruct’ its leading edge only to secrete the necessary lytic enzymes, and this would imply that the particle somehow or other decided beforehand the direction in which it was about to move; (iii) there is one unlikely possibility, which has to be entertained. Whereas cells in tissue culture and unicellular organisms do show intracellular movements - indeed, they are an important criteria that the cells are alive - it is just conceivable that such intracellular movements do not occur in the generality of cells in intact metazoa. This has yet to be examined, but I believe that it is a reasonable assumption to regard movements found in unicellular organisms and in tissue cultures as likely to be the same as those in most intact cells. This could only be disproved by demonstration that intracellular movement does *not* occur in cells in the intact animal. There is indirect evidence that it does, for example, from secretion of saliva, diapedesis, phagocytosis and pinocytosis, all seen in living intact animals.

The fibres composing the asters in dividing cells are widely named microtubules (Inoue and Sato, 1967). They are seen by light and electron microscopy (Dustin, 1984; Hyams and Lloyd, 1994). However, by electron microscopy, the microtubules appear to have diameters of 15-30 nm, which is well below the

resolution of 200-250 nm of the light microscope. The only way the two appearances could represent the same structures would be if the electron-microscopically visible fibres were so close together in such a way that the total width of a whole bunch were to exceed 200 nm. This is unfortunately not the case. Electron micrographs show that the microtubules seen in cell division are single strands.

There is much written in the literature about the ‘function’ of the cytoskeleton. The idea that molecular motors move it will be discussed. It is also believed to be involved in movement of whole cells. This is mainly because of the finding of the presence of actin and myosin in many non-muscle cells (Tilney, 1975; Lackie, 1986; Preston, King and Hyams, 1990; Lazarides and Weber, 1994; Remedios and Thomas, 2001), and their role in muscle contraction. It is argued that since muscles move, then actin and myosin probably move structures within cells or the whole cells. This will be discussed under the heading ‘Movement’ (Chapter 43). It has also been suggested that the cytoskeleton is ‘structural’, in that it represents the internal beams which support the shape of the cells, especially during movement.

One must conclude that the cytoskeleton, like the Golgi body and the endoplasmic reticulum, appears as a consequence of precipitation of the cytoplasm. If the immunocytochemists are right (Carraway and Carraway, 1992; Isenberg, 1995), the substances found in the fractions of the networks are present in the cytoplasm; if they are wrong, their complex empirical procedures show up artifactual fibrous precipitates, not ‘specifically’ representing the antigens they believe they are labelling. Important experiments still remain to be carried out in simple unfixed cells, with only antibodies present.

If the explanation is not accepted that the cytoskeleton is a precipitate of cytoplasm, one has to ask where all the solutes and submicroscopic particles go, when the cytoplasm is dehydrated for histology or electron microscopy.

Chapter 29

The Lysosomes

Lysosomes are believed to be spherical bodies, surrounded by membranes. They can be separated from liver, kidney, brain, etc. by complex procedures (Table 69). They are believed to contain a large number of acid hydrolytic enzymes. When the fractions were first separated by high speed centrifugation, they were found to have very little enzyme activity, but they were then subjected to the following: homogenisation with a Waring-Blendor; ‘inadequate’ osmotic protection; freezing and thawing; sonic vibrations; autolysis; exposure to lecithinases; proteinases; fat solvents and detergents. After this, they exhibited many enzyme activities, (Table 70). De Duve, (1969) described the finding that they had little activity, before these treatments as, “structure - linked” due to “a membrane - like barrier of lipoprotein nature restricting the accessibility of their internal hydrolases to external substrates”. This concept was generally accepted among biochemists. He nicknamed the lysosomes ‘suicide bags’; the fraction breaks down proteins, peptides, nucleotides, glycoproteins, glycolipids, glycosaminoglycans and lipids. Indeed, the lysosomes are believed to be the main sites of catabolism in cells (Segal and Doyle, 1978). De Duve and Wattiaux (1966) produced complicated diagrams of how particles entered cells, were digested, or were expelled. These diagrams have been widely reproduced (Figure 31). By electron microscopy, the lysosomes appear as darkly stained spheres containing darker centres and surrounded by cell membranes, or, simply empty. (Sawant et al, 1964; Smith and Farquhar, 1966; Baudhuin, Evrard and Berthet, 1967; Hers and Van Hoof, 1973). Many electron microscopists designated both the dark centred bodies and the vacuoles as lysosomes (Novikoff, 1963; De Duve and Wattiaux, 1966; Gordon, 1973).

Preparation of rat liver lysosomes

Male rats (200 - 250 g) were fasted for 24 - 48 hr

They were decapitated

Livers were excised

They were washed with cold 0.25 M sucrose

(All subsequent operations were carried out at an ambient temperature of 0 - 4°C)

The livers were minced and homogenised in 0.25 M sucrose

They were homogenised at top speed in a Waring blender

The pH was adjusted to 7.2 by adding 5 N KOH

The homogenate was filtered through muslin
 (Lysosomal enzymes, acid phosphatase, ribonuclease, aryl sulphatase and cathepsin were monitored at each stage)

The homogenate was centrifuged at 750 g for 10 min in a refrigerated centrifuge, then at 3,300 g for 10 min

The pellet was discarded

The supernatant was centrifuged at 16,300 g for 20 min

The supernatant was discarded

The pellet (the 'light mitochondrial fraction, F1') was resuspended in 0.3 M sucrose

It was centrifuged at 17,000 g for 20 min

It was washed again in sucrose

It was centrifuged to give purified lysosomes ('FIV')

Table 69. This is the procedure of Sawant, Shibko, Kumta and Tappel, (1964), by kind permission of Elsevier Science Ltd. Others are discussed by Beaufay, (1969).

Enzymes believed to be present in lysosomes

<i>Enzyme</i>	<i>Major natural substrate</i>
<i>Protease and peptidases</i>	
Cathepsin D	proteins
Cathepsin E	proteins
Collagenase	collagen
Cathepsin A	proteins; peptides
Cathepsin B	proteins; peptides
Cathepsin C	peptides
Aryl amidase	aminoacid arylamides
Peptidase	peptides
<i>Nucleases</i>	
Acid ribonuclease	RNA
Acid desoxyribonuclease	DNA
<i>Phosphatases</i>	
Acid phosphatases	most o-phosphoric monoesters
Phosphoprotein phosphatases	phosphoproteins
Acid pyrophosphatases	flavin adenine dinucleotide; ATP
Phosphodiesterases	oligonucleotides; phosphodiesters
Phosphatidic acid phosphatase	phosphatidic acids

<i>Enzymes hydrolyzing the carbohydrate chains of glycoproteins and glycolipids</i>	<i>Substrates</i>
β - N - acetylhexosaminidase	β - N - acetylhexosaminide in glycoproteins and glycolipids
α - N - acetylhexosaminidase	heparan sulphate
β - galactosidases	β - galactosides, galactosides in glycolipids, glycoproteins and mucopolysaccharide protein complexes
β - glucosidases	β - glucosides in glycoproteins
α - glucosidases	glycogen
α -mannosidase	α - mannoside in glycoproteins
β - xylosidases	β - xylosides in glycoproteins and mucopolysaccharide - protein complexes
α - fucosidase	fucosides
Sialidases	glycoproteins; glycolipids
Aspartylglucosamine amido-hydrolase	aspartylglucosamine link in glycoproteins
O - seryl - N - acetyl galactosaminide glycosidase	seryl acetylgalactosaminide link in glycoproteins
Neuraminidases	gangliosides
<i>Enzymes degrading glycosaminoglycans</i>	
Lysozymes	mucopolysaccharides; bacterial cell walls
Hyaluronidases	hyaluronic acid; chondroitin sulphates A and C
β - glucuronidase	polysaccharides; mucopolysaccharides; steroid glucuronides
Arylsulphatases A and B	arylsulphates; cerebroside sulphates; chondroitin 4 - sulphate
<i>Enzymes degrading lipids</i>	
Acid lipases	lipids
Triglyceride lipases	triglycerides
Phospholipases	lecithin; lysolethichin; phosphatidyl ethanolamine
Esterases	fatty acid esters
Glucocerebrosidase	glucocerebrosides
Galactocerebrosidase	galactocerebrosides
Sphingomyelinase	sphingomyelin
Ceramidases	Ceramides

Table 70. The data came from Tappel, (1969) and Holtzmann, (1989)

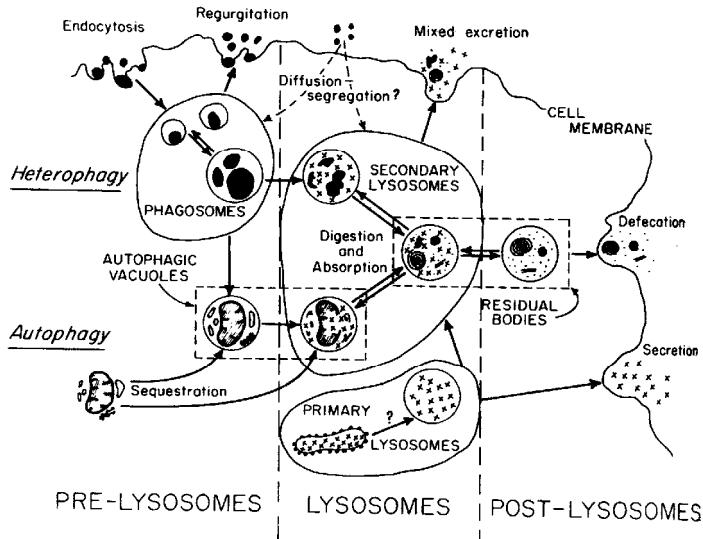


Figure 31. This figure is from De Duve and Wattiaux, (1966) by kind permission of the Annual Review of Physiology. (See also Marzella and Glaumann, 1987).

Elaborate schemes of how particles enter, are “digested”, or are expelled from cells, have been proposed. These have been expressed in diagrams (Figure 31). A number of new terms, such as phagosomes, autophagosomes, primary and secondary lysosomes, sequestration, regurgitation and defacation (from cells) was adumbrated.

Figure 31 raises the following questions: Are there any anatomical differences between phagosomes, autophagic vacuoles, primary lysosomes, secondary lysosomes, residual bodies, storage granules and glycogen? If not, how can one distinguish them? How can one know their direction? Do they occur in all cells, or just in phagocytes? How can one distinguish between autophagy, heterophagy and crinophagy? What is the difference between exocytosis, regurgitation, mixed excretion, defecation and excretion? How can one distinguish anatomically or in any other way between endocytosis, digestion and absorption? Is there a difference between a lysosome and a vacuole? What is ‘segregation’? If membranes surround phagosomes, primary lysosomes and secondary lysosomes, do the bodies within them also have membranes around them? If this diagram represents a series of hypotheses, what parts of them are provable? If they are not provable and disprovable, what place

can such a diagram have in cell biology?

After the enzymes believed to be located in the lysosomes had been listed, it was noticed that a large number of diseases, particularly hereditary and paediatric, showed deficiencies or excesses of the substrates, enzymes or precursors of these enzymes, and these were called 'Lysosomal Storage Diseases' (Table 71). The deficiencies are recognised by examining the biochemistry of the blood, and they are treated by adjusting the patients diets accordingly. It is not at all clear whether malfunction of the particular organelle is believed to be the cause, or the result, of the particular disease.

Summary of lysosomal storage disorders

<i>Disorder</i>	<i>Primary deficiency (secondary deficiency)</i>	<i>Substrate</i>
<i>Disorders of sphingolipid degradation</i>		
Fabry disease	α - galactosidase	gal-gal-glu- ceramide
Farber disease	Ceramidase	ceramide
Gaucher disease	Glucocerebrosidase	glucosylceramide
G_{M1} gangliosidosis galactosyl subscript 1	β - galactosidase	GM1 ganglioside, oligosaccharides keratan sulfate
G_{M2} gangliosidoses		
Tay-Sachs disease	β - hexosaminidase, α - subunit, (hexosaminidase A)	G_{M2} ganglioside
Sandhoff disease	β - hexosaminidase, β – subunit, (hexoseaminidase A,B)	G_{M2} ganglioside, oligosaccharides
Activator deficiency	G_{M2} activator	G_{M2} ganglioside
Krabbe disease	Galactosylceramidase	galactosylceramide, galactosylsphingosine
Metachromatic leucodystrophy		
enzyme-deficient form	arylsulfatase A	galactosylsulfatide
activator-deficient form	sulfatide activator/saposin	galactosylsulfatide
Mucolipidosis IV	primary defect unknown (ganglioside sialidase)	
Multiple sulfatase deficiency	primary defect unknown	(sulfatase substrates)
Niemann-Pick disease	sphingomyelinase	sphingomyelin
Schindler disease	α -N-acetylgalactos-aminidase	α -galNAc glycolipids glycoproteins

<i>Disorder</i>	<i>Primary deficiency (secondary deficiency)</i>	<i>Substrate</i>
<i>Disorders of glycoprotein degradation</i>		
Aspartylglycosaminuria	aspartylglycosaminidase	N-linked- α oligosaccharides
Fucosidosis	α -L-fucosidase	α -L-Fuc oligosaccharides
Galactosialidosis	protective protein/cathepsin (β -galactosidase and sialidase)	(substrates of S-galactosidase and sialidase)
α -Mannosidosis	α -mannosidase	α -Man oligosaccharides
β -Mannosidosis	β -mannosidase	β -Man oligosaccharides
Sialidosis	sialidase	sialyl oligosaccharides
<i>Disorders of glycosaminoglycan degradation</i>		
Hunter syndrome	iduronate sulfatase	dermatan sulfate, heparan sulfate
Hurler and Scheie syndromes	α -L-iduronidase	dermatan sulfate, heparan sulfate
Maroteaux-Lamy syndrome	GalNAc 4-sulfatase	dermatan sulfate
Morquio syndrome		
A-subtype	Gal 6-sulfatase	keratan sulfate, chondroitin 6-sulfate
B-subtype	β -galactosidase	keratan sulfate
Sanfilippo syndrome		
A-subtype	heparan N-sulfatase	heparan sulfate
B-subtype	α -N-acetylglucosaminidase	heparan sulfate
C-subtype	AcetylCoA; glucosamine acetyltransferase	heparan sulfate
D-subtype	GlcNAc 6-sulfatase	heparan sulfate
Sly syndrome	glucuronidase	dermatan sulfate, heparan sulfate, chondroitin 4, 6- sulfates
<i>Other single enzyme deficiency disorders</i>		
Pompe disease (glycogenolysis II)	α -glucosidase	glycogen
Wolman disease	acid lipase	cholesteryl esters, triglycerides
<i>Disorders of lysosomal enzyme biosynthesis</i>		
I-cell disease and pseudoHurler polydystrophy	{ 6-phospho-N- acetylglutosamine transferase (mislocation of many lysosomal enzymes)	nascent hydrolases (substrate of mislocalised enzymes)
<i>Disorders of lysosomal membrane transport</i>		
Cystinosis	cystine transport	cystine

Sialic storage and Salla disease	sialic acid transport	sialic acid
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Table 71. Most of these are listed by Neufeld, (1991) pages 257-280, and this table is reproduced by kind permission of the Authors and Publishers. References are given in the original Table. Other lists are found in Novikoff, Beaufay and de Duve, (1956); Hers and Van Hoof, (1973, pages 197-216); Hoogwinkel, (1974); and Watts and Gibbs, (1986).

One can now examine the lysosome both as a structure, and then as a site of acid hydrolytic activities in the intact cells of the living animal.

Both the apparently filled, and the vacuolar, lysosomes are sometimes difficult to distinguish by electron microscopy from microsomes, peroxisomes, glycogen granules and synaptosomes.

The lysosomes have diameters of 0.2 μm to 0.5 μm , and sometimes they appear as large as mitochondria or nuclei. Their dimensions are within the resolution of the light microscope, but they are rarely seen by light in healthy cells, and never in unfixed cells.

Electron micrographs show membranes around the individual lysosomes. It seems likely that since heavy metals are deposited to stain particles, any particle will appear to have a single membrane around it. Milk globules appear to be surrounded by membranes, when examined by electron microscopy (Wooding, 1974). It is not clear whether the membranes in milk are due to membranes around the milk globules, or pieces of the mammary glandular epithelium, which have been shed (Stewart, Puppione and Patton, 1972). My interpretation would be that the heavy metal salts deposit on an interface. Whereas the metal depositing on both sides of a membrane must appear as two lines, (a 'unit' membrane), the salt deposited around a single particle will appear as single ring, and be considered to be a membrane.

In respect of the lysosomal enzymes, there are other problems. As with other organelles, the lysosomes would have to house the precursors, substrates and the co-factors of all the enzymes within them. The pHs at which the enzymes, (Table 71), have their 'optimal' activities are very low, but the cytoplasm would be much more neutral, and so their enzymes activities at physiological pHs might be very low indeed.

The subcellular fractionation procedure for lysosomes (Table 69) is just as

problematic and empirical as all the others, and its practitioners share the assumptions and lack of controls of all such procedures. Nevertheless, they do seem to indicate that a large number of enzyme activities end up in the same fraction. But did they co-habit in their earlier lives? Are they the only enzymes which survive the huge injection of energy?

The concept of 'latency' of the 'membrane-linked' enzymes seems rather strange. The isolated lysosomal fraction had little enzyme activity, so it was subjected to a number of grossly unphysiological manoeuvres or reagents, and then the enzyme activity appeared. It seems rather difficult to extrapolate from such findings to the ideas that the 'latent' enzyme activities are 'released', and that they break down the proteins, lipids, carbohydrates and nucleotides in living intact cells. If the lysosomes require such powerful non-physiological agents to 'liberate' their enzymes activities - in addition to the powerful battery of agents to which they have also been subjected to fractionate them - how are such hydrolytic activities exhibited in the living intact cells?

The hypothesis that lysosomes can break down ingested particles could be proved, if they were observed in living cells actually causing organelles, particles or granules, to disappear in real time. Unfortunately, living processes can not be seen in the fixed, stained, histological or electron microscopic, sections. It will remain an unproved hypothesis, until the process can be shown, *while it is occurring*.

Chapter 30

Peroxisomes

Peroxisomes were isolated from the liver and kidney. They appeared in electron micrographs as spheres or ovals, 0.1 to 1.5 μm in diameter (Figure 11, De Duve, 1965b; De Duve and Baudhuin, 1966); there are 400 to 600 of them in a single cell. Like lysosomes, they are believed to be surrounded by a single membrane. Whereas they are spherical or oval in section, reconstructions show them to have sessile spheres protruding from them (Masters and Crane, 1995; page 12; Latruffe and Bagaut, 1997). They can be seen after staining with aminobenzidine under the electron microscope (Novikoff and Goldfischer, 1969; Gibson and Lake, 1993).

A subcellular fractionation procedure has been described by Leighton et al (1968). The rats were injected with the detergent WR 1339 (85 mg / 100 g body weight), and they were killed 3½ days later. As with other procedures, the liver was homogenised, frozen, thawed, sonicated and centrifuged isopycnically. The purity of the fraction was assessed by the numerical proportion of the structures which could be identified as peroxisomes, compared with the total *number* of particles present. The separation procedure took 5-6 hours.

Masters and Crane (1995) listed more than 30 enzymes believed to be located in peroxisomes, of which catalase, L- α -hydroxyl oxidase, D-aminoacid oxidase, urate oxidase and acyl CoA oxidase, have been examined most intensively. The enzymes present in the fractions are involved in metabolism of peroxides, hydroxyacids, aminoacids, purines, pyrimidines, prostaglandins and β -oxidation of fatty acids. They also take part in the biosynthesis of ether lipids, cholesterol, bile acids and dolichols, the metabolism of glyoxylate, and photorespiration. After separation of the peroxisomal fraction, further freezing, thawing, sonifications and centrifugations, separated it into membranous core, matrix and marginal fractions, each with its own selection of enzyme activities (Böck, Kramer and Pavelka, 1980; Alexson et al, 1985; Fahimi and Sies, 1987; Zaar, Völkl and Fahimi, 1991).

Peroxisomes are believed to be ‘involved’ in a number of diseases (Table 72).

Diseases in which peroxisomes are believed to be involved

Acetalassaemia	Obesity
Alcoholic hepatitis	Peroxisomal B-oxidation deficit
Carcinoma of the liver	Primary hyperoxaluria type 1
Conradi-Hanemann syndrome	Pseudo Zellweger syndrome
Cyclosporin nephrotoxicity	Rhizomelic chondroplasia punctata
Drug induced hepatitis	Starvation
Fatty acyl-CoA oxidase deficiency	Thiolase deficiency
Hyperpipecolic acidæmia	Toxic hepatitis
Hyperthyroidism	Tumours in rats
Infantile Refsum's disease	Viral hepatitis
Neonatal seizures	X - linked adrenoleukodystrophy
	Zellweger's (cerebro-hepato-renal) syndrome

Table 72. The data came from Goldfischer and Reddy, (1984), Harkness, Pollitt and Addison, (1987), Pollitt et al, (1987), Roels, (1991), Masters and Crane, (1995), Reddy et al, (1996). *These diseases have a wide range of aetiologies.*

There are some difficulties about the existence of peroxisomes in intact cells:

- (a) although their dimensions in stained cells appear to be within the resolution of the light microscope, they are rarely seen under the latter instrument;
- (b) by electron microscopy, they are a name given to a shape, which is not always distinguishable from a lysosome, residual body or glycogen particle;
- (c) the method of counting them as a proportion of particles of particular structure compared with the total number of visible particles, does not take into account aggregates of unknown particles, particles broken down and ones not recognisable;
- (d) the 'core' of the peroxisomes often appears as crystalline hexagonal lattices (Baudhuin, Beaufay and de Duve, 1965), but these are only seen in one orientation. (The interested cytologist is invited to examine original micrographs to judge this);
- (e) the fundamental difficulties of all subcellular fractionation procedures apply here.

Chapter 31
The Nucleus

The nucleus is an oval, spherical or lobular, body, surrounded by a membrane, and floating in the cytoplasm. By time lapse photography in tissue cultures, its profile can be seen to be changing continuously and rotating, while the cell is alive. By light microscopy, the nuclear membrane appears as a single thickness, and by electron microscopy as two or four thicknesses. The nucleus can vary in maximal diameter from 1 - 2 μm in Schwann cells, to 20 - 30 μm in neurons. In living cells, the nucleoplasm is translucent compared with the cytoplasm. Of course, the nucleus can be seen by electron microscopy (Hnilicka, 1972; Harris, 1978). The nucleus contains the nucleoplasm and the nucleolus (q.v.). The nucleus contains the genes, which are encoded in the DNA, and histones are found in the nuclear fraction. In frog oocytes, there is a high concentration of Na^+ in the nucleus (Allfrey et al, 1961).

Pores have been seen in the nuclear membrane by electron microscopy (Callan and Tomlin, 1950; Watson 1954 a,b). A more recent view is shown (Figure 32). Originally simple, they have become much more complex and, are now called ‘nuclear pore apparatuses’. They became decorated by annular granules with wispy hairs (Harris, 1974; Franke et al, 1981; Unwin and Milligan, 1982) (Figure 33). The biochemists have separated a subcellular fraction of pores, and characterised its subunits (Franke, 1966, (in onions); Monneron 1974; Dwyer and Blobel 1976; Harris 1978). The pores occupied 3–32% of the nuclear membranes then reviewed (Feldherr, 1972). Any conclusions about the chemistry of the pores would have to take this into account. The pores are believed to be the sites at which material pass from the nucleus to the cytoplasm (Anderson and Beams, 1956), and, presumably also in the opposite direction.

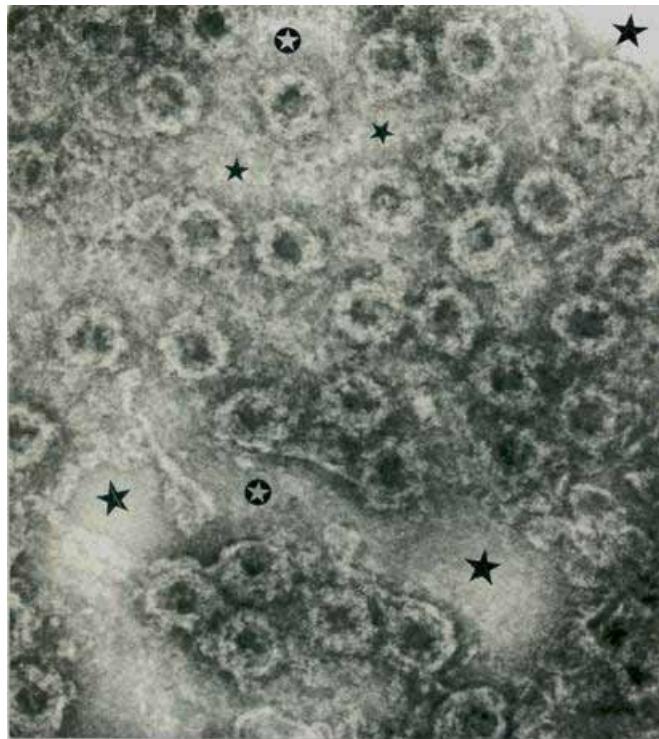


Figure 32. Nuclear pores from isolated rat liver. The preparation was negatively stained with ammonium molybdate. The electron micrograph was kindly sent as a Christmas Card in 1978 by Harris R, Stubbs G, Marshall P, Bonbee D, Ludlough R and Stokes R, of the Electron Microscopy Unit of North East London Polytechnic, to whom I express my thanks. *Please note that nearly all the pores are circular.*

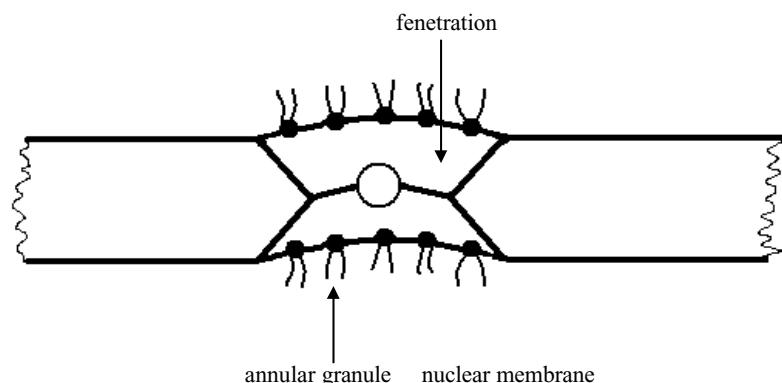


Figure 33. A diagram of a nuclear pore apparatus, after Franke W, and Scheer U, (1974). This is reproduced by kind permission of the Academic Press.

Whereas in living cells the nucleus appears to have a lower refractive index than the cytoplasm, fixation causes the nucleus to darken. In living cells, the nucleoplasm appears clear and uniform, but when the cells are stained, a deposit appears around the periphery of the nucleolus (Hyden, 1960). The ‘nucleolus - associated chromatin’ is seen in stained cells (Caspersson and Schultz, 1940; Caspersson, 1950). One of the big mysteries of cell biology is where the chromatin, which will constitute chromosomes during cell division, is located during the resting phase of cells. Chemically, DNA is about 1.08 nm to 1.11 nm wide and several hundred μm long (Watson and Crick, 1953; Wilkins, Stokes and Wilkins, 1953). It has a molecular weight of 1×10^6 to 120×10^6 (Michaelson, 1963; Fassman, 1976). It is rarely seen by the electron microscope during the resting phase of a cell, unless it is extracted (see, for example McKnight, 1977; Beyer, Bouton and Miller, 1981). The facts that nuclei can be transplanted and that they reproduce the characteristics of the parent cell (Bromhall, 1975; Hochedlinger and Jaenisch, 2002) is *prima facie* evidence that DNA is present in the nucleus. It seems likely that it is present in the *nucleolonema*, not in the nucleoplasm. The nucleus is also believed to contain histones (Hnilicka, 1972).

A nuclear fraction has been separated (Table 16) by a procedure, which uses less reagents and requires less energy than any other fraction. It contains Col pyrophorylorylase, and, possibly other enzyme activities (Mirsky and Osawa, 1961; Dixon and Webb, 1964).

The nuclear membrane appears imperforate by light microscopy. However, by electron microscopy, a number of cracks 40 - 120 nm in diameter are seen on transverse section, and circles or pores on plan view (Figure 32), (Callan, Randall and Tomlin, 1949; Afzelius, 1955; Wischnitzer, 1958; Gall, 1967; for review, please see Maul, 1977). The membrane is believed to be attached to the endoplasmic reticulum. On transverse section, the pores sometimes have oval edges. The earlier simple view was superceded by the description of ‘nuclear pore apparatuses’ (Figure 33). The pores were closed by ‘fenestrations’, in the middle of which plugs (‘annular granules’) were present.

Messenger RNA is believed to pass from the nuclei through the nuclear pores to the ribosomes (Maul, 1977; Gerace and Blobel, 1981).

Large neuron cell bodies can be cut open, and their nuclei dissected out. (Cummins and Hyden, 1962). (Figure 34).



Figure 34. A nucleus isolated by hand from the hypoglossal nucleus of a rabbit. The cell is not fixed. The maximum diameter of the nucleus is approximately 20 μm . *Note the nucleolar membrane.* Phase contrast.

A nuclear fraction has been prepared, and a potential difference has been recorded across the nuclear membranes (Naora et al, 1962; Lowenstein and Kanno, 1963, a,b; Mazzanti, Bustamante and Oberleithner, 2001). The isolation is likely to lower the potential difference, but, nevertheless, it is encouraging that these nuclei are sufficiently intact to be polarised at all.

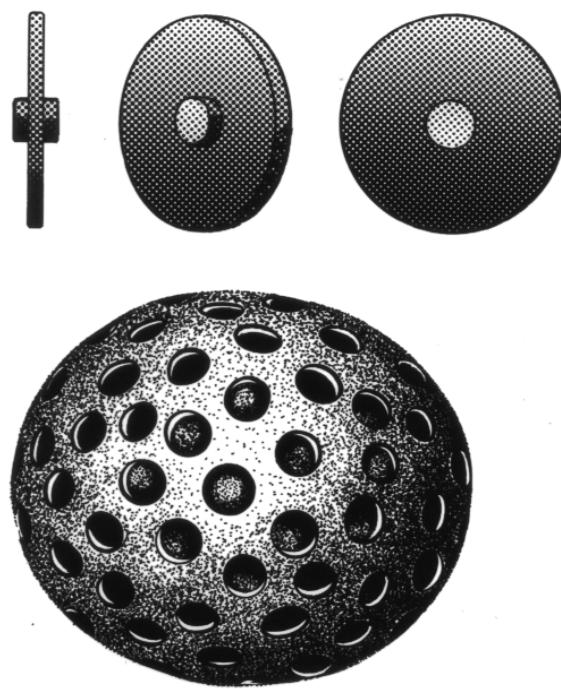
A fraction of nuclear pores has been prepared (Dwyer and Blobel, 1976).

In view of the relatively ‘mild’ treatment for the isolation of the nuclear fraction (Table 16), it would be a useful control experiment to subject this fraction further to the procedures for isolation of microsomal and lysosomal fractions (Table 64 and 69), in order to find out which enzyme activities it then displayed, and with what intensity.

There are several anomalies concerned with the nuclear pores, although there is no doubt that ions and small molecules cross between the cytoplasm and the nucleoplasm. These are as follows:

(i) the nuclear pores nearly always appear as clear circles on plan view and cracks on transverse section of the nuclear membranes. They never appear in the whole range of orientations which would be dictated by solid geometry, namely (from the centre outwards): circles, ovals, ellipses, disks and indentations on a golf ball (Figure 35). This means that the pores are two dimensional and their appearance is

created after the sections have been cut;



H.K. Teh.

Figure 35. Drawings of expected appearance of nuclear pore apparatuses, *upper left*, transverse view, *middle*, oblique view and *right*, plan view, *lower* drawing shows the invaginations on the nuclear membrane should appear circular in the middle, and gradually more oval and elliptical as one reaches the edge. This would apply whether one were looking at the whole nucleus or at sections of it.

(ii) since the sections of the pores should cut them at different angles, they should appear to have a variety of diameters, not a single one even if they were all the same diameter;

(iii) if the nuclear pores were continuous with the cisternae of the endoplasmic reticulum, that would make the nuclear 'envelope' four layered, composed of two trilaminar membranes, plus another two around the nucleus, since the former and the latter must be separate to permit nuclear rotation. As has been pointed out previously, the attachment of the reticulum to the pore is much easier to find in diagrams than in

micrographs;

(iv) nuclear rotation is seen in living cultures, but could not occur if the cytoplasm and nucleoplasm were riveted together by several nuclear pore apparatuses;

(v) messenger RNA has a molecular weight of approximately 4×10^6 , so that every time a pore opens to permit its exit from the nucleus, the many smaller ions and molecules would then be able to pass through the pore;

(vi) if the pores are blocked by fenestrations and plugs, the latter would have to open to permit the passage of messenger RNA. Therefore, there would have to be some way by which the messenger RNA induced the plugs to open. If the pores contain plugs, where do the latter go, when the pores are open?

(vii) the diagrams of the 'nuclear - pore apparatus (after Franke et al. 1981); (Figure 33) can be seen to be rather imaginative interpretations of what can be seen in the electron micrographs;

(viii) whereas the opening of ion channels and the discharge of transmitters are believed to be associated with miniature potential differences, these have not been reported for the nuclear pores;

(ix) if there were pores in the nuclear membrane, it seems likely that the potential differences across the membrane would be short circuited, whenever the pores opened.

If there are no nuclear pores, small molecules and ions could still cross the semipermeable nuclear membranes, as they cross dialysis tubing. If, indeed, *large* molecules do exchange between the nucleus and the cytoplasm, one needs to explain how and where they do this. One wonders how macromolecules for the synthesis of enzymes in the nuclei, mitochondria, lysosomes and transmitters, obtain entry to these phases. The following possibilities may be envisaged: only small molecules cross, and the larger molecules are synthesised in the organelles; large molecules may enter compartments whole, as in phagocytosis, pinocytosis and diapedesis. (Of course, this statement begs the question, because one does not know whether these phenomena occur in the generality of cells, nor indeed, the mechanism by which they occur at all).

If the nuclear pores are artifacts they probably represent cracks in the deposit around the nuclear membrane, when seen on transverse section, and bubbles when seen on the surface of the nucleus. The electron micrograph is an image of an area containing some membrane covered by a heavy metal deposit in an embedding

medium. The heavy metal, the membrane, the embedding medium, and any other substances added during the preparation, are each grossly different to each other, in respect of physico-chemical properties, such as temperature co-efficient of expansion, homogeneity of constituents, volatility, heat conductivity and heat capacity. Therefore, its sudden subjection to low pressure, massive bombardment by electrons and x-irradiation, in conditions in which heat can not dissipate rapidly, is likely to result in evaporation of all biological tissues - possibly at an explosive rate - , and severe mechanical stress, causing the metal deposit to crack. (Chapter 9).

The appearance of the pores as two-dimensional circles on the surface of the nuclear membrane, (Watson, 1954 a, b; Barnes and Davis, 1959; Wischnitzer, 1958, 1974) makes it certain that they occur after the sections have been cut. They are extremely uniform in diameter and in the same plane, as are air droplets at the bottom of a saucepan, when the water begins to boil. They may well be of similar origin, that is, due to gases coming out of the specimen on rapid heating (or cooling). The gas may have originated from volatilised substances from the biological material, or water or gases previously present in the material, such as nitrogen, oxygen, carbon dioxide or ammonia, when much heat is dissipated by the bombardment of electrons. The heat could not diffuse away quickly, due to the vacuum in the microscope chamber and the rapid dissipation of energy in the beam - although specialised stages have been designed to allow the heat to escape as quickly as possible.

One must conclude that in life, the nucleus is surrounded by an imperforate membrane. It is translucent. The DNA is probably present in the nucleolonema. The nuclear membrane is probably semi-permeable, and is of different chemistry than the cytoplasm or the nucleus (q.v.). There is probably a potential difference across the nuclear membrane. If the nuclear pores are artifacts, this leaves open the question of how macromolecules cross the nuclear membrane. The membranes may open, like self-sealing tanks, or only small molecules can cross it as they do a dialysis membrane, while the larger molecules are synthesised in the compartments to which they move.

Chapter 32
The Nucleolus

The existence of the nucleus had been recognised in a wide variety of cells (Remak, 1838) by the time Schleiden and Schwann, (1847) made their generalisation that all tissues are composed of or are produced by cells. Although several often rather amorphous nucleoli can be seen in plant and unicellular organisms, the few nucleoli in mammalian cells are generally spherical or oval, and have diameters of 0.5 to 5 μm in unfixed cells. In tissue cultures, they move and change their shapes slightly. They have a higher refractive index than the nucleoplasm. Ruzicka, (1899) and Estable and Sotelo, (1951) noted in nucleoli of nerve cells the presence of a fibrillar skein, which the latter authors called, the ‘nucleolonema’ (Figure 36). The rest of the nucleolus, without visible structure, was called the ‘pars amorpha’. When the cells were fixed and stained for histology or electron microscopy, the fine structure of the nucleolus was largely lost, and it appeared blurred (Bernard et al, 1955; Bernard, 1958; Vincent and Baltus, 1960; Sirlin, 1960; 1962; Vincent and Miller, 1966; Busch and Smetana, 1970; Ghosh, 1976).

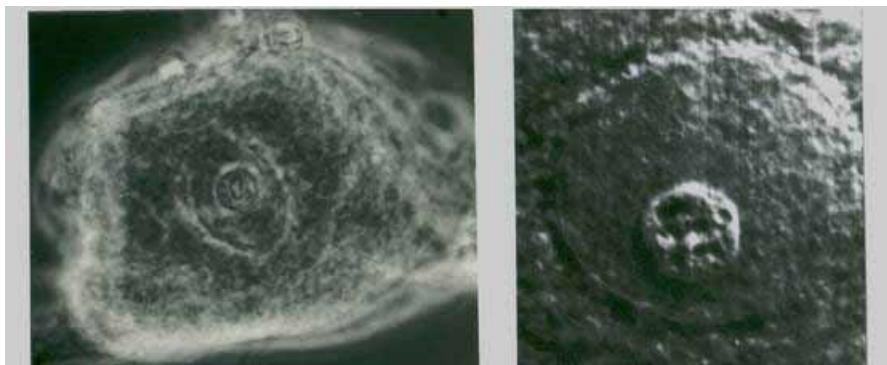


Figure 36. Unfixed neuron cell bodies from the rat vestibular nucleus, showing the nucleolus containing the nucleolonema. Oil immersion. The maximum diameters of the nucleoli are approximately 5 μm .

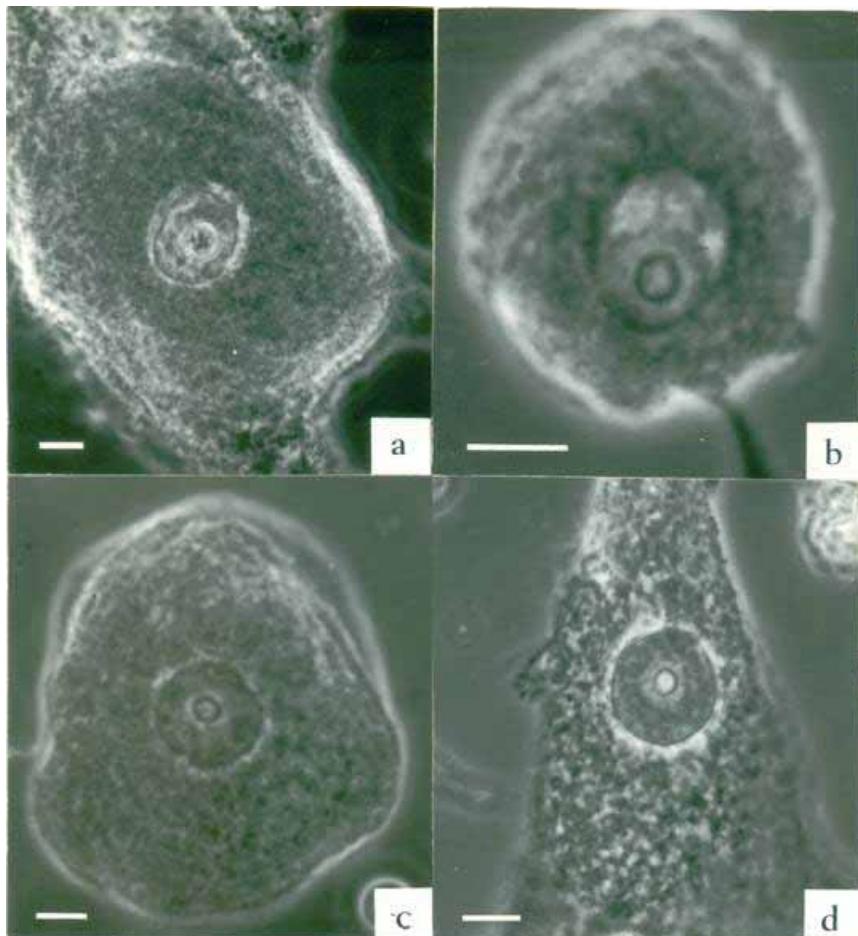


Figure 37. The nucleolar membrane in (a) a rabbit Deiters' neuron, (b) a rat sympathetic ganglion cell, (c) a guinea pig ventral horn cell, (d) a frog anterior horn motor neuron, all in '199' medium of Morgan, Morton and Parker, (1950). The bars are 10 μm . The micrographs come from Hussain, Hillman and Sartory, (1974). *The nucleolar membrane can not be seen after preparation for electron microscopy, which precipitates the nucleolus.*

There is an indentation on some chromosomes adjacent to the nucleoli, and this locus has been called the 'nucleolus organising region'. It is believed to be the site at which cell division is initiated (Caspersson, 1950). The nucleolus disappears during prophase at about the same time as does the nuclear membrane, and reappears during telophase just before the latter.

Ring shaped nucleoli have been seen mainly in egg cells (Montgomery, 1898; Austin and Braden, 1953). However, Hussain, Hillman and Sartory, (1974) noted a ring around nucleoli in several nerve cells and regarded the structures as nucleolar *membranes* rather than as *ring-shaped* nucleoli (Figure 37). They saw this appearance, if the nerve cell bodies were dissected out in normal saline, and not in the 0.25 M sucrose, which Hyden used (Hillman, 1986b). The same appearance was also seen in a cancer line T-47D of human breast carcinoma origin (Hillman, 1982). It is too early to say how widespread the nucleolar membrane is. We found it by phase contrast in all unfixed neuron cell bodies we examined from frog, rat, rabbit, sheep, ox, and human, brains and spinal cords.

High power phase contrast microscopy of nucleoli shows that the nucleolonema consists of small moving parachute-shapes, which appear and disappear (Estable, 1966; Sartory, Fasham and Hillman 1971). These observations have rarely been made by other research workers, examining these or other cells, (Lettré 1955); such observations should be repeated. At the same time, we observed that small strands of the nucleolonema passed into the nucleoplasm at intervals.

The nucleoli may be examined in sections by cytochemistry, or in enriched fractions (Table 17). The enzyme activities, - alkaline phosphatase, nucleotidase, ATPase, glucose-6-phosphatase, guanosine triphosphatase, succinic dehydrogenase, cholinesterase, RNA polymerase, NAD phosphorylase, NAD synthetase and ribonuclease - have been found in mammalian nucleolar fractions (Siebert et al, 1966; Busch and Smetana, 1970, pages 66-341).

Mainly nucleolar fractions have been found to contain RNA, DNA and proteins. The concentrations of these were referred to the concentration of protein in the fraction or in the tissue (Monty et al, 1956; Busch et al, 1963; Muramatsu et al, 1966).

Vincent (1955) summarised what he believed to be 'functions' of nucleoli, and some discussion of these is desirable. They are, as follows: (1) The 'shielding' of the chromosomes from the cytoplasm during mitosis. This seems very unlikely, since the nucleoli disappear at the beginning of the cell division. (2) The transfer of chromosomal influence to the cytoplasm. This depends upon the chromosomes taking up the role of the nucleolus (please see below). (3) A reservoir of materials produced by the chromosomes. Again this is dependant upon the nucleolar material being transferred to the chromosomes. (4) The site for a synthetic reaction necessary for

cytoplasmic synthesis. This is unprovable. (5) An accumulation of unused chromosomal or intranuclear products. One has to believe that nucleolar materials ends up in the chromosomes. One can not prove that intranuclear products end in the nucleolus. (6) An accumulation of unutilised or unusable materials of cytoplasmic origin, which enter the nucleus but can not return to the cytoplasm. It is difficult to know how one can judge what materials are ‘unutilised’ or ‘unusable’, or whether these unidentifiable materials can or can not return to the cytoplasm. (7) A reservoir of energy for nuclear activities. The energy sources have not been measured, and probably could not be measured, in the nucleoli in living intact cells. Nor can one know to which nuclear activities reference is made.

The following findings seem to be beyond doubt: (a) in life the nucleolonema is fibrillar and moving; (b) the nucleolus disappears before the chromosomes appear; (c) DNA can not be seen in the resting nucleoplasm; (d) the chromosomes disappear before the nucleolus reappears (Schrader, 1953).

A new hypothesis may be proposed. During the resting phase of cells, the DNA is located in the nucleolonema. Pieces of the nucleolonema protrude into the cytoplasm. During prophase, when the nuclear membrane dissolves, the cytoplasm mixes with the nucleoplasm, and the DNA in the nucleolonema attracts messenger RNA. The uncoiled nucleolonema breaks up into the chromosomes. Anaphase takes place, and the daughter chromosomes aggregate into the nucleolus, before the nuclear membrane is restored in late telophase. If correct, this hypothesis explains why the DNA is not *seen* in the nucleoplasm, and where the chromosomes go during the resting phase of the cell. It can probably be tested experimentally. Ultraviolet irradiation of the nucleoli in grasshopper neuroblasts inhibits their mitosis (Lettré, 1955; Peters, 1963). The nucleoli may have a genetic function (Berns, Olson and Rounds, 1969; Deak, Sidebottom and Harris, 1972).

Junctions Other Than Synapses and Neuromuscular Junctions

Various junctions between cells have been characterised in glands, stomachs, hearts, intestines, pancreases, gall bladders, livers, kidneys, uteri, capillaries, lenses, etc., from frogs, rats, mice, guinea pigs, rabbits and dogs. They have been seen in thin sections by electron microscopy, freeze etching, negative staining, low angle diffraction, immunocytochemistry, molecular biology, intracellular pipettes and subcellular fractionation (Farquhar and Palade, 1963; Kelly, 1966; Staehelin, 1974; Unger et al, 1977 a,b; Feldman, Gilula and Pitts, 1978; Bock and Clark, 1987; Dhein, 1998 and Novartis, 1999). They do not appear to have been seen in unstained cells.

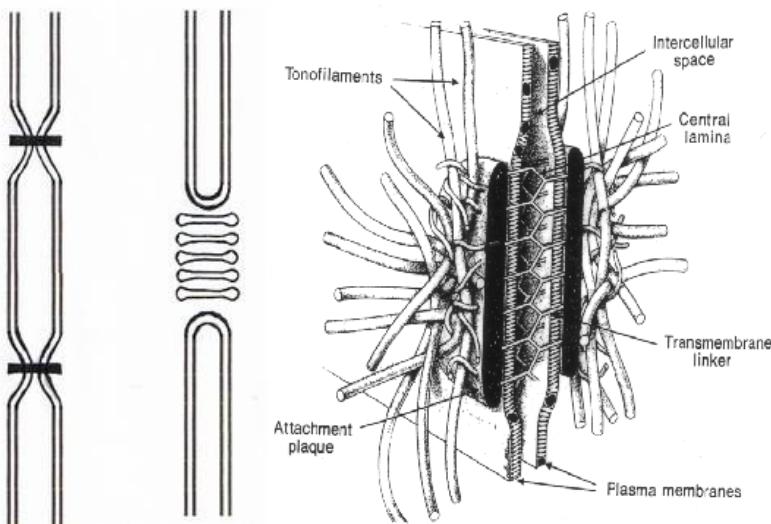


Figure 38. *Far left*, tight junction. *Middle*, gap junction. *Right*, desmosome, from Staehelin, LA and Hill, BE (1978). Cell junctions, *Scientific American*, 238, No 5, by kind permission of the artist, Ms. Patricia J. Wynne, and the Publishers.

Four types of non-synaptic junctions have been described, ‘tight’, ‘gap’ and ‘intermediate’ junctions, and ‘desmosomes’ (Figure 38). The tight junctions are characterised by regions at which the membranes of the two adjacent cells are close to each other, (Unwin and Zampighi, 1980) and are bound by ‘claudins’ and ‘occludins’. Freeze fracture electron microscopy shows that the tight junctions are part of a three

dimensional network. The regions at which the membranes touch are believed to prevent fluids moving between the cells (Staehelin, 1974). Lanthanum salts fill the extracellular space partially at the tight junctions (Revel and Karnovsky, 1967; Hanna, Ornberg and Reese, 1985). Freeze etching is believed to split membranes across their hydrophobic planes and knobs are seen between them. Similar knobs are seen in a fraction of liver prepared with egta, and extracted with sodium deoxycholate, and this is regarded as evidence that the fraction contains tight junctions (Schneeberger and Lynch, 1984; Stevenson and Goodenough, 1984).

Revel and Karnovsky, (1967) also described 'gap' junctions between cardiocytes and between hepatocytes (Figure 38). They appear as gaps in the cell membrane with bars, called 'connexons' at right angles to the membranes, joining the cytoplasm, of two adjacent cells. These gap junctions or the connexons have now been seen in many tissues: heart; liver; stomach; artery; lens; thyroid; teeth; pancreas; cerebrum; neuroglia; retina; myelin sheath; inner ear; skin; lacrimal glands; cartilage; fibroblasts; smooth muscle; ovaries; placenta (Bock and Clark, 1987; Hertzberg and Johnson, 1988; Hoyt, Cohen and Saffitz, 1989; Dhein, 1998; Novartis, 1999). The transmembrane connexins are believed to be composed of a multigene family of 19 α and 6 β subunits, and to permit molecules of less than 1000 KD to pass between adjacent cells (Kumar, 1999). The resistance between cells, especially the epithelial and gut cells, which they have been studying, is low, especially in cells in tissue culture. It is measured by inserting intracellular pipettes into adjacent cells, and also by observing fluorochromes passing from one to another. In effect, adjacent cells are coupled together by these connexins.

Dhein, (1998) adopted Manjunath, Going and Page's, (1982) procedure for isolating pellets of gap junctions and then C43 connexins from the junctions (Table 73). The procedure involves 42 steps to separate the pellet, and subjects this to discontinuous gel electrophoresis. The whole procedure involves the use of 18 different unphysiological reagents, and 30, mostly highly energetic, manoeuvres. This particular connexin recovered is one of several, each requiring its personal recipe to isolate. The assumption inherent in this, like all biochemical procedures, is that it does not change the chemical properties of the original structures significantly.

Simplified description of the procedure of Manjunath, Going and Page, (1982), modified by Dhein, (1998) for separating a pellet of gap junctions from rabbit heart, and CX43 connexins from it.

The heart is cooled to 0 - 4°C in 1 mM NaHCO₃
 The fat and blood vessels are removed
 The heart is cut into small pieces and added to 1 mM NaHCO₃
 Phenylmethyl sulphonyl fluoride (PMSF), is added to a final concentration of 1 mM
 It is stirred for 15 min at 4°C
 It is homogenised for 60 sec in a Virtis homogeniser
 It is further homogenised in a tissue mixer SDT 100 EN
 The homogenate is diluted to 300 ml with 1 mM NaHCO₃
 It is filtered through 6 - 8 sheets of medical gauze
 The filtrate is centrifuged at 33,000 g for 15 min
 The supernatant is removed and the pellet is dissolved in 300 ml of 1 mM NaHCO₃
 It is centrifuged at 33,000 g again for 15 min
 The supernatant is removed, and the pellet is resuspended in 100 ml of 0.6 M KCl, 6 mM Na₂S₂O₃ in 1 mM NaHCO₃
 PMSF to 1 mM is added
 The suspension is stirred overnight at 4°C
 It is filtered through 6 sheets of medical gauze
 It is centrifuged at 27,000 g for 30 min
 The supernatant is removed, the pellet is resuspended in 0.6 M KCl, 6 mM Na₂S₂O₃ in 1 mM NaHCO₃ and homogenised for 3 strokes
 The homogenate is centrifuged at 27,000 g for 30 min
 The supernatant is removed, and the pellet is washed in 70 ml of 0.6 M KI, 6 mM Na₂S₂O₃ in 1 mM NaHCO₃
 The washed pellet is centrifuged at 27,000 g for 15 min
 The supernatant is removed, and the pellet is washed in 30 ml of 5 mM tris at room temperature
 The solution is homogenised with 3 strokes
 It is stirred, while being added to 30 ml of 0.6% of N laurylsarcosine in 5 mM tris
 It is stirred for 10 min at room temperature
 A density gradient is prepared in a centrifuge tube and the solution is added from top to bottom: 20 ml sample: 8 ml of 35% sucrose, 0.3% deoxycholate in 5 mM tris and 5 ml of 44.5% sucrose, 0.3% deoxycholate in 5 mM tris
 It is stirred for 10 min at room temperature
 A density gradient is prepared in a centrifuge tube and the solution is added from top to bottom: 20 ml sample: 8 ml of 35% sucrose
 It is allowed to stand for 20 min at room temperature
 It is centrifuged at 65,000 g for 60 min at 15°C
 The band at 35 - 44.5% interface is dissolved in 45 ml of 0.3% deoxycholate in 5 mM tris
 It is centrifuged at 65,000 g for 60 min at 15°C
 The band at 35 - 44.5% interface is dissolved in 45 ml of 0.3% deoxycholate in 5 mM tris
 15 ml of the solution is added to the density gradient again

The band at the 35 - 44.5% interface is dissolved in 1:1 0.3% deoxycholate in 5 mM tris
 The solution is centrifuged at 106,000 g for 30 min at 15°C
 The pellet is washed in 5 mM tris, and is believed to contain gap junctions and non-junctional membranes
 A density gradient is prepared and the solution is added from top to bottom: 9ml 31.5% sucrose, 0.3% deoxycholate in 5 mM tris, and 9 ml 35% sucrose, 0.3% deoxycholate in 5 mM tris
 It is centrifuged at 65,000 g for 90 min at 15°C
 The material at the 31.5 - 35% interface is centrifuged at 106,000 g for 30 min at 15°C
 It is washed again in 5 mM tris
 The pellet contains the gap junctions
 The connexins are separated using discontinuous gel electrophoresis.
 The gels had the following constituents:

Stacking gel

acrylamide 30%, 0.8% bisacrylamide in water
 tris / HCl 0.625 M
 Na dodecyl sulfate, 0.5% in water
 ammonium persulfate 10%
 N, N, N, N tetramethylene diamine (TEMED) 6 µl

Separating gel

acrylamide 30%, 0.8% bisacrylamide in water
 tris / HCl
 SDS 0.5%
 ammonium persulfate 10%
 TEMED 30 µl

Sample buffer

tris / HCl 0.5 M
 SDS 20%
 glycerin
 β-mercaptoethanol 5%
 0.1% biomoethanol blue in ethanol

Running buffer

tris / HCl 0.15 mM
 glycine 0.1 mM
 SDS 3 mM

Staining solution

methanol 500 ml
 concentrated acetic acid 500 ml
 water 100 ml
 Coumassie brilliant blue R-250 2.5 g

Destaining wash solution

methanol 50 ml
 concentrated acetic acid 75 ml

Table 73. Steps and reagents used to isolate CX43 connexins. *This Table, as others*

showing detailed procedures, is given: (a) to emphasise the large number of strong reagents and physical agents used; (b) to consider how each reagent or manoeuvre could affect significantly the chemistry of the final fraction; (c) to ask to what extent the properties of the final fraction might reflect the procedure used rather than the particle after which the fraction is named; (d) to consider what control experiments could be carried out to examine the latter; (e) to pose the question as to why the latter have not been carried out. This table from Dhein, (1998) is reproduced by kind permission of Karger Publishers.

Conditions in which gap junctions and connexins are believed to be involved

Ageing (Spach and Dolber, 1986)	Gliomas
Asthma	Hepatomas
Arteriosclerosis	Herpes simplex
Breast cancer	Huntington's chorea
Metastases	Infective heart disease
Cancer therapy	Ischaemic heart disease
Cardiac arrhythmias	Mental retardation
Cataracts	Pemphigus
Chagas disease	Rhabdomyosarcoma
Charcot - Marie - Tooth disease	Sensori-neural deafness
Congenital heart disease	Temporal lobe epilepsy
Diabetic cataracts	Wolff-Parkinson-White syndrome
Experimental metastases (Pauli and Weinstein, 1981)	

Table 74. The data comes from Ciba Foundation, (1987), Hertzberg and Johnson, (1988), Dhein (1998), Novartis, (1999) and Purpura and Magistretti, (2000).

The connexins can also be identified by immunocytochemical procedures, using 'specific' antibodies, and molecular biological techniques (Rozental, Campos de Carvalho and Spray, 2000; Purpura and Magistretti, 2000). It was suggested that the gap junctions close as a result of fixation (Bennett, Spira and Pappas, 1972). The truth of this hypothesis is difficult to demonstrate, since the junctions can only be seen in fixed tissues.

The molecular biology of connexins has been studied (Kumar, 1999). Intermediate junctions were first seen and named by Farquhar and Palade, (1963), and were subsequently studied extensively and characterised (Kelly, 1966; Staehelin, 1974). By electron microscopy, they are seen in epithelial cells, about 20 nm apart.

A fuzzy 'terminal web' is seen on each side of the junction, and it is believed to be composed of 7 nm actin filaments (Unger et al, 1977a). The cell membranes appear about 8 nm thick, composed of a 3 nm inner membrane, 2.5 nm middle, and a 2.5 nm outer layer (not shown). The intercellular region is said to consist of a 'family' of Ca^{2+} dependant adhesion molecules (CAMS). On the cytoplasmic sides of the junctions, there are molecules of tenuin, vinculin, ubiquitin and phakoglobin. Glycoproteins of 135 kD are also found in fractions containing intermediate filaments, and are believed to be 'specific' for them.

Desmosomes are the most complex junctions, usually between epithelial or skin cells (Figures 11, 38). They are 200 - 300 nm long, with a space of 20 - 35 nm in between the thickened cell membranes of the adjacent cells (Drochmans et al, 1978). Dense 'attachment plaques', 15 - 20 nm wide, are on the cytoplasmic face of each membrane, and 'tonofilaments' of keratin, 10 nm wide, radiate from the same face in all directions (Kelly, 1966). The intercellular space contains a dense line, called the central lamella (Staehelin, 1974). All electron micrographs show the same structures (Farquhar and Palade, 1963; Kelly, 1966; Staehelin, 1974 and Bloom and Fawcett, 1994). The micrographs differ from the diagrams in the following respects (Figure 11). The micrographs show the opposing membranes of the desmosomes as thickenings of, and continuous with, the membranes of the adjacent cells; they do not show the 'adhesion plaques' of the diagrams; the 'tonofilaments' of the micrographs are almost always fairly normal to the surfaces of the thickened membranes, not radiating from them in all directions; the central lamellae are much less defined in micrographs than in diagrams. The name 'hemidesmosome' is given to the appearance when it appears only on one face of the junction (Kelly, 1966).

The biochemical properties of parts of the junctions are derived from subcellular fractionation and immunochemistry, using colloid gold markers and polyclonal antibodies against cytokeratins and 4 protein families (Skerrow and Matoltsy, 1974, a, b; Steinberg et al, 1987; Schwartz et al, 1990). Different fractions were separated by treatment with trypsin, deoxycholate and edta. Two kinds of glycoproteins, related to cadherins, were found in the desmosomal fractions: desmogleins were believed to be part of the adhesion plaques; desmocollins are similar to cadherins believed to be present in the central laminae; desmoplakins I and II, which are larger molecules, and smaller phakoglobin are believed to be components of the intracellular plaques; the plaques are believed to be covered by a

very large protein, a desmoyokin.

Powell (1981) proposed that molecules could take two possible routes across an epithelium. They could cross the cells by the transcellular route. Fluid and particles could pass through the external membrane of the epithelial cell, and through that cell, to reach the extracellular compartment of the next layer of cells. Alternatively they could by-pass the epithelial cells by infiltrating between them extracellularly at the junctions, where their ingress could be controlled. Unfortunately, in cells in the intact animal, one can not know, the rate of passage of fluid or particles across individual epithelial cells, nor the proportion of the flow that takes each of the above routes.

Regrettably, one must entertain doubts about the existence in life of all these junctional structures, for the following reasons;

- (i) all these structures are seen by electron microscopy, but not by light microscopy, despite the membranes adjacent to the junctions being seen by light;
- (ii) nearly all micrographs show the junctions in perfect transverse section with the membranes of the adjacent cells of uniform distance apart. The only exceptions I could find in the literature were the oblique views seen after staining with lanthanum salts (Revel and Karnovsky, 1967; Staehelin, 1974);
- (iii) measurements of any dimensions of the junctions are made in dehydrated tissues, so that one can not know the thickness that any structure had in the intact animal;
- (iv) when one examines a section of a junction or an interface, one does not know its dimensions in other planes. One has to assume symmetry in three planes, even in a dehydrated tissue;
- (v) much of the chemistry of junctions, such as those in skin and heart, is examined in tissues in culture, which are not necessarily the same as those in intact tissues (please see Chapter 12);
- (vi) ‘negative’ staining is often used to show the junctions. Of course, all electron microscopic staining is negative. The stains precipitate on the membranes. They do not dissolve them. Therefore, the concentration of stain on either side of the membrane is much higher than it is within the membrane itself.
- (vii) when freeze etching is used, it is assumed that the planes of cleavage go between the two solid lines of the ‘trilaminar’ unit membrane. If the membrane is one thickness, as is argued here (Chapter 20), one can not split it in the latter plane.

However, there are no objective criteria whatsoever for deciding in which plane the ‘cleavage’ occurs;

(viii) detergents are used unsparingly in isolating junctions (Venter and Harrison, 1984; Goni and Alonso, 2000). They are extremely active chemically. The ones used are mainly polyethylene glycol, polar or non-ionic detergents, or bile salts (Le Maire, Champeil and Møller, 2000). They are believed to solubilise membrane proteins and lipids, initially by inducing the formation of micelles. The common assumptions are that the solubilisation does not affect: the naturation of the proteins; the affinity of stains, antibodies and ligands for the membranes; the chemical properties of the fractions and subtractions; the enzymes associated with the alleged structures; the chemical constitution of the membranes; or the entropy of the reactions associated with the membranes. Nor is it known how reversible any of the latter actions are when the detergents are removed.

(ix) a wide range of disorders is attributed to lesions of the gap junctions or connexins (Table 74). As in other biochemical lesions, one can not distinguish between the causes and the effects of the diseases. The crucial test of the belief in the usefulness of the understanding of these well known diseases, is whether studies of these structures lead to such insights in the diseases, as will result in rational treatment of them;

(x) many other mechanisms within one can cell influence neighbouring cells (Table 44).

Chapter 34

Neurons and Neuroglia

It is useful to identify the anatomical findings, which have led to the current consensus in order to understand the cellular structure of the nervous system. There are extensive historical studies on the question, (for example, Golgi, 1906; Ramon Y Cajal, 1906, Penfield, 1932; Ramon Y Cajal, 1909-1911; Clarke and O'Malley, 1968; Shepherd, 1991; Finger, 1994). My views on this topic have already been published in extenso (Hillman, 1986a; Hillman and Jarman, 1991; Hillman, 1996a), where full references may be found, and this chapter represents a summary of these. A film has also been made, and it is available from the author.

Probably, the first microscopic observations on the brain were made by Hodgkin and Lister, (1827), who saw a granular material in unfixed brain (Figure 39), Purkinje, (1838), Hannover, (1844), and Von Deiters, (1865), teased out cell bodies and their connections in both unfixed and fixed tissues. Hannover introduced chromic acid for fixation, and Deiters found nuclei, which were subsequently called Hortega cells (Figure 41), and are now called neuroglial nuclei.

Von Virchow, (1846) noticed that there was a substance between the neurons, and he gave it the name 'neuroglia', meaning nerve glue. He regarded it as an amorphous ground substance.

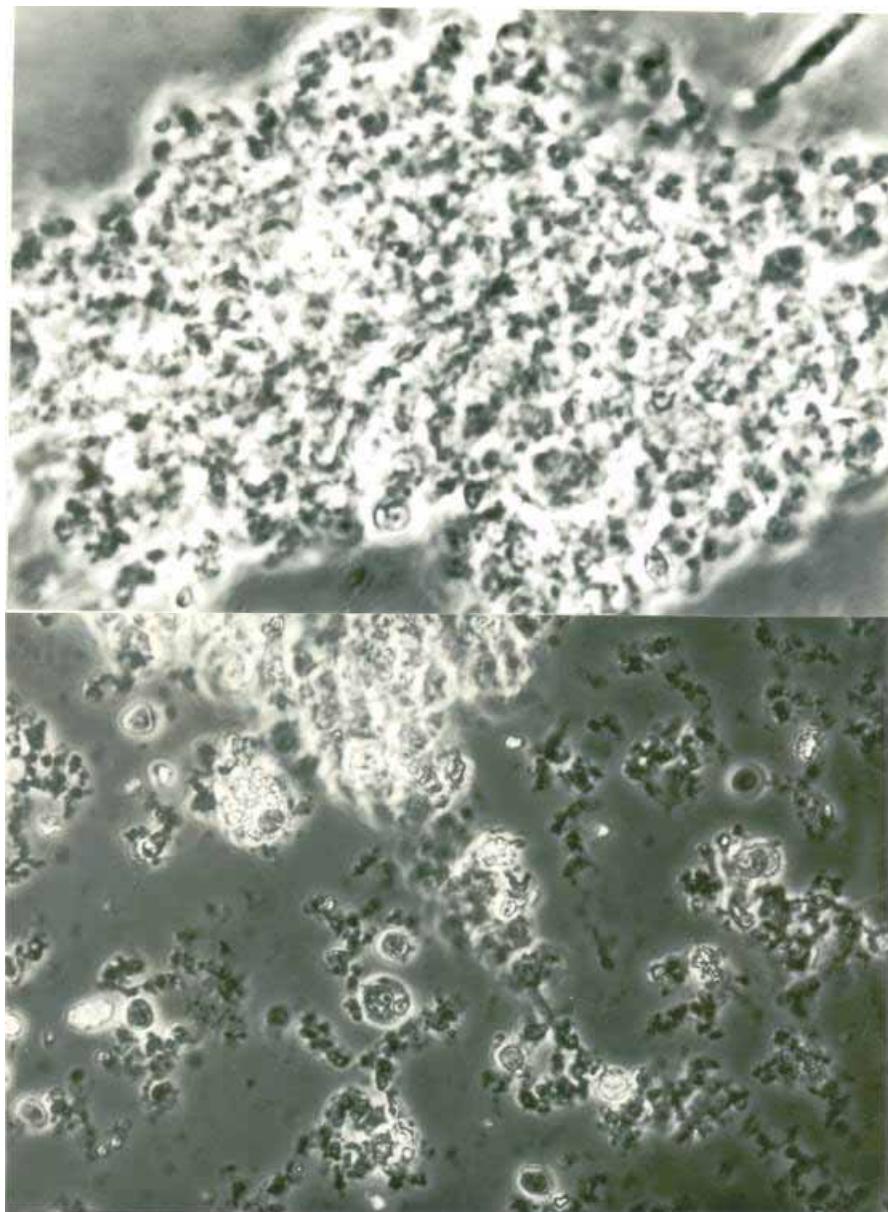


Figure 39. Fine granular material from *upper*, human putamen, *lower*, rat parietal cortex. Unfixed. Phase contrast, oil immersion, x750.

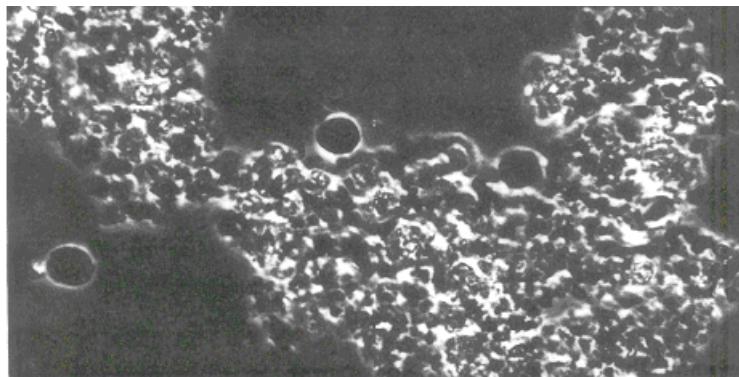


Figure 40. Neuroglial nuclei and fine granular material in human caudate nucleus. Unstained, phase contrast, x750.

Golgi, (1879) introduced a silver staining technique which showed up cell bodies and their many processes. The earlier histologists believed that most neurons were connected to each other (Von Gerlach, 1858; Apathy, 1897; Held, 1897; Golgi, 1906). This became known as the ‘reticularist’ view (Figure 41). Golgi, (1906) said that “the transmission of nerve impulses is conducted from the protoplasmic extensions, and the cell body towards the nerve extensions; consequently, each nerve possesses a receiving apparatus constituted by the body and the protoplasmic processes, a conducting apparatus - the nerve process (now called the axon) and a transmitting or discharging organ (now called the synapse or neuromuscular junction)”.

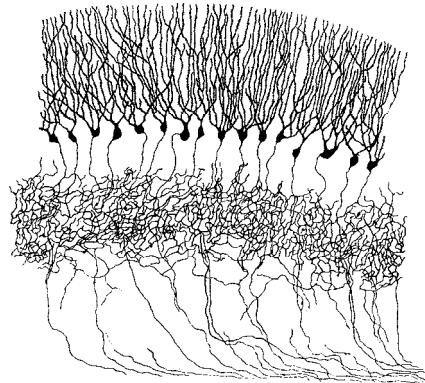


Figure 41. The dentate gyrus of the cerebellum, from Golgi, (1906), illustrating the multiple connections which each cell was believed to have.

The other view, called the ‘neuron doctrine’ was due, inter alia, to His, (1886), Forel, (1897) and Ramon y Cajal, (published in English in 1954). In summary, this stated that each neuron was a discrete body and was not attached to any other directly. Kühne, (1862) (Figure 42), Held, (1897) (Figure 43), and Auerbach, (1898), showed ‘end-fusse’ on muscle and nerve cells. They were regarded as the sites of transmission of signals from one neuron to another muscle or nerve, (Sherrington 1897). See also Kühne (1887).

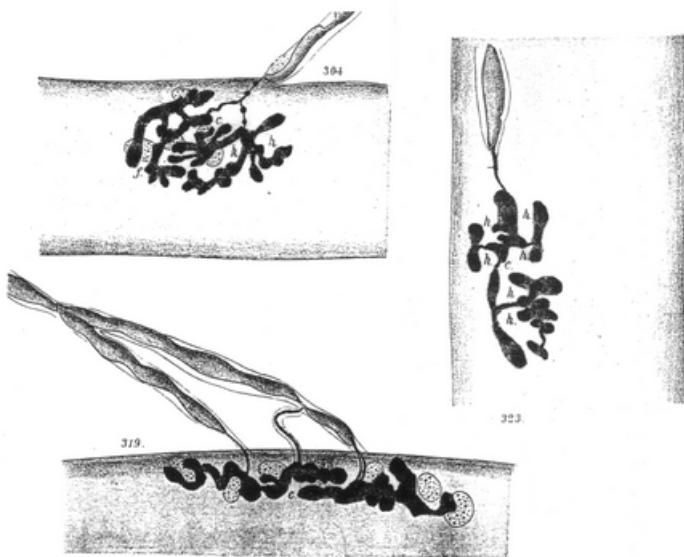


Figure 42. Neuromuscular junctions from, 304, mouse; 319, cat; 323, human, from Kühne, (1862). Magnification x 1000.

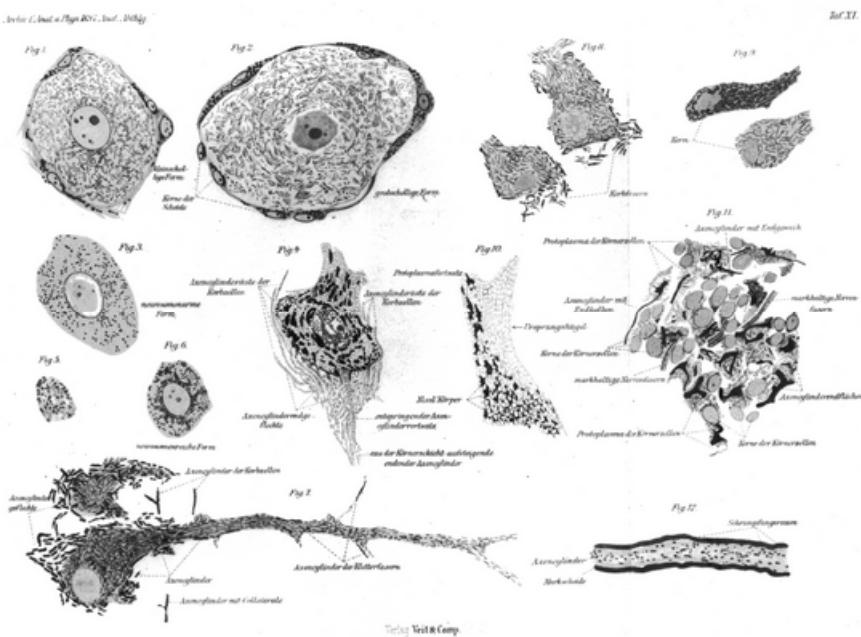


Figure 43. Drawings of neurons from rabbit trapezoid nucleus showing silver stained end-feet, which are considered one of the first demonstrations by light microscopy of synapses. Note particularly figures 1, 2, 4, 7, 8. These illustrations are from Held, (1897) his plate XI.

Although there were fierce controversies about whether the brain and spinal cord were reticular or neuronal, this difference was somewhat overstated. The former now puts the dendrites and axons all in direct communication with each other or with other bodies, while the latter view puts a synaptic cleft in between them. Ramon y Cajal (1954) listed the following kinds of connections: axo-somatic by nerve nests rich in fibres; axo-somatic by chalices or baskets poor in fibres; axo-somatic by terminal tubercles; axo-somatic by pericellular nerve bushes; axo-somatic by large nests (e.g. end-fusse of Held); axo-dendritic by climbing fibres; axo-dendritic by gears; axo-dendritic by oblique or cross-like long nerve branches; axo-dendritic by means of flat and parallel overlapping plexuses; axo-dendritic by diffuse arborisations; connections and terminations of peripheral nerve arborisations (e.g. motor end plates). In summary, all fibres seemed to have synaptic connections with all others.

Del Rio-Hortega, (1919, 1932) and Ramon y Cajal, (1913, 1928) stained what they believed to be three kinds of neuroglial cells, which were called astrocytes, oligodendrocytes and microglia. It should be noted that they assumed: (a) that neurons were not stained by their procedures, (b) that the different kinds of neuroglia occupied the space between neurons seen by Von Virchow; (c) the different staining systems were assumed to be showing different populations of cells, rather than determining the different appearances of what might have been one population.

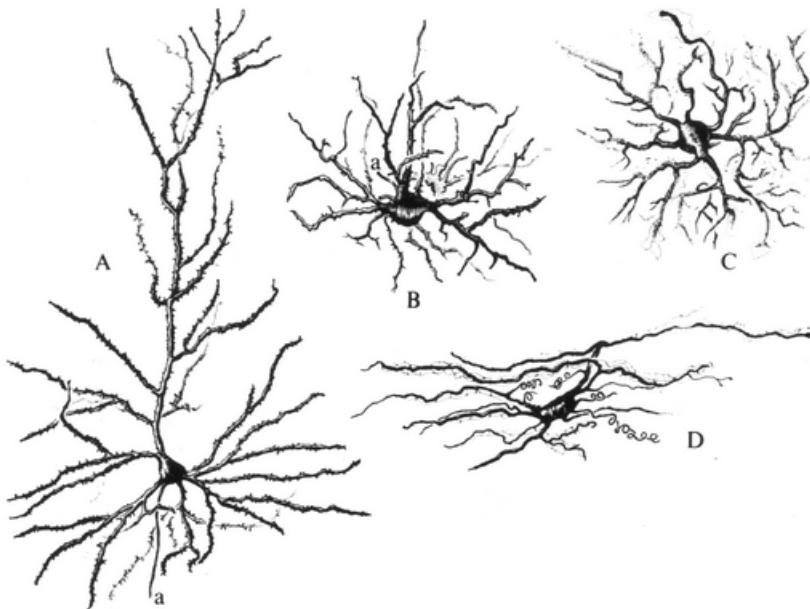


Figure 44. Diagrams of Golgi-stained: A, pyramidal visual neuron; B, dentate cerebellar neuron, C, grey protoplasmic astrocyte; D, white matter oligodendrocyte, from Peters, Palay and Webster, (1998) frontispiece, by kind permission of the Authors and Oxford University Press, New York.

After the light microscopists and the histologists had determined the 4 kinds of cells, neurons, astrocytes, oligodendrocytes and microglia (Figure 44), the electron microscopists examined what were believed to be the same types of cells, (Palade, 1954; Palay, 1956; Palay and Chan-Palay, 1977; Peters, Palay and Webster, 1998). Later, the immunocytochemists, mostly examining cells in tissue culture, identified antibodies to the antigens they believed to be present in the different types of cells (Eng et al, 1971; Pevzner, 1979; Raff et al, 1979; Björklund, Hökfelt and Kumar, 1984-92). Eng et al's tissue was derived from a neuroblastoma, and Raff et al concentrated on astrocytes from the optic nerve. The criteria used by the histologists antedated and were different from those used by the electron microscopists, and also those used by the immunocytochemists (Pevzner, 1979; Weisinger, 1995). Furthermore, most of the tissues studied have been cultures, in which the original histological criteria for the different kinds of cells have been completely ignored. It is difficult to know whether this is because the immunocytochemists have tested the 'specific' marker and histological neuroglial stains on the cell types in tissue culture, and have *not* found that they show up the particular cells, and, therefore, have not published their findings, or they have not attempted to examine whether an astrocyte, oligodendrocyte or microglial cell, stained by the classical stains, was the same cell type as that identified by the markers. Of course, if it were not, it could be argued that the conditions of tissue culture *changed* the staining properties. Nevertheless, the fundamental question must be asked about whether the histologists, the electron microscopists and the immunocytochemists *do* mean the same cells, when they use the particular terms.

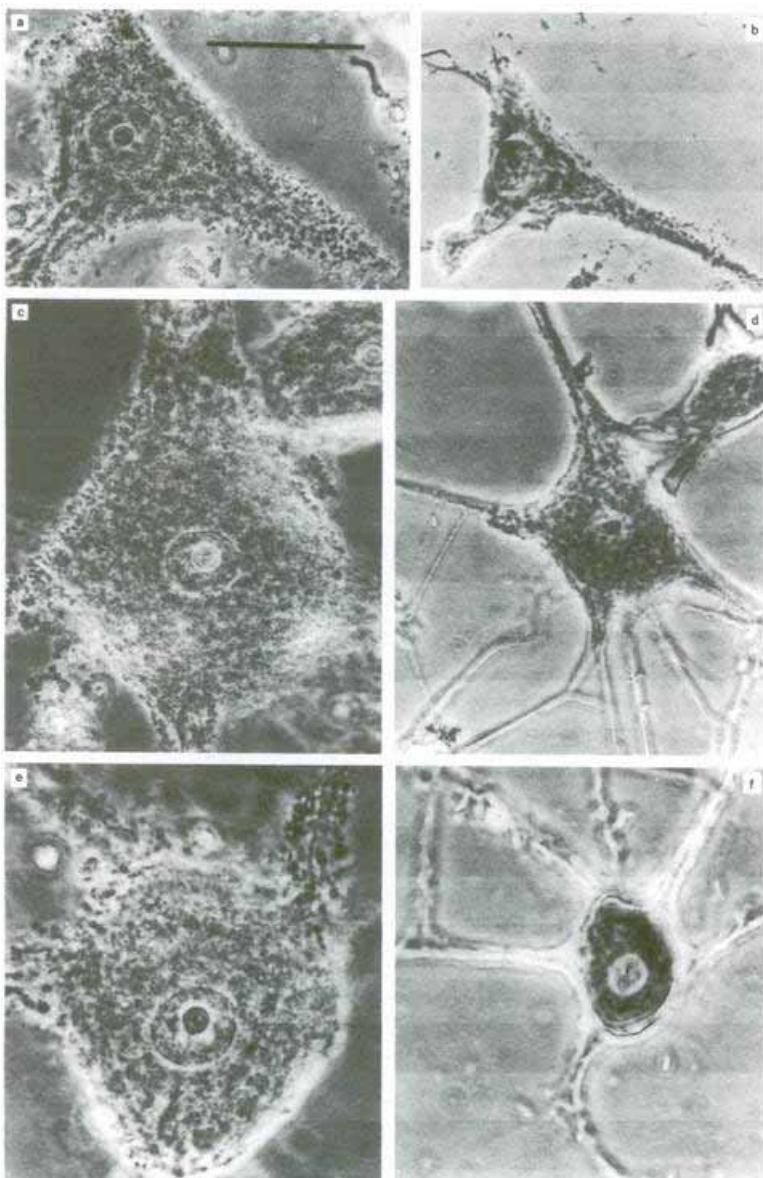


Figure 45. The effects of staining on the projected area and appearance of isolated rabbit medullary neurons, *left*, unfixed before staining, *right*, same cells after staining. *Upper*, stained with haematoxylin and eosin, *middle*, with Palmgren's stain, *lower*, with osmic acid. This figure comes from Chughtai, Hillman and Jarman, (1987), by kind permission of the Quekett Microscopical Club. Phase contrast. The bar is 50μm.

Hydén and his collaborators compared the properties of the neuron cell bodies with those of the neuroglia (Figure 39, 40). If the quantity of an enzyme or nucleotide went up in the cell body, and down in the neuroglia, or it went up in both, or down in both, all these were taken as evidence that the neuroglia was related to neurons. They were designated ‘satellite’ cells, ‘supporting’ cells, ‘nutrient’ cells, and other second-class names. In fact, neuroglia were only studied in fractions believed to be enriched in them, or in tissue cultures.

Human neuron cell bodies isolated by the technique of Hydén (1959; 1960) are shown (Figure 46). Obviously, the isolation involves breaking off many dendrites (Figures 47, 48). However, they are very fine, and the axoplasm is viscous. After isolation, the cell bodies still had resting membrane potential differences (Hillman and Hyden, 1965). The dendrites broke several microns away from the cell bodies. Myelinated axons can be stripped off their sheaths (Figure 48), and unstained peripheral axons look rather elegant (Figure 49). Lipofuscin can be seen in sections of the brain (Mann and Yates, 1974; Brizzee, 1975; Brizzee, Kaac and Klara, 1975), and in human unfixed isolated human neuron cell bodies (Hillman and Jarman, 1991). It increases with age (Reichel, 1968; Reichel et al, 1968; Brizzee and Cancilla, 1972).

Cell bodies were separated from bovine brains by filtration (Roots and Johnston, 1954).

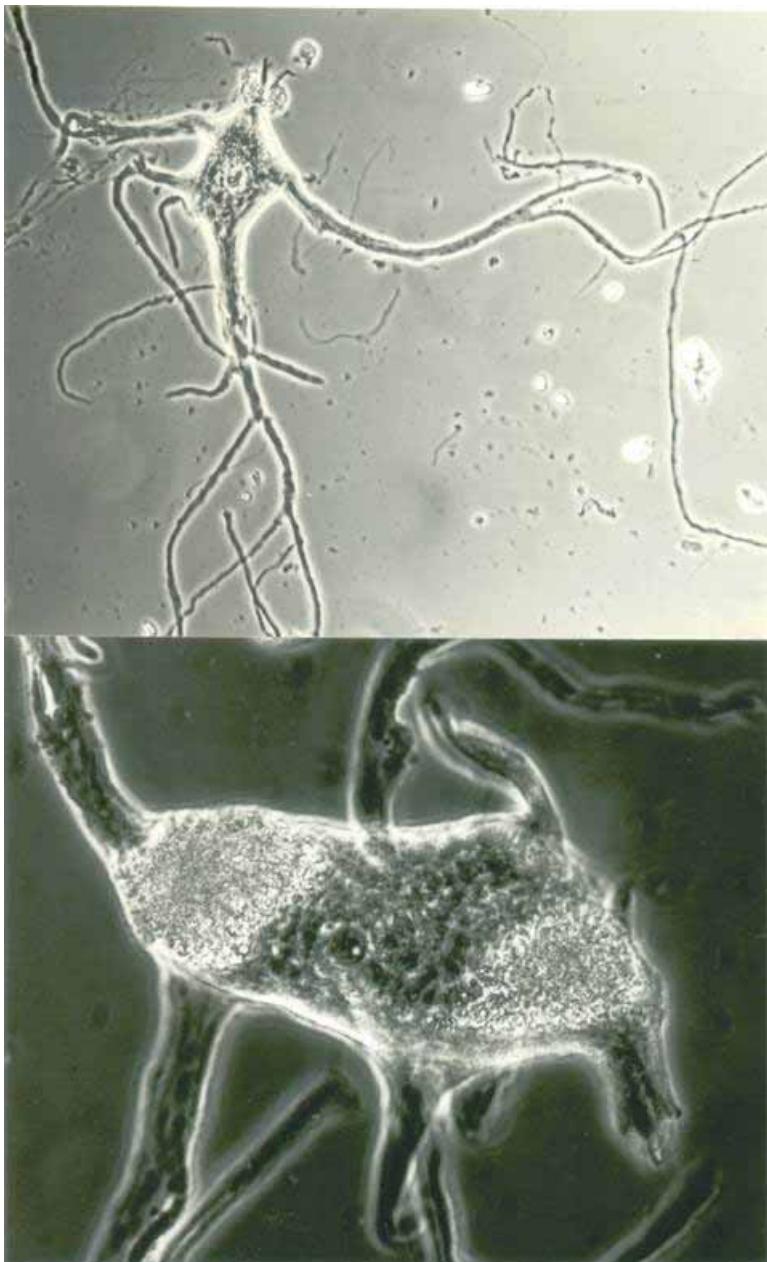


Figure 46. Unfixed human neurons, *upper*, from the hypoglossal nucleus. Phase contrast, x 300; *lower*, anterior horn cell cervical cord, phase contrast x 750. Note, in the *upper*, the extent of the dendrites which may be dissected out, *lower*, the lipofuscin at both ends of the cell body.

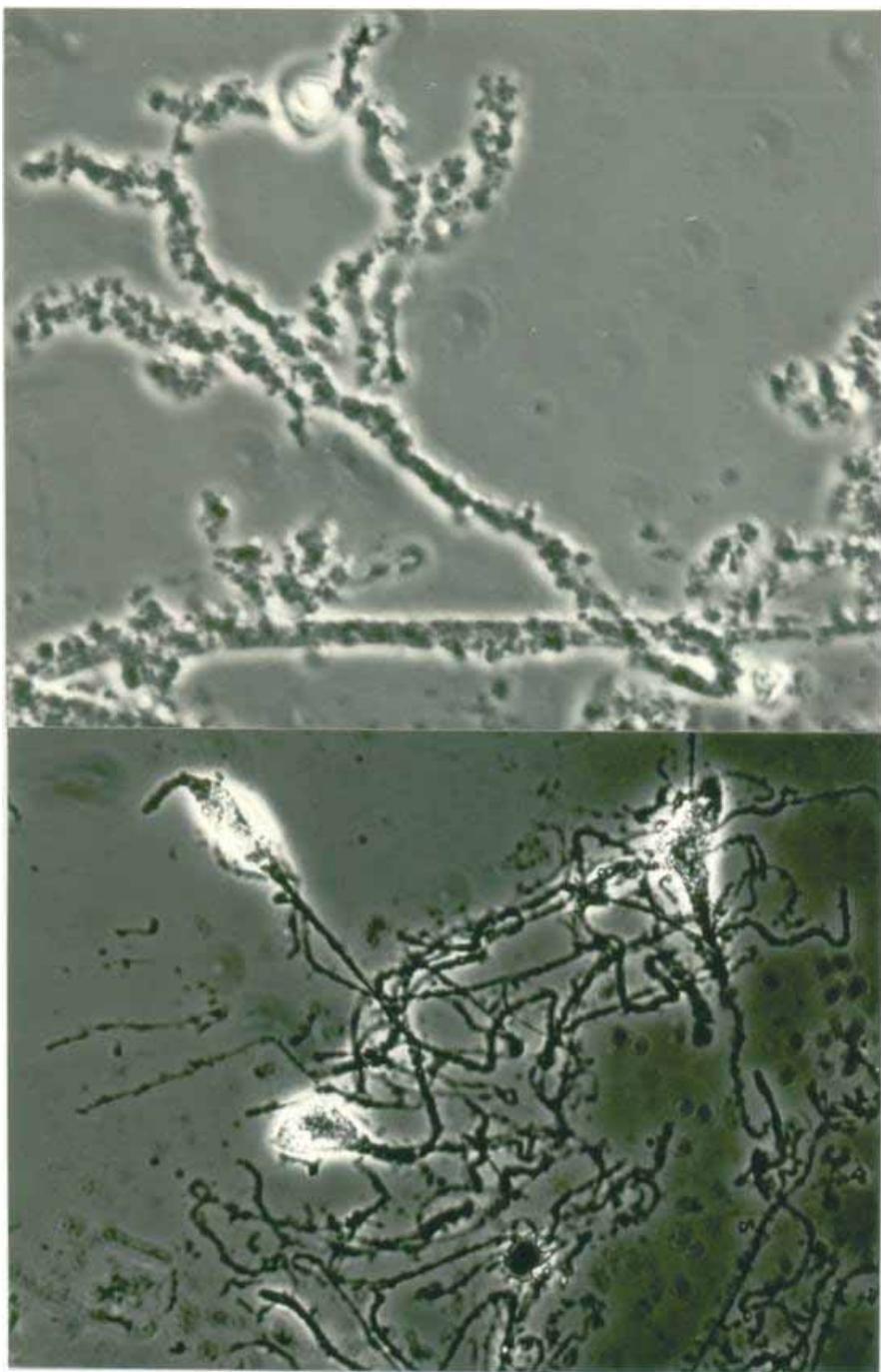


Figure 47. Human unfixed dendrites, *upper* parietal cortex, *lower*, frontal cortex.
Phase contrast, $\times 750$

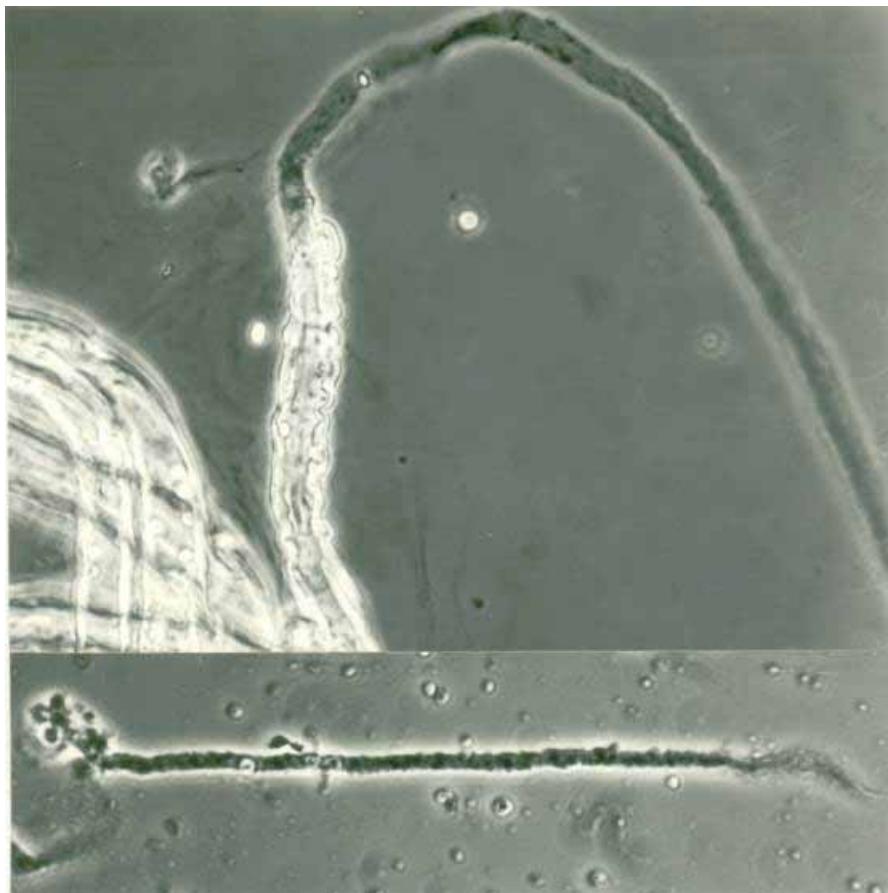


Figure 48. Rat sciatic nerve, *upper*, sheath stripped away to the right, *lower*, unmyelinated fibre. Phase contrast, x 750.

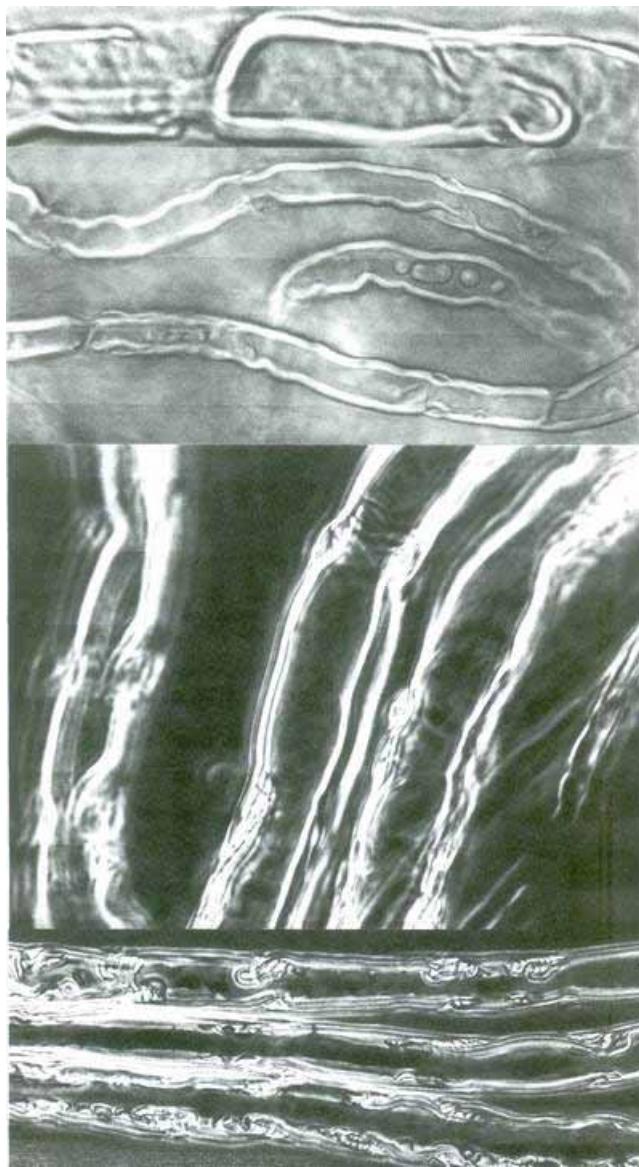


Figure 49. *Top*, rat myelinated nerve fibres, by phase contrast microscopy, showing the low refractive index of the myelin sheath, *lower*, similar showing intraaxonal inclusions. The *upper* and *lower* preparations were made in co-operation with Mr James Dutton; *third down*, teased rabbit vagus nerve by phase contrast microscopy, showing Schmidt-Lanterman clefts and axonal particles, which can be seen moving under high power microscopy; *bottom*, rabbit sciatic nerve fibres, showing Schmidt-Lanterman clefts and intraaxonal particles. Phase contrast, x 600.

The dendrites appear non-myelinated but covered in granules in the central nervous system (Figure, 47, 48), and most of them seem to be invested with myelin sheaths in the periphery (Figure 49). Whereas by phase contrast microscopy the myelin sheath appears to have a low refractive index and to be transparent (Figure 49) electron microscopy shows the sheath to be full of a spiral of lamellae (Figure 50). These are regarded as being a scroll of the membranes of Schwann cells.

Conel, (1939-1967) carried out extensive Golgi-Cox staining of thousands of sections of neonatal and young cerebral cortices. His studies assumed that the staining procedures used showed up neurons only, and no neuroglial cells (Figure 51).

The capillaries can be separated by teasing (Figure 52). Nuclei, believed to be those of Schwann cells can be seen in culture (Dubois-Dalq et al, 1981).



Figure 50. Electron micrograph from the peripheral myelinated nerve fibre of a baboon. Adjacent is a Schwann cell with a large nucleus. Magnification x30,000. This micrograph was kindly provided by Professor D Landon, formerly of the Institute of Neurology, London. Please see also Landon, (1976).

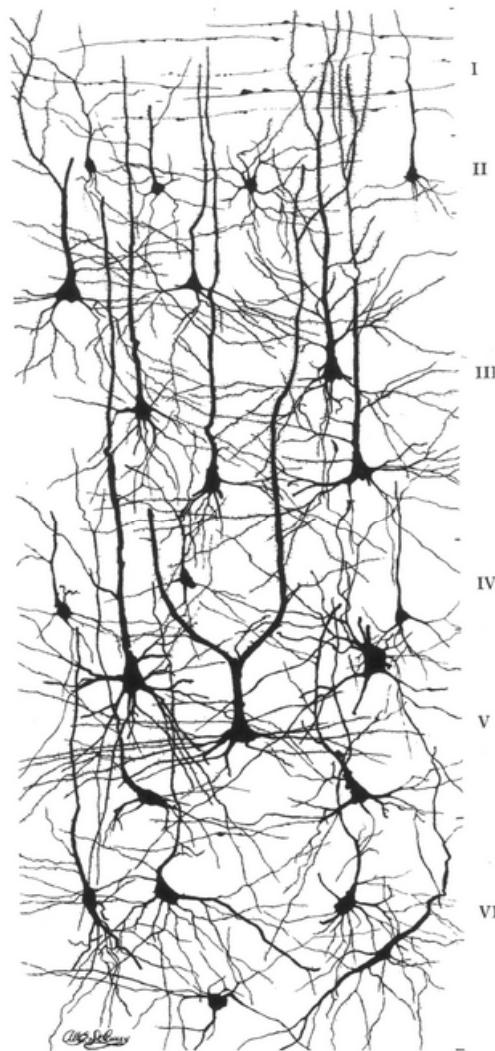


Figure 51. Golgi-Cox staining of 45 μm section of central posterior gyrus of 6 year old child. Plate 48 from Conel JR, (1967). *The Post Natal Development of the Human Cerebral Cortex*, Vol. 6, *The Cortex of a 6 Year Old Child*, by kind permission of Harvard University Press. Please note that (i) the space between the neurons is believed to be filled with neuroglial cells; (ii) the contacts of dendrites are believed to be the sites of the synapses, although, of course, one can not tell from a micrograph whether the apparent overlaps represents contacts; (iii) a section does not show the full image of a cell body or dendrite unless the former is within the

thickness of the section; (iv) many of the dendrites do not appear to end on any other dendrite or cell body; (v) fine 'dendritic spines' may be seen. They are believed to be the sites of synapses.

One may now summarise the current consensus about neurons and neuroglia. (Kettenman and Ranson, 1995; Jessen and Richardson, 2001).

*1. There are 4 kinds of cells in the nervous system, the neurons and 3 kinds of neuroglial cells, astrocytes, oligodendrocytes and microglia.

*2. The neurons are star shaped, and have dendrites which end on neuromuscular junctions, or synapses on other dendrites or cell bodies. Neurons communicate with muscle or other nerve cells at junctions by chemical transmission; they stain with MAP 2.

*3. The astrocytes are star-shaped, are adjacent to neurons, and stain with Ramon Y Cajal's gold stain, Del Rio Hortega's silver, Weil and Davenport's and other procedures (Hillman 1986a, pages 74-77). Nowadays they are identified by antiglial fibrillary acid protein.

*4. The oligodendrocytes have little cytoplasm and few processes. They are stained by Marsland, Glees and Erikson's (1954) and other procedures, and also contain other enzymes and proteins, believed to be markers.

*5. Microglial cells appear to be mainly nuclei; they have little cytoplasm, and continuous membranes around the cytoplasm are difficult to see by light or electron microscopy (Figure 53).



Figure 52. Capillary in human frontal lobe, unstained. Phase contrast, x 700.

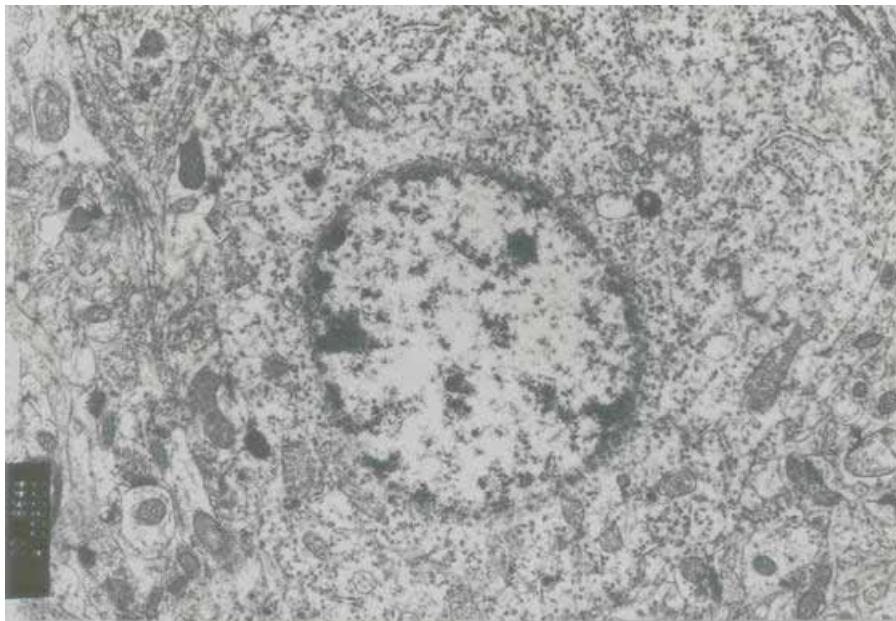


Figure 53. Electron micrograph of human parietal microglial cell, stained with osmium tetroxide. Note the absence of continuous cell membrane around the cytoplasm. Magnification x 1644.

*6. The apparent space between neurons is filled by various kinds of neuroglial cells and by blood vessels.

*7. The neuron ‘doctrine’ is correct.

*8. Neurons have single axons, which have an initial, non-myelinated part and are invested with myelin sheaths, interrupted by nodes of Ranvier, (1889).

9. The myelin sheath consists of rolled tubes of membranes (Robertson, 1955).

10. The ventricles and the spinal canal are lined by ependymal cells, and cerebrospinal fluid circulates through them.

*11. Signals pass from a neuron to another neuron or muscle by chemical transmission.

*12. The Geren model describes the development of the myelin sheath.

*13. Memory is stored in synapses of the central nervous system.

*14. Neurons, astrocytes, oligodendrocytes and microglia have the same properties in tissue and organ culture, as the same named cells did in the parent tissue in the intact animal.

15. The myelin sheaths in the spinal cord are continuous with those in peripheral nerves.

Since the conclusions of this chapter may be regarded unpopular, it would be useful to restate the main principles used here. I believe that these would be generally uncontroversial, and, therefore, would be grateful if anyone who disagrees with them would communicate with me.

A. One should not accept hypotheses, which are not provable *and* not disprovable.

B. One can not measure dimensions, establish structure, or determine the number of processes in a single histological section.

C. Any structure must be visible, or a three dimensional model must be possible, if it is claimed to exist in life.

D. An *explanation* for failure to see a structure claimed to be present can not be used as evidence that it is present.

E. The *specificity* of a reaction, an inhibitor, activator, enzyme location, protein location, stain or marker can only be claimed if it has been demonstrated experimentally not to affect or show up *any* other reaction, enzyme, protein or cell.

F. If the presence or absence of one of these is determined genetically, in the absence of the gene, no significant quantity should be measurable in the tissue.

G. Diagrams, in the absence of micrographs showing them, have little epistemological value.

As pointed out, most of the arguments presented below in disagreement with statements *I to 15*, can be found in full in Hillman (1986a).

The distinctions between neurons, astrocytes, oligodendrocytes and microglia are based on the following properties: histological appearances in section; staining properties; electron microscopy; pathological appearances; appearances in tissue culture; media in which the cells grow; specific biochemical markers; expectation from clinical diagnosis; characteristic ion channels; electrical properties; susceptibility to virus infections (Table 75).

Criteria for defining neuroglia

- Appearances in stained sections by light microscopy and electron microscopy
- Tissue adjacent to neurons
- Dimensions in stained sections by light and electron microscopy
- Biochemical conditions under which cells are cultured
- Appearances of teased unfixed cells by light microscopy
- Sections stained specifically for cell types
- Appearances of cells in tissue culture by light and electron microscopy
- Histochemical markers in tissue sections
- Protein and enzyme markers in cells in tissue culture
- Electrical activity of cultured cells
- Enriched fractions of central nervous system
- Clinical knowledge of the history of the lesion
- Age of person afflicted
- Knowledge of experimental lesion of animal
- Location of tumour in the nervous system
- Presence of mitochondria in cells
- Schwann cells
- Presence of ion channels
- Ground substance of central nervous system
- Presence of neuronal-glial junctions
- Fine granular material

Table 75.

Distinctions based on histological appearances depend on the shapes, dimensions and distribution of cells and their organelles. In an extensive comparison of histological publications going back to 1865 (Hillman, 1986a, pages 52-61), the histological appearances were found to overlap considerably. In particular, the appearances of neurons and astrocytes overlap, as do oligodendrocytes and microglia. The particular staining procedures which are believed to show up the particular cell types depend very largely upon the opinions of particular authors (*ibid*, pages 75-78). Neuroglial clumps were isolated and stained with the following procedures: Mallory's phosphotungstic acid haematoxylin; Weil and Davenport's; Marsland, Glees and Erikson's, and Gallyas's. They stained well, so did the rabbit neuron cell bodies which had been adjacent to them (Hillman and Deutsch, 1979). Of course, different histological procedures shrink and distort cells differently, so that it is likely that there would be *some* variation, if the same cells were stained by a number of different procedures.

Another demonstration of the lack of specificity of the stains was shown by staining 6 μm serial sections of rat frontal cortex with the following staining procedures: Gallyas; Weil and Davenport; Patay; Mallory's phosphotungstic acid haematoxylin; haematoxylin and eosin (Hillman, 1986a, pages 99-108). Usually, one could trace one cell body by its position and shape from one serial section to the next. On average, 38% to 65% of the cells seen in one section could be identified in the next. Of course, in a 6 μm section, a cell of 25 μm diameter would be expected to appear in about 4 sections. It was found that in some of the sections, the cells might be too small to recognise. However, the percentage of cells seen in successive sections was high, indicating that the stains were not very specific. It was also found that Purkinje cells of the cerebellum, cells from the cranial nuclei, and anterior horn cells - all undoubtedly neurons - stained with all procedures tried. Most staining procedures were pioneered when the concepts of specific staining, control experiments and statistical analyses were less current than the respect for illustrious histologists.

Electron microscopy permits such high magnification, that it is difficult to know the shapes of whole cells. However, even using this technique, the differences between morphological descriptions of neurons and astrocytes, and between oligodendrocytes and microglia, so overlap that is difficult to tell them apart. Nevertheless, it is clear from Figure 53, as from all sections of mammalian nervous system which I have seen under the electron microscope, as well as in the micrographs published by Palay, Palade, Chan-Palay, Jones, Peters and Webster, and in light micrographs, that no membranes can be seen clearly around the cytoplasm of microglia. The same also applies to: granule cells of the cerebellum; inner and outer nuclear cells of the retina; astrocytes; neuroglial cells of autonomic ganglia; Schwann cells; reactive astrocytes; cells in malignant tumours, as well as most cells in syncytia (Table 25). The expected locations of the elusive membranes are indicated in Figure 54. The argument has been advanced that these membranes are not seen because they are not cut normal to the direction of the electron beam. This view is untenable in view of the clarity with which the cell membranes, the nuclear membranes and the mitochondrial membranes are seen clearly and completely, using the electron microscope.

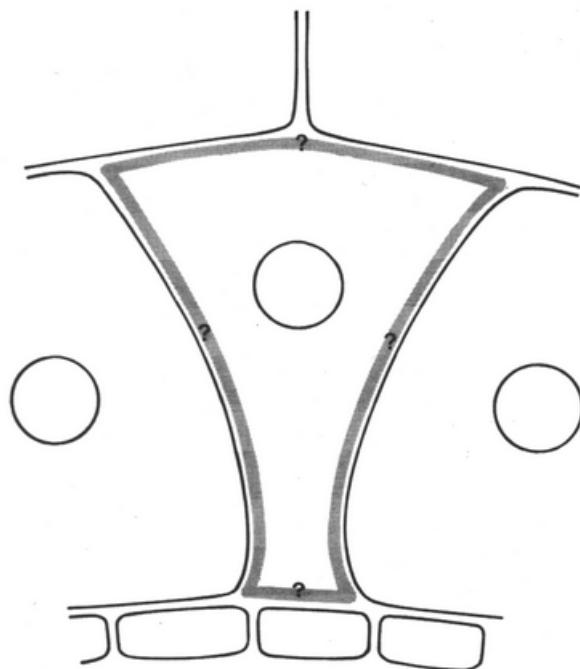


Figure 54. The thickened line shows where a neuroglial cell membrane should be. A neuroglial nucleus is seen sandwiched between two neurons and capillary cells, but where is the membrane around the neuroglial cytoplasm?

The pathological appearances, for example, of the infected or tumourous nervous system are often shown, particularly in relation to neuroglial cells. This assumes that their appearances would not be changed by the diseases. Indeed, most illustrations of astrocytes and oligodendrocytes in the literature come from ill tissues. It is a simple observation of histology that reactive astrocytes, and all the other cells listed in the latter paragraph appear as spherical or oval nuclei in syncytia.

The appearances of cells in tissue culture also represent a number of well known problems: the shapes of the cell change between excision from the parent tissue, going through a latent stage, then putting out processes, and finally growing; they are in completely different environments (Table 37) than they were in the native animal; their shapes and biochemistry are totally dependant on their conditions of culture; different types of cells are separated and cultured in such different environments, that it is not at all surprising that they appear different from each other,

especially if they grow for several generations. Thus, identification of cells in tissue culture depends upon belief in their origins and the procedure for separating and culturing them. Once again, one may ask the question, 'If one took, say, fibroblasts or epithelial cells, and grew different batches of the same cells in media believed to grow neurons, astrocytes, oligodendrocytes or microglia, would they each have different appearances and chemical properties?'

Ion channels have been detected in most cells, membranes and organelles (Chapter 21), including neurons and neuroglia by clamping patches (Bevan et al, 1985; Sontheimer and Kettenmann, 1988; Somjen, 1995; Sontheimer and Ritchie, 1995; Kettenmann and Ranson, 1996; Gallo and Ghiani, 2000, Table 61; Hille, 2001). These ion channels in the neuroglia include those for acetylcholine, Na^+ , K^+ , gaba, Ca^{2+} and Cl^- (Sontheimer and Ritchie, 1995). The more investigations carried out on neuroglial cells in tissue culture, the more channels are found. Furthermore, neuroglia in culture show very slow electrical transients (Hild and Tasaki, 1962; Physiological Society Transactions since the 1990's). The cells in which the channels were found were usually identified in culture as astrocytes, which are rarely seen in the central nervous systems of intact mammals. Receptors of many kinds have been reported in cultured astrocytes (Hansson and Rönnbäck, 2004).

Thus, one sees that with the possible exception of physiological properties in tissue culture, it is extremely difficult to differentiate *neurons* and the different kinds of what are believed to be *neuroglial cells* by any objective criteria. The microglia, neuroglial nuclei, or naked nuclei, are exceptions to this.

Perhaps it is appropriate here to utter a few doubts about some of the named neuroglial cells. The astrocytes are always said to have endings on capillaries. This is on the basis of light micrographs, in which the capillaries have been stained. When an ending is seen on a capillary, the cell from which it originates is thereby characterised as an astrocyte. Similarly, a tube shaped structure, upon which a process believed to originate from an astrocyte is seen, is thus concluded to be a capillary. Furthermore, since one looks at *sections* of tissues, and especially because sections are chosen which illustrate the endings, one does not know whether the incidence of endings from what are believed to be astrocytes, is higher than the incidence on the surfaces of neurons, oligodendrocytes and microglia or, they are, indeed, quite random. It would be useful to test the incidence of endings from astrocytes relative to those from other cells, both objectively and statistically.

It used to be said that only neurons were excitable, so that when a brain or spinal cord was penetrated by a micropipette, and the cell could not be stimulated electrically, it was *concluded* to be an astrocyte. Of course, that is a circular argument.

Much work on the immunocytochemistry of astrocytes has been carried out by Raff et al, (1979, 1980) and his co-workers. They have seen star shaped cells in the optic nerve, and assumed that they were astrocytes. The results generalised from their work seem to be regarded then as the properties of astrocytes in general, in terms of morphology, immunology, markers, etc. Of course, retina is an outgrowth of the brain. The optic nerve seems to be just as likely to contain neurons as astrocytes, or, perhaps, more rigorously, one should ask, “On what criteria should one decide that this cell is a astrocyte, rather than a neuron?”

Oligodendrocytes are believed to produce myelin in the central nervous system (Ross, Bornstein and Lehrer, 1962; Bunge, 1968). This is mainly based on the proximity of nuclei believed to be oligodendrocytic near developing myelin. This is an unproved hypothesis. In order to prove it, one would have to produce presumably a culture of pure oligodendrocytes and watch myelin arising from it. Alternatively, one would have to identify oligodendrocytes unequivocally in a whole developing animal, and then show myelin arising more frequently from the region of those cells, than from other regions. Furthermore, it is not at all clear why cells adjacent to axons should be called neuroglia.

It is widely believed that microglia are macrophages (Peters, Palay and Webster, 1998, page 308, and sources quoted there.) This is mainly on the grounds that microglia proliferate around infections. However, neither by light nor electron microscopy can membranes be seen around their cytoplasm, nor can particles or granules be seen in their cytoplasm, so that it is difficult to characterise them as phagocytes.

From the earliest descriptions of the cells, authors since Von Deiters, (1865) have indicated that they can not distinguish between: neurons and astrocytes; astrocytes and oligodendrocytes; oligodendrocytes and microglia. However, a much larger number of authors, including pathologists, use the term neuroglia, glia or gliomata, clearly implying that they can not distinguish on any grounds between astrocytes, oligodendrocytes and microglia (Hillman, 1986a, pages 38-39).

The following research workers: Holger Hyden, Anita Palm, Inger Augustsson, Peter Sartory, Jocelyn Fasham, Karl Deutsch, Tasawar Hussain, Iffat Chughtai and David Jarman worked with me between 1962 and 1995. We dissected out tens of thousands of neuron cell bodies of cranial nerve nuclei and spinal grey matter, from humans, cows, sheep, cats, rabbits, guinea pigs, rats and mice. The cell bodies were not fixed or stained and they were viewed by phase contrast microscopy (Hillman and Jarman, 1991). During the whole of this period, we found only *one single* cell body, which resembled a neuroglial cell (*ibid*, page 172). There were plenty of naked nuclei and the ubiquitous fine granular material.

Neuron cell bodies are much more spherical before fixation than after staining (Figure 45).

There is plenty of good physiological evidence that stimulation of a cell body, or the axon of a motor nerve, causes contraction of a muscle. A single axon or dendrite does not appear to be myelinated at its origin on the cell body, and this has been called the ‘non-myelinated’ part. Similarly, one does not see myelination just before a neuromuscular junction or synapse. It is generally tacitly assumed that these unmyelinated axons pass through to be invested with myelin sheaths, which they lose at each end. Although such transitions are possible, and illustrated in many drawings and diagrams (see, for example, Figure 42), I have been unable to demonstrate them myself or to find publications showing *micrographs* of such junctions. However, the connections between spinal fibres and peripheral nerves have been examined (Carlstedt, 1977). When viewed by electron microscopy, the myelin in the former was much more broken up than in the latter.

It will be proposed that oligodendrocytes and microglia are naked nuclei in a syncytium (please see below).

The transformation of Von Virchow’s concept of neuroglia, as a jelly between neurons, to the current concept that it is full of all the three kinds of neuroglia, plus blood vessels, started with the characterisation of the different kinds of neuroglial cell, and the natural conclusion that they would all fill up the non-neuronal space. This was based on the beliefs: firstly, that the types of neuroglia were separate populations, because the staining procedures were specific; secondly, that the ‘terra incognita’ (Campbell, 1905), although it did not stain for neurons, could contain other cells, which would be shown up by other staining systems. It was believed that if one could stain for all the different neuroglial cells at the same time, the whole central nervous

system would be shown to be solid with cells; this was regarded as proved, when electron microscopy showed the central nervous system to be solid with cell constituents. Mitochondria could be seen both within and beyond neuron profiles.

This problem was addressed directly. Neuroglial clumps between neurons were taken out under direct vision. All neuron cell bodies were removed. The neuroglial clumps were teased and viewed by phase contrast microscopy. Like the neurons, they were found to be packed with mitochondria, (Hillman, Deutsch, Allen and Sartory, 1977). In later experiments, the neuroglial clumps were sandwiched between liver or between kidney, and stained with haematoxylin and eosin, cresyl violet, Weil and Davenport, or the phosphotungstic acid haematoxylin procedure. The sections were then viewed by phase contrast microscopy. In liver and kidney, membranes could be seen around all cells, while the neuroglia did not show any membranes. There were no membranes around the cytoplasm surrounding the neuroglial nuclei (Figure 54), and the clumps were full of mitochondria (Hillman, 1986, pages 96-99).

Further experiments were carried out. Sections of rat frontal, parietal, occipital, medullary tissue and spinal cord, were cut, and stained with a variety of procedures: haematoxylin and eosin; Marsland, Glees and Erikson; Weil and Davenport; Patay's triple stain; and phosphotungstic acid haematoxylin. In each case, 20 sections were made from 2 different brains, using each procedure. The stained sections were then scanned automatically with a Quantimet. The sections were cut and stained meticulously by the late Mr James Kirby, and analysed by Mr David Jarman, to both of whom I express my profound gratitude.

Each procedure stained between 3% and 6% of the nervous tissue. Those for nerve cells and their processes, astrocytes, oligodendrocytes and microglia, added together, made up from 14% to 20.5%. This is, of course, a minority of the tissue, and is, itself, an overstatement of the situation, since the procedures could not be considered specific (*ibid*, pages 105-109). It was concluded that most of the brain did not stain at all, despite sections being stained with *all* the specific stains.

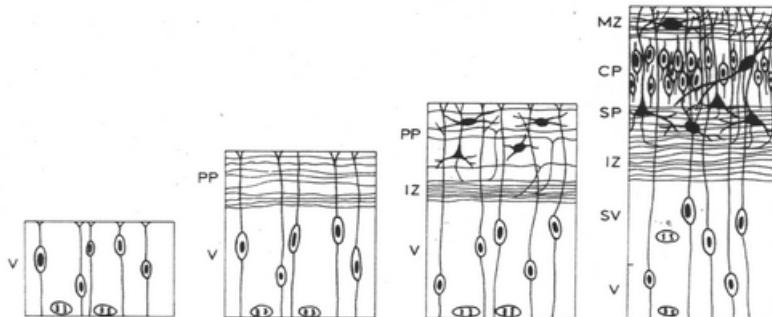


Figure 55. Four panels illustrate the layers of the cerebral wall at different stages of development VZ, ventricular zone; PP, preplate; IZ, intermediate zone; MZ, marginal zone; CP, cortical plate; SP, subplate; SV, subventricular zone. This figure is from Parnavelas, Alifragis and Nadarajah, (2002), and is reproduced by kind permission of the Authors and Elsevier Science Ltd.

As brain develops in the embryo, neurons migrate through the tissue, as seen in the diagram (Figure 55, from Parnavelas, Alifragis and Nadarajah, 2002). At this stage, the neurons definitely pass through syncytia (Tyler, Von Borstel and Betz, 1957). Such movement could not occur, if the central nervous system were solid with neuroglial *cells* at that stage.

The simplest explanation for all these findings is that in the material between neurons, there are no neuroglial *cells*, only nuclei and mitochondria, in a syncytium. This explains *inter alia*:

- (a) why neurons stain with neuroglial stains;
- (b) why it is so difficult to find in unfixed, or in stained intact tissues, astrocytes or oligodendrocytes in the same sections as neurons;
- (c) how gliosis occurs within hours of an infected focus, as the ‘reactive astrocytes’, aka neuroglial nuclei, travel through the syncytium not separated by membranes;
- (d) the malignancy of gliomas, because the nuclei can move freely and rapidly in the syncytium in life;
- (e) a recent catalogue from the Chemicon International Corporation, (2005), entitled, ‘Stem Cell Biology,’ listed 30 neuronal, 2 astrocytic and 10 oligodendrocytic, markers. Interestingly, the catalogue differentiated between

embryonic and adult cells, and between the membranes, nuclei, cytoplasm and axoplasm of neurons, clearly implying that each of these from the same types of cells were immunologically different. Nearly all the markers had been found and tested in tissue culture. There was no indication as to whether they had been tested on intact adult animals, or upon their specificity in the latter circumstances. No markers for microglia are listed. It is also of interest that histology textbooks generally talk about the stains claimed to show up particular kinds of central nervous cells, but do not claim that the stains are 'specific'. In the practical textbooks, one is warned that 'overstaining' may colour other cells. Furthermore, an extensive examination of the classical literature showed enormous disagreements as to which staining procedures should be used to show up particular cells (Hillman, 1986a, pages 44-91). Of course, it is not surprising that cells grown in different culture media exhibit different chemical properties (Chapter 12). The validation of the use of markers in culture to identify different cell types in intact animals will not be justified until a battery of experiments has been carried out upon the specificities of staining procedures and markers, and on the effects of reagents used during culture on the staining and marking properties. Furthermore, the use of the term 'oligodendrocyte' for both the cells in the central nervous system and the peripheral nerves is in urgent need of justification;

(g) a considerable volume of evidence has accumulated recently that stem cells from the central nervous systems of many animals, including human beings, are precursors of all the different alleged kinds of cells. These experiments have mainly been carried out on tissue cultures, using 'specific' markers (Johé et al, 1996; McKay, 1997; Temple and Alvarez-Buylla, 1999; Ushida et al, 2000);

(h) the fluid nature of brain in life is observed daily by neurosurgeons, when taking biopsies, and the 'contre-coup' phenomenon is well known in casualty departments, and among neuropathologists;

(i) when the brain or cerebral slices are penetrated by intracellular pipettes, only a small proportion of their travel goes through polarised elements, (Hillman, Campbell and McIlwain, 1963; Julian and Diacoumako, 1977), compared with their penetration into muscle.

Some idea of the difficulties in identifying neuroglial *cells* may be appreciated by perusing Table 76.

Neuroglial identifications

Neuroglia in sense of Von Virchow, (1854)	a viscous fluid between neurons in the central nervous system
Cells with long and short processes	in brain stained by Golgi's stain (De Felipe and Jones, 1988)
Astrocytes	star shaped cells with long processes
Oligodendrocytes	spherical or oval cells with few processes
Microglia or Hortega cells	large nuclei; no processes; cell membranes not visible;
Ependymal cells	columnar cells lining ventricles
Bergman's glia	mainly fibres stretching into cerebellar cortex
Müller cells	elongate cells at right angles to the retina
Satellite cells	small, mainly nuclei, found near cell bodies
Reactive astrocytes	same appearance as microglia seen around inflammation
Fine granular material	seen in unfixed brain and spinal cord
Schwann cells forming myelin	have large flat nuclei, found near peripheral nerve fibres, are very small
Schwann cells, no myelin	have large flat nuclei, found near peripheral nerve fibres, are very small
Teloglia	found on somatic motor nerve terminals
Autonomic ganglion glia	small cells adjacent to neurons in ganglia
Cells marked by anti-gfap	mostly in cell cultures and optic nerves
Enteric ganglion glia	small cells adjacent to neurons in ganglia
Leech neuroglia	star shaped cells next to neurons
Neuroglia or glial cells	general term for cells not believed to be neurons, but not distinguishable as astrocytes, oligodendrocytes or microglia
Gliomas	malignant tumours

Table 76. The identifications come from: von Virchow, (1854), Penfield, (1932); Glees, (1955); Schoffeniels, Frank and Tower, (1978); Raff et al, (1979); Jessen and Richardson, (2001); and Castonguay, Levesque and Robitaille, (2001).

One may simplify the reticularist versus neuron doctrine by saying that the first view is that all neurons are joined together, while the neuronal view is that they are all connected by synapses or by neuromuscular junctions. In electron micrographs, the pre- and post-synaptic membranes appear to be less than 1 μm apart. The synaptic clefts may be regarded as separating the pre- and post-synaptic membranes, or, as joining them together. By analogy, when a wire is connected to another in an

electrical contact, there is probably a very thin air layer between the two pieces of metal. Is it more or less thick than the synaptic cleft? Does one say that the cleft joins the elements on either side, or separates them? One could propose that when the two elements approximate sufficiently to influence each other electrically or chemically, they are contacting each other. Until then, they are separate. The problem then yields its micro anatomy to the discipline of semantics.

The structure of the neuromuscular junction will be considered later (please see Chapter 35), but at this stage, it is important to urge the distinction between this and the synapse.

A summary will follow of why the anatomical synapse is an artifact, the full evidence having been published previously (Hillman, 1985b):

(i) Synapses are not seen on the cell bodies in unfixed unstained neuron cell bodies (Hillman and Jarman, 1991). The ‘end-fusse’ of Held, (1897) and Auerbach, (1898) were only seen after silver staining. The granular appearance of the unfixed cell bodies is *not* due to the synapses, as is illustrated in Figure 56. One can tell if discrete granules are on the membrane surface or in the cytoplasm. In the former case, they will be seen on the profile of the cell, and will only be distributed slightly less frequently over the nucleus, as in the left hand diagram. If the structures are in the cytoplasm, they should not be seen on the contour of the membrane, and should be seen much less densely over the nucleus. These geometrical imperatives can be seen best in whole cells in cultures, but also apply to sections. In illustrations of neurons in tissue cultures, the latter circumstances are seen to apply (Hillman, 1986, page 148). In other words, the granular appearance is due to cytoplasmic particles, probably mitochondria, and not synapses. This assertion represents an invitation to research workers who culture neurons to examine their preparations, their micrographs and their publications, to support or gainsay it. The decision about whether particles are on the outside of the cell membranes, or within the cytoplasm can not be made on tissue sections.

Boycott, Gray and Guillory (1960) gave reasons why they could not see synapses in the cerebral cortex.

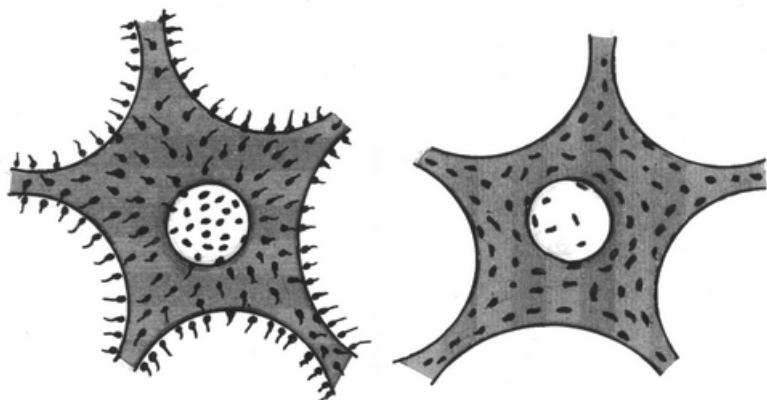


Figure 56. Diagrams of whole neuron cell bodies, *left*, with synaptic endings on the surface, *right*, with intracytoplasmic particles.

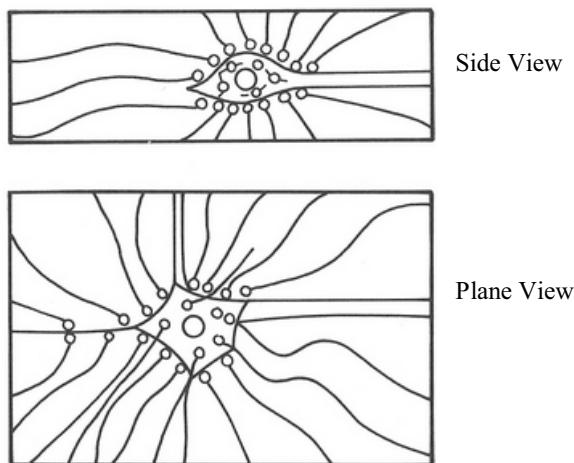


Figure 57. Expected views of synapses seen by light microscopy on the soma of a neuron. Please note that some synaptic connections ('telodendroglia') should spread to the edge of the field.

(ii) another feature which is found in all micrographs of synapses, is that the presynaptic fibres are extremely short. They do not join presynaptic dendrites originating in the cell bodies of other neurons (Figure 56, 57). This failure to join up is seen throughout the literature on the histology of neurons (see, for example, Golgi, 1906; Ramon Y Cajal, 1909-1911; Wyckoff and Young, 1956; De Robertis, 1959), *inter alia*. It is even more dramatically illustrated in the dendritic spines, which are

widely believed to be synapses (Figure 51). They are within the resolution of the light microscope, since cerebellar white matter fibres of similar diameters can be seen travelling across the section quite clearly (Hillman, 1986a). Of course, one would not see many fibres cut normal to the surface of the cell body, but those arising in the maximum diameter, in the plane of section of the cell bodies, should be seen travelling long distances, as they are in all Golgi stained sections (Figure 51) (for example, Conel, 1939-1967). Obviously, a synapse not joined to the dendrite of another cell is hardly more useful than a premobile telephone, not connected to an exchange;

(iii) both under the microscope and in the micrographs, one sees two-dimensional images, so that one can not know from a perusal of the enormous number of micrographs showing the histology of the central nervous system, whether two dendrites apparently crossing each other, are actually in contact and touching at synapses, or not. In preliminary observations I have carried out on Golgi preparations (to be submitted for publication), I have usually been able to measure significant vertical distances in the sections between two fibres appearing two dimensionally to be crossing, until one focuses on the individual fibres. It would be useful, if these experiments were repeated elsewhere, particularly in preparations, stained for fibres, dendrites and processes. However, if it should turn out to be a true generality that apparently crossing fibres do not actually touch, this throws considerable doubt on the existence of anatomical synapses;

(iv) when one examines any of the latter histological preparations in regions where the fibres are not crossing, the vast majority of fibres seem to be ending nowhere; they are *culs de sac* in the ‘terra incognita’. This is particularly true in neonatal and developing brains (Conel, 1939-1967; Uylings et al, 1990; Goldin, Segal and Avignone, 2001).

(v) tens to hundreds of synapses are seen by light microscopy, but thousands by electron microscopy (Hillman, 1985b). Both sets of observations were carried out on stained sections, each stained by different procedures. Of course, one would expect that the higher resolution of the electron microscope would detect more synapses than the light microscope would. Yet these discrepancies should not be ignored:

(vi) more serious, perhaps, is the discrepancy between the diameters of the synapses using the two instruments. The light microscopists saw objects much larger

in diameter while the electron microscopists find them to have much smaller diameters (Tables, 77, 78). One would be tempted to explain this as being due to the higher resolution of the electron microscope. Unfortunately, the 1 to 5 μm synapses are never seen on electron micrographs of mammalian neuron cell bodies. It has been suggested that the staining for electron microscopy shrinks the synapses more than do the histological reagents, but it seems rather unlikely that the former would shrink the tissue to one tenth of the diameters, that the latter procedure would. However, this discrepancy is very serious, and rather indicates that the two kinds of microscopists were looking at different objects;

Diameters of synapses seen by light microscopy

<i>Animal tissues</i>	<i>Dimensions (nm)</i>	<i>References</i>
Human spinal cord	2,000 to 5,000	Held, (1897)
	500 x 1,000 to 3,500 x 5,000	Minckler, (1940)
Human cerebral cortex	500 to 7,000	Ramon y Cajal, (1909)
	800 to 2,500	Szentagothai-Schimert and Albert, (1955)
Cat cerebral cortex	2,500	Entin, (1954)
Rat visual cortex	2,000 to 4,000	Boyceott, Gray and Guillery, (1960)
Monkey spinal cord	800 x 960 to 2,600 x 3,300	Phalen and Davenport, (1937)
Cat spinal cord	800 x 1,000 to 1,000 x 2,200	Hoff, (1932)
	2,000 x 2,000	Gibson, (1937)
Cat spinal cord	500 x 600 to 4,000 x 4,000	Barr, (1939)
	500 x 500 to 3,000 x 3,000	Barnard, (1940)
	1,000 to 5,000	Bodian, (1942; 1952)
	1,500 to 8,000	Szentagothai-Schimert and Albert, (1955)
	1,000 to 4,000	Gray and Guillery, (1966)
Rabbit spinal cord	1,200	Wyckoff and Young, (1956)
	2,000 to 5,000	Auerbach, (1898)
Cat reticular formation	500 to 3,000	Brodal, (1981)
Cat sympathetic ganglia	5,000	Gibson, (1940)

Table 77. These diameters were generally given by the authors, sometimes as two coordinates, sometimes as one.

Diameters of synapses by electron microscopy

<i>Animal tissues</i>	<i>Dimensions (nm)</i>	<i>References</i>
Monkey visual cortex	600	Somogyi and Cowey, (1981)
Monkey lateral geniculate nucleus	1,500	Hasan and Glees, (1973)
Monkey oculomotor nucleus	2,500	Pappas and Waxman, (1972)
Monkey spinal cord	1,000 to 2,000	Bodian, (1964)
	300	Akert et al, (1972)
Monkey sympathetic ganglia	2,000	Bodian, (1972)
Cat sensorimotor cortex	650 to 1,000	Pappas and Waxman, (1972)
Cat oculomotor nucleus	1,000 to 1,500	Pappas and Waxman, (1972)
Rat vestibular nucleus	1,300	Brodal, (1981)
Cat hippocampus	1,000	Pappas and Waxman, (1972)
Cat subfornical region	500 to 800	Akert et al, (1972)
Cat lumbar spinal cord	600 to 1,000	Gray, (1959); Gray and Guillery, (1961)
Cat sympathetic ganglia	300 to 500	Elfvin, (1963)
Rat cerebral cortex	250 to 1,200	Pappas and Waxman, (1972)
	300	Jones and Brierley, (1972)
Rat visual cortex	400-900	Peters, Palay and Webster, (1998)
Rat auditory cortex	500 to 750	Peters et al, (1998)
Rat prepyriform cortex	800	Gray and Guillery, (1966)
	1,300	Johnston and Roots, (1972)
Rat hippocampus	500	Blackstad, (1967)
Rat olfactory glomerulus	600 to 1,000	Reese and Shepherd, (1972)
Rat medial septal nucleus	500 to 2,500	Raisman and Matthews, (1972)
Rat mammillary nucleus	800	Heimer and Ekholm, (1967)
Rat ventral cochlear nucleus	250 to 600	Peters et al, (1998)
Rat cerebellar cortex	1,500	Lumsden, (1968)
	500 to 2,000	Bloom, (1972)
Rat superior cervical ganglion	500	Tamarind and Quilliam, (1971)
	400 to 1,500	Gabella, (1976)
Ox brain	500	Roots and Johnston, (1964)
Guinea pig ileal mesenteric plexus	600 to 2,000	Gabella, (1976)

Table 78. The descriptions and dimensions are as stated by authors, or measured from their micrographs to the nearest 50 nm. The earlier references are in Fulton, (1949, pages 447-484). See also Pappas and Purpura, (1972).

(vii) an observation which has probably been overlooked, so that its implications were ignored, is that when black granules appear on silver or osmic acid stained sections on cell bodies or dendrites, they are identified as synapses, but a very large number are also seen in the absence of cell bodies or dendrites, or between them, without apparent attachment to them. Of course, histological preparation could have pulled them off, but that is an untestable hypothesis. Perhaps, it is a bit naive to regard any body showing these granules as only synapses. If the granules seen *between the cell bodies and the dendrites* are, indeed, deposits of heavy metals, it casts some doubt on the belief that they are synapses, when they are seen *on* cell bodies or dendrites;

(viii) pre- and post- synaptic membranes are believed to have ‘thickenings’ on electron microscopy (Sjöstrand, 1953c; Palade and Palay, 1954; Kirsche, 1977). These nearly always appear normal to the planes of section, and to have clefts between them uniformly distant apart (Hillman, 1986a, page 150, and references quoted there). Of course, if such structures existed in three dimensions, one would expect to see dark circles as frequently as synaptic clefts, and ellipses much more frequently than these. I can find no micrograph or publication showing the latter orientations at all (Figure 58, 59). If the latter appearances are not found, one must conclude that the synaptic thickenings are two dimensional and, therefore, appear after the sections have been cut. In short, they are artifacts.

(ix) there is plenty of physiological evidence going back to Sherrington that the pyramidal tract activates (synapses with) the spinal cord, both reflexly and voluntarily. It is extremely surprising that no one has ever shown by light microscopy the pyramidal tract connecting by synapses to anterior horn cells (Waldeyer-Hartz, 1888; Ziehen, 1899; Ramon y Cajal, 1909-1911; Peters, Palay and Webster, 1998). No explanation has been offered for this missing observation. Of course, this should not be confused with the presence of granules, sometimes with short stalks in the immediate region of anterior horn neuron cell bodies.

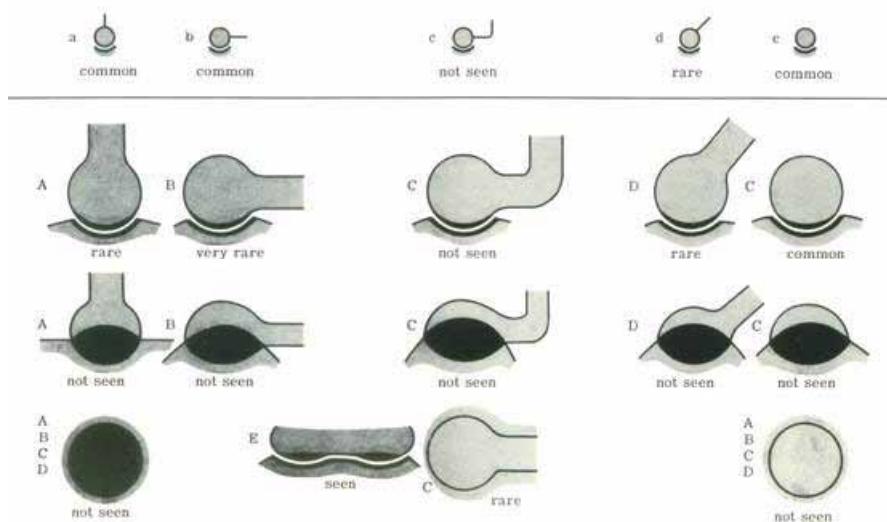


Figure 58. Drawings of possible appearances of synapses by light microscopy (top line, a, a, b c, d, e) and electron microscopy (lower lines, A, B, C, D, E); the same letters refer to the expected views of the same shaped synapse at different magnifications and orientations. The black lines and shading indicate cell membranes and pre- and post-synaptic thickenings; the grey shading indicates the cytoplasm of the synapses and the neurons. These diagrams are based on the assumption that the synapses are randomly distributed over the surface of the neurons, as shown, for example, in the diagram of Barr, (1939). The appearances of the synapses by light microscopy should be completely compatible with their appearances by electron microscopy. Geometry dictates unequivocally that *all* the appearances of the electron microscopic images indicated by a particular capital letter should be seen, *and* that they should occur in approximately equal frequencies. Examination of the considerable literature on the structure of synapses fails to show a significant incidence of any of the images of the third line down (with oval pre- or post-synaptic membranes), nor of the segmental sections of the synapses as seen in the lower extreme left and extreme right. Light microscopic images corresponding to the 'double' synapse seen on electron microscopy (E) do not seem to have been reported either. It should also be noted that the common views by light microscopy a, b, do not correspond to the common view by electron microscopy, C, in the right hand column.

Brain and spinal cord were prepared to produce a fraction identified as consisting largely of synapses, and called the 'synaptosomal' fraction (Whittaker, Michaelson and Kirkland, 1964; Whittaker, 1972; for reviews, see Jones, 1976; Cottrell and Usherwood, 1977; Bradford, 1986, pages 311-352). The preparation consisted of the following steps: anticholinesterase was added to preserve the acetylcholine; the tissue was homogenised; it was centrifuged for 10,000 g. min; the supernatant was drawn off, and centrifuged for 3000 g. min; the pellet was added to a sucrose gradient of 0.32 M to 1.2 M; the 1.2 M layer contained the synaptosomes.

Bradford, (1986) discussed the 'purity' of the preparation. Purity may mean either that the particular fraction only contains synapses, or that it has the same chemical composition after separation as the synapses have in the intact animal. Bradford estimated the 'contamination' from glial elements as about 20%, but he did not mention unidentifiable particles, broken synapses, other neuronal components, lost parts of cells, etc. One can not know what percentage of synapses *in vivo* survives to the synaptosomal preparation. For the reasons stated in Chapter 7, it is extremely unlikely that the chemistry of the preparation is not affected by the fractionation procedure.

The difficulties of the synaptosomal fraction are, firstly, the preparation procedure; secondly, the belief based on no evidence that the pulled-off synapses 'reseal' after breaking off. That means that the holes created would have to be closed by the shrinkage of the membranes around them, or new membrane would have to be synthesised. Both of these would require mechanisms and energy, operating between the homogenisation and the centrifugation of the tissue - not, perhaps the optimal chemical conditions. The synaptosomes appear to be surrounded by a single membrane, rather than a double 'unit' membrane, as micrographs of 'exocytosis' and 'endocytosis' seem to imply. 'Stalks', that is, pre-synaptic fibres do not seem to be visible on electron micrographs.

Spherical or oval synaptic 'vesicles' of about 50 nm in diameter can be seen on electron micrographs of the 'presynaptic' regions of neurons of the central nervous system (Peters, Palay and Webster, 2001). They appear to be dark balls with clear interiors. Fractions have been prepared by further treatment of synaptosomes (De Robertis et al, 1963); they are subjected to hypotonic solutions and density gradient centrifugation (Farnebo and Hamberger, 1971; Arbilla and Langer, 1980). The resultant fraction is also called 'synaptic vesicles' because the bodies are believed to

be identical with those seen in the presynaptic region of synapses. However, whereas the latter are seen to be approximately empty and clear (for example, Patrick and Barchas, 1974; De Belleroche and Bradford, 1980; Cheung, 1980), the bodies in the fractions are amorphous and dark stained, (Giorguieff, Kemel and Glowinski, 1977).

There are other problems with the synaptic vesicles. They appear to be all of approximately the same diameter in single electron micrographic sections, but, of course, solid geometry dictates that even if, *in life*, they were all of uniform diameters, successive sections should show a range of diameters, which they do not. Furthermore, it has been shown that the number of vesicles seen on electron micrographs depends upon the concentration of Mg²⁺ used in staining, hardly encouraging for the appreciation of quantitative studies on them.

It will be shown that the chemical theory of transmission is too uncertain to be acceptable.

The Schwann sheath is easily seen by light microscopy (Figure 60); (Causey, 1960). In electron micrographs, the myelin sheath appears to be layered in lamellae, (Robertson, 1959). The Geren model is believed to be the mechanism by which nerve fibres become myelinated (Geren and Rashkind, 1953; Geren, 1954). The nucleus of the Schwann cell is generally thought of as being located in a balloon, which collapses and subsequently wraps around the axon. The sheath is interrupted by nodes of Ranvier (1889). The wrapping continues accumulating scrolls of Schwann cell membranes, which thus form the myelin sheath. The hypothesis was originally conceived after observations in tissue culture, and is now generally accepted and represented in diagrams in textbooks (see, for example, Sunderland, 1991, pages 5) - 6). This hypothesis has several problems;

(a) the myelin lamellae do not obey the laws of solid geometry (please see Figures 3, 13);

(b) every internode is not seen to have its own Schwann cell, although it is generally believed that it has;

(c) each lamella should appear 4-layered by high-power electron microscopy, but it appears two layered, explained as the layers 'fusing together';

(d) the ratio of 2:2:1 for phospholipid: cholesterol: cerebroside, which is believed to characterise myelin lipids from peripheral nerves and brains, was measured on extracted myelin, since it can not be measured *in situ*. Many of the low-angle diffraction studies have been made on stained, dehydrated or extracted tissues

(ibid);

(e) a widening membrane spreading from the Schwann cell nucleus is not seen, except in diagrams;

(f) the winding of the Schwann cells was simply not seen in young rat sciatic nerves during the first days of development (Hillman and Jarman, 1989). It is difficult to know where it could occur in the circumstances of a whole peripheral nerve packed as it is with developing fibres (Figure 60).

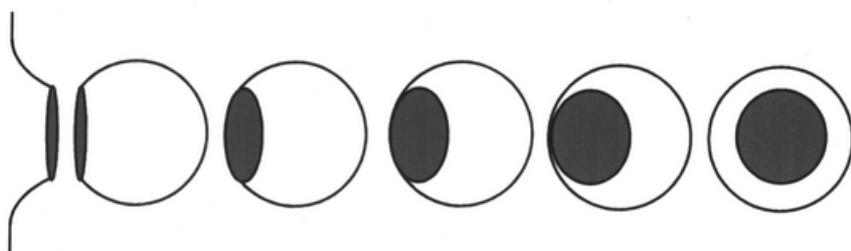


Figure 59. Computer graphics of the expected appearances of pre- and post-synaptic thickenings, at 22.5° orientations of the synaptic cleft, from *upper*, perfect transverse section to *lower*, section through the synaptic cleft. This graphic was kindly prepared by Dr. Nick McKay, of Surrey University.

(g) the idea that learning, memory and recall, reside in the synapses, can not be warranted, if the anatomical synapses are artifacts.

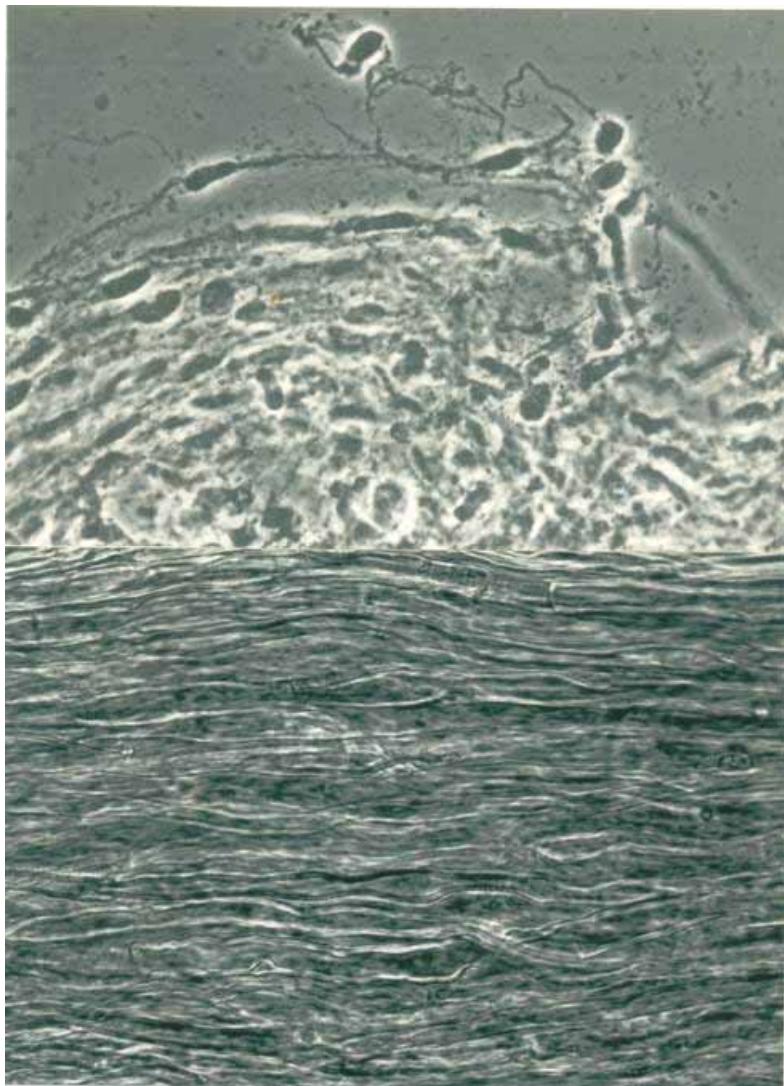


Figure 60. Unfixed rat sciatic nerves by phase contrast microscopy, *upper*, one day old, before myelination, *lower*, 8 days old, myelination almost complete.

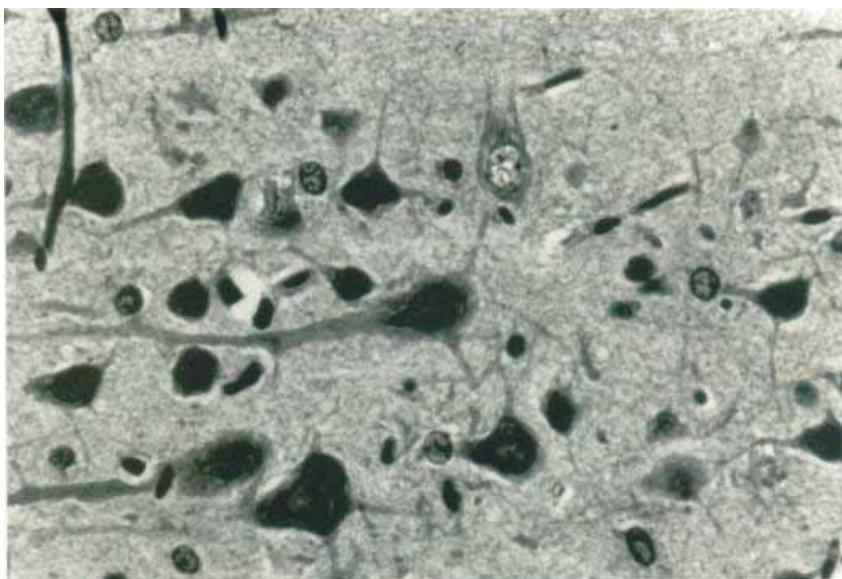


Figure 61. Rat parietal cortex stained with Gallyas's stain, x 600

Note the cells with processes and naked nuclei

A summary of the cellular structure of the nervous system can now be made in the light of the above considerations. The central nervous system consists of neurons with cell bodies, axons and dendrites. The network of neurons is embedded in a much larger volume of a syncytium (the neuroglia) containing nuclei in a viscous fluid suspending fine granular material. In addition to the blood vessels, there are only two kinds of cells in the central nervous system (Figure 61); these are neurons, which are all cells with processes, now called neurons and astrocytes, and naked nuclei, now called neuroglial nuclei, oligodendrocytes, microglia, reactive astrocytes and neuroglial nuclei in malignant cells.

Chapter 35

Neuromuscular Junctions

The neuron, the neuromuscular junction and the muscle constitute the motor unit (Buchthal, 1960). The distinction between neuromuscular junctions (end-plates, or motor end plates) and synapses (nerve-nerve junctions) has been rather blurred, especially since Katz, (1969) and his colleagues proposed the theory of chemical transmission as a result of experiments on neuromuscular junctions (mainly in frogs). It has been generally assumed that synapses operate by the same mechanisms. Neuromuscular junctions are much easier to study, and their physiological properties are much clearer than those of synapses. So there has been a tendency in the recent literature to use the term 'synapse' for both junctions.

Yet there are significant differences between the two, which are summarised in Table 79. The two structures are being considered separately, because whereas the anatomical synapse has been shown to be an artifact of staining, the neuromuscular junction can be seen in unstained fresh tissue.

Differences between neuromuscular junctions and synapses

<i>Neuromuscular junctions</i>	<i>Synapses</i>
Peripheral nervous system	Central nervous system
Lobulated	Spherical or egg shaped
Basal plate between nerve ending and muscle	Synapses abut directly on neurons
45 - 120 nm between nerve and muscle	20 - 40 nm synaptic cleft
Approximately 32 folds	No folds
No pre or post - synaptic thickenings	Pre and post - synaptic thickenings on electron microscopy
Tens of neuromuscular junctions in whole muscle	100's to 1000's of synapses
Generally larger	Generally smaller
Connections of motor fibres to muscles seen	Long pre-synaptic fibres never seen
Motor	Motor and sensory
Acetylcholine and adrenalin act on them	Many different transmitters
Slow and 'fast' muscles	Thicker and thinner nerve fibres
'Blocked' by curare	Blocked by cholinesterases
Denervation causes immediate hyperexcitability	Denervation prevents transmission

Table 79. These properties are not necessarily agreed by the present author.

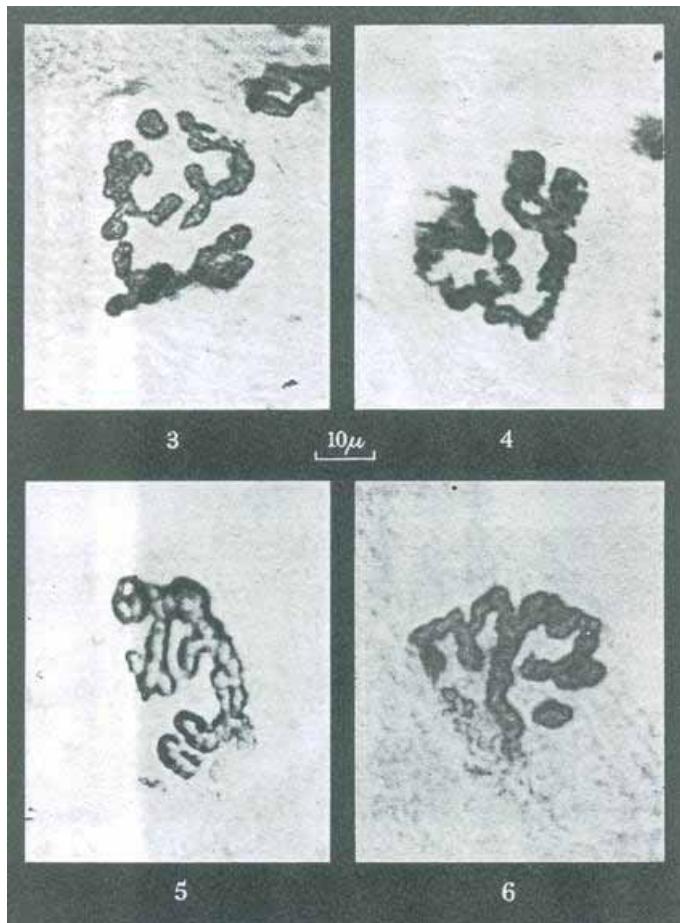


Figure 62. Neuromuscular junctions in rat diaphragm stained for cholinesterases from Denz FA, (1953), reproduced by kind permission of Blackwell Scientific Publications.

The neuromuscular junction has been seen under the light microscope, using a number of different staining systems including gold, Golgi, methylene blue, Gomori and cholinesterase (Zacks, 1964). Its shape varies from that of a sphere to that of a crab (Figure 62). There appear to be very few of them in a whole frog muscle. It has a basal plate across which a transmitter or its messenger must diffuse before it reaches the muscle. Are the receptors for the transmitter located at the nerve face or the muscle face of the basal plate? The real problem with the latter structure is that it is only seen in stained, and, therefore, shrunken tissues. Thus, one can not know whether it is a total artifact resulting from the staining of the tissue, or whether its size

or shape has been increased or decreased by the procedures used to study it. This question could be studied using confocal, phase contrast or quantum dot microscopy of unfixed tissues. If it could be established that the basal plate exists in unfixed cells, one could examine the sites of the receptors to the transmitters, and the rates of movement of the transmitters, their receptors and electrical excitability across the neuromuscular junctions. Nevertheless, the excitability, itself, does *not* originate from the junction. The latter only directs it to the muscle fibres. The passage of excitability from the neuromuscular junction to every single fascicle of muscle, muscle fibre and sarcomere must be by conduction, whether or not T-tubules really exist in living muscles.

It should also be noted that cholinesterase staining (for example, Koelle, 1950; Denz, 1953), show that the acetylcholine is confined clearly to the neuromuscular junctions, and does not appear to be escaping into the muscle itself. Therefore, one must conclude that, either, another mechanism or an electric current, takes the wave of excitability to the individual sarcomeres, but not the acetylcholine itself. Alternatively, one could consider whether it was not the biochemical environment around the neuromuscular junction, which favoured the appearance of the enzyme activity in that region, rather than the monopoly of the presence of the transmitter there. Muscle is normally stimulated through the neuromuscular junction from the motor nerve, but it can be stimulated directly in experimental conditions. When the muscle is denervated, the region from which it can be excited either by electrical stimulation, or by the application of acetylcholine, spreads to all over the muscle (Erb, 1868; Frank, Northmann and Hirsch-Kauffmann, 1922; Brown, 1937). These experiments show that the neuromuscular junction only *funnels* the excitability to the muscles.

There are normally tens to hundreds of neuromuscular junctions on particular muscles (Vrbova, Gordon and Jones, 1978), but there are hundreds to thousands of muscle fibres. There have been many estimates of the number of muscle fibres served by a single motor nerve (for review, see Shields 1995). For example, the ratio of human nerve to muscle fibres varied between 5 and 1,934 (Enoka, 1995). Each muscle fibre contains many individual sarcomeres in series and in parallel. Furthermore, fibres coming out of the neuromuscular junctions can not be seen going to every fibre or sarcomere by light or electron microscopy. It thus follows that the excitability must be *conducted* to between 5 and 1,934 muscle fibres and to several

times that number of sarcomeres. It has been suggested that 'T-tubules' are the route of this conduction, and these will be dealt with under muscle contraction.

The excitability to both electrical stimulation and acetylcholine of denervated muscle (Magladeny and Solandt, 1942) shows that the neuromuscular junction itself does not confer excitability. The consensus view is that the neuromuscular junction is replete with acetylcholine receptors. By this view, when the muscle was denervated, one would expect the appearance of new crops of receptors all over the muscle. Although botulinus toxin and bungarotoxin, which are believed to bind acetylcholine receptors, now bind across the whole muscle (Axelsson and Thesleff, 1959; Thesleff, 1960; Bader, 1981), there is no evidence of new end plates or receptors visible by light or electron microscopy.

The width of neuromuscular junctions seen by light and electron microscopy are compared (Table 80).

Width of neuromuscular junctions by light and electron microscopy

<i>Light microscopy</i>	μm	<i>References</i>
Toad sartorius	12	Anderson, Cohen and Zorychta, (1977)
Mouse gastrocnemius	5	Bowden and Duchen, (1976)
Rat diaphragm	5	Denz, (1953)
Rat muscle	3	Csillik and Savay, (1958)
Rat soleus	1-2	Vrbova, Gordon and Jones, (1978)
Rat superior oblique	5-6	Peters, Palay and Webster, (1991)
Rabbit gastrocnemius	2-4	Coërs, (1967)
Cat gracilis	15-20	Coërs and Durand, (1957)
Human, various	3-4	Coërs, (1955), Coërs and Woolf, (1959) Coërs, Reske-Nielsen and Harmsen, (1973)
Human, intercostal	20	Zacks, (1964)
<i>Electron microscopy</i>		
Frog pectoralis	1.5	Hirokawa and Heuser, (1982)
Frog sartorius	2-6	Robertson, (1960)
Frog sartorius	2	Gray, (1959)
Frog gastrocnemius	0.7	Reger, (1958)
Mouse extraocular	6	Salpeter, Fertuck and Salpeter, (1977)
Rat superior oblique	12	Peters, Palay and Webster, (1998)

Table 80. Many other neuromuscular junctions can be seen in Kühne, (1862; 1887) Andersen-Cedergren, (1969), Zacks, (1964) and Salpeter, (1987). *The discrepancies*

between the dimensions by light and electron microscopy have not been noted previously.

At the moment, one must keep an open mind as to the precise structure and dimensions of the neuromuscular junction. The hypothesis that transmission is chemical will be considered later (please see Chapter 44).

Chapter 36

Muscle Structure

Traditionally, muscles are classified into (i) skeletal, voluntary, striped or striated; (ii) visceral, involuntary, smooth, unstriated; and (iii) cardiac. Their most obvious common characteristic is that they contract and do work. They convert chemical energy to mechanical energy (Table 81). They are elastic, and contain muscle and tendon fibres which store potential energy. They can be stimulated by nerves, transmitters and the local presence of particular substances. The activities of groups of muscle fibres are co-ordinated by nerves.

The large skeletal muscles and cardiac muscle are reddish brown, due to the presence of myoglobin and blood in the capillaries. They contain muscle spindles and stretch receptors, which feed back to ‘inform’ the central nervous system about their degree of tension and the position of joints. In all types of muscle, the local chemistry and the mechanical properties of the muscle fibres, also act as feedback mechanisms.

Similarities between skeletal, smooth and cardiac muscles

- contract when stimulated or spontaneously do work
- cells have resting and action potentials
- have neuromuscular junctions
- the junctions can be fatigued and ‘blocked’
- can be stimulated through nerves, electrically, by high K^+ , transmitters or hypoxia
- require Ca^{2+} and Mg^{2+} to contract
- believed to contain T-tubules, which conduct excitability
- believed to contain sarcoplasmic reticulum, which ‘sequesters’ Ca^{2+}
- contract when ATP is added
- the fatigue and blockage can be affected by drugs
- can be stimulated directly, bypassing neuromuscular junctions
- denerivated muscles can contract after direct stimulation
- contraction causes liberation of energy and heat
- contain nuclei and mitochondria
- require substrate for prolonged contraction
- contain myosin, actin, tropomyosin and other proteins
- contain filaments of various diameters and lengths
- the latter are believed to be connected by cross bridges
- they are believed to contract by sliding filaments

Table 81. The data for this and the next table come from Huxley AF and Niedergerke, (1954), Huxley, HE and Hanson (1955; 1959). Huxley AF, (1956), Huxley HE, (1957), Slayter and Lowey, (1967), Needham, (1971), Somlyo et al,

(1973), Huddart, (1975), Elliott, Offer and Burridge, (1976), Shoenberg and Needham, (1976), Bülbbring, Brading, Jones and Tomita, (1981), Gabella, (1981), Stephens, (1984) and Somlyo et al, (1987).

Differences between skeletal, smooth and cardiac muscle

<i>Skeletal</i>	<i>Smooth</i>	<i>Cardiac</i>
reddish brown	colourless	reddish brown
long fibres with tendons	circular or longitudinal	imbricated
have tendons and ligaments	do not	do not
voluntary	reflexly controlled	automatic
A and I bands present	no A or I bands	A and I bands present
Z lines present	no Z lines	intercalated disks
H zone present	no H zone	H zone present
thick filaments (20-30 nm)	not agreed	thick filaments (20-25 nm)
no intermediate filaments	intermediate filaments (7-10 nm)	no intermediate filaments
thin filaments (5-8 nm)	also present	also present
thin/thick ratio 6/1	thin/thick ratio 11-15/1	not known
sarcomere length 1.7-2.8 μm	length 50-70 μm	length 1.5-2.5 μm
peripheral nuclei	central nuclei	central nuclei
rapid response	slow response	slow response
short action potential	long depolarisation	long plateau
acetylcholine stimulates	acetylcholine stimulates	acetylcholine inhibits
no caveolae	caveolae in sarcoplasmic reticulum	no caveolae
movement and posture	tone	pumping

Table 82.

Muscle of the different kinds have many properties in common (Table 81) and this has led to the assumption that the sliding filament hypothesis, which was derived from observations on skeletal muscle, is also true for smooth and cardiac muscle.

Smooth muscles are usually composed of the circular and longitudinal fibres in the walls of the intestines, respiratory systems, blood vessels, uterus, bladder, etc. They appear white, because they are supported by fibrous connective tissue, including collagen. Their neuromuscular junctions tend to be very thin and translucent. Their tone determines the pressure in the organs, in which they are found. In life, these muscles are contracted to a variable extent, and relax when an animal dies. They are

involved in digestion, maintenance of blood pressure, uterine contraction and micturition, and are controlled by the autonomic nervous system.

Cardiac muscle is spontaneously excitable. An excised, denervated, mammalian heart beats at about 50 - 60 per minute, and the vagal and sympathetic innervation decreases or increases the frequency and force of the contraction, respectively. Some muscle fibres of the Purkinje system act as nerves, and conduct the excitation from the atrio-ventricular node down the bundle of His to the ventricles. They can thus be regarded as modified heart muscle. They exhibit plateau-shaped action potentials, and have their own repertoire of physiology, including channels, conductors, activators, inhibitors, etc. (Draper and Weidmann, 1951; Noble, 1975; Eisner, Lederer and Vaughan Jones, 1983).

The absence of neuromuscular junctions from the AV node down the bundle of His to individual cardiac cells makes it certain that the major proportion of the journey of excitation of an action potential is by conduction. The heart is a 'functional syncytium', in that the intercalated discs do not seem to be relays in the excitation, as junctional tissues are believed to be.

If one wishes to propose that the sliding filament hypothesis is the mechanism of contraction of all three kinds of muscle, the mechanism must be located only in those properties, which the three kinds have in common (Table 81). This chapter is concerned with structural aspects of muscle. The mechanism of contraction will be dealt with later (please see Chapter 46).

Voluntary muscles are attached to bones or other tissues. The tendons and ligaments are fibrous, and contain much collagen; they are flexible and slightly elastic, but only passively contractile. Tendons, and ligaments attach spindle-shaped muscles to bone, and aponeuroses attach flat muscles to each other.

The sarcoplasmic reticulum is believed to be a network of T-tubules attached to Z lines and longitudinal tubules. It was seen in the electron microscope (Rosenblith, 1969; Smith, 1971; Franzini-Armstrong, 1980), and was also recognised to have been seen by the light microscope (Verratti, 1902, subsequently republished in 1961). Recent reviews have appeared (Martonosi, 2000; Eisner, 2002). Of course, all these authors have regarded it as a real network located between muscle fibres. They also hold that the T-tubules conduct excitability from neuromuscular junctions to individual sarcomeres, and that the light microscopic images represent the same structures as are seen under the electron microscope. As far as I can find, no one

hitherto has proposed that they might be artifacts. One may summarise the evidence that this is the case:

- (i) The illustrations which Martonosi, (2000) used on a book cover, which originated in Verratti, (1899; 1902), show striations (presumably in transverse section). Unfortunately, neither in the references cited above, nor the very extensive literature between the 1960's and 2000's has anyone published *oblique* views of the T-tubules, the Z lines or the longitudinal tubules. One must await such important corroborative evidence with hope;
- (ii) the drawings of light micrographs, showing the lines of sarcoplasmic reticulum, (Verratti, 1961) depict striations, and one must ask how these can be distinguished from A bands;
- (iii) in the elegant electron micrographs, for example, of Smith, (1971), which are typical of all stained muscle, one sees disjunctures between the T-tubules and between the Z lines of adjacent muscle fibres, when they contract. However, when AF Huxley observed contraction in living cells by interference microscopy, he saw clearly that adjacent fibres contract simultaneously with the Z-lines all remaining as if attached in parallel with each other;
- (iv) if one looks at *living* muscle fibres - which, of course, have not been stained for histology or electron microscopy, - one does see occasional mitochondria between the fibres, but the spaces containing the sarcoplasmic reticulum and terminal 'cisternae' are simply not seen between individual muscle fibres;
- (v) Rosenblith (1969) showed the sarcoplasmic reticulum, interspersed with myofibrils, in motor muscle of a lobster's antenna. In his micrographs, the section seems to have gone through a large number of tubules, and there are no oblique or side views of the reticulum, in three quarters of the muscle. The other quarter contains myofibrils, *every one* of which appears in the plane of the section. Such very obvious defiance of the dictates of solid geometry makes it certain that both of these apparent structures must have appeared on the surface of the field, after the sections were cut, and placed on the stage of the electron microscope. The simplest explanation for them is that they are dried-out sarcoplasm, which has reacted with heavy metal stains.

Differences between types of muscle are noted (Table 82).

Voluntary muscle

A whole muscle, such as the biceps, psoas, sartorius or soleus, usually takes origin from a bone, and is inserted by a ligament into another bone, which it moves. Each real muscle consists of bundles of fasciculi in parallel. These may be dissected apart, and have elongated and flattened nuclei lying on them. In turn, they consist of tens of muscle fibres, each comprising longitudinal strings of sarcomeres.

Voluntary muscles are called striped because of the appearance of dark lines (anisotropic or the A band) and light lines (isotropic or the I band). An array of a large number in parallel gives the appearance of striations.

The sarcomere is considered to be the unit of contraction, although the muscle fibre must be considered to be the cell, since it has the single nucleus and mitochondria (and, perhaps, T tubules) on its surface, between it and the next cell. A diagram of the consensus view of the thick and thin filaments is given (Figure 63). This view derives from the electron microscopic observations of Huxley HE and Hanson, (1955; 1959), Huxley AF, (1956), and Huxley HE, (1960).

A number of important observations were made on this system. By treating a few fibres with 0.6 M KCl for 18 hours - a reagent considered to extract only myosin - it was concluded that the A-band consisted of myosin (Hasselbach, 1953; Hanson and Huxley, 1953). Actin can be extracted with 0.3 M KCl, plus 0.15 M phosphate buffer (Guba-Straub solution) or 0.47 M KCl, plus 0.01 M pyrophosphate (Guba and Straub, 1943; Straub and Feuer, 1950; Hasselbach and Schneider, 1951). Further extractions showed that the A-band and the I-band both contained actin and tropomyosin (Perry and Corsi, 1958; Corsi and Perry, 1968). The mixture of both had previously been called myosin, (Straub, 1943). It was concluded that the A-band consisted of thin filaments made of myosin, while the I-band consisted of thin filaments made of actin.

Reiser (1949) found that the viscosity of living muscle was low, and small droplets could be injected, and they flowed in the muscle mass.

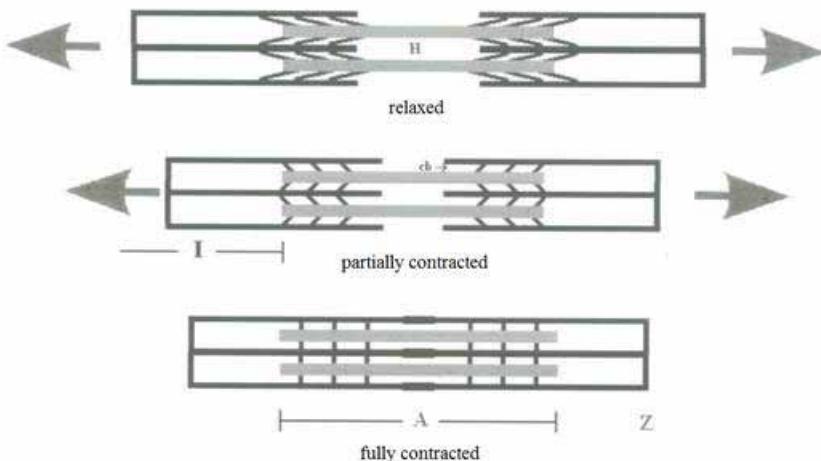


Figure 63. The sarcomere during contraction, believed to originate in the cross bridges. The thick filaments are grey, and the thin horizontal filaments are darker. The A band is anisotropic; the I band is isotropic; the H band is between the thin filaments; the Z band is at the end of the sarcomere, and cb indicates cross bridges. In vertebrate muscle measured after staining, sarcomere lengths are from 1.7 μm to 2.9 μm and the I bands from 0.75 μm to 1.3 μm (Huddart, 1975, page 10).

The cross bridges are not simply struts, but appear in diagrams as hinged; they are beaded, two-headed, triangular, roller or golf club in shape; their distal ends are applied to the thin filaments (see Pollack, 1990, pages 9-37). In micrographs, the cross bridges appear as spheres or bars, (Güth, 1980; Cooke, 1981; Huxley, HE and Kress, 1986; Toyoshima, Toyoshima and Spudich, 1989).

The interpretation of the fine structure of sarcomeres by electron microscopy has several, mainly geometrical, difficulties.

1. All measurements are carried out on electron micrographs of metal deposits on dehydrated tissue. Indeed, Huxley, HE (1953) pointed out in respect of filaments that "It is not known how thick they are in living muscles as the images seen in electron micrographs are of protein filaments which have been dehydrated and stained".

2. The thick and thin filaments, and the Z lines are virtually never seen over whole fields in *oblique view* either under electron micrographs or in published

electron micrographs. The filaments are seen apparently in near perfect longitudinal or transverse section, and the Z-lines nearly always appear edge on. The only possible explanation for this is that the electron microscopist is looking at images of a two-dimensional deposit.

3. The thick and thin filaments appear to have remarkably uniform diameters in longitudinal sections of particular muscles. Of course, they should appear to have a variety of thicknesses - even if in life they are of uniform thickness - depending on the thickness of the section and the depth of the fibres within the section.

4. Cross bridges are seen in longitudinal sections of muscles between the filaments but do not appear on transverse even in thin sections, of frog sartorius and rabbit psoas (Hodge, Huxley and Spiro, 1954; Huxley, HE 1972).

5. Cross bridges should appear more oblique in relaxed muscle, than they do in the contracted muscle (Figure 63), and at right angles to the filaments during maximal contraction, whereas they appear as random fibres or granules with no particular orientations in either the relaxed or contracted muscle (op. cit.).

6. Appearances of similar structures also called cross-bridges are also seen in saponin - extracted rapidly frozen rat sciatic nerves (Hirokawa, 1982) (Figure 64).

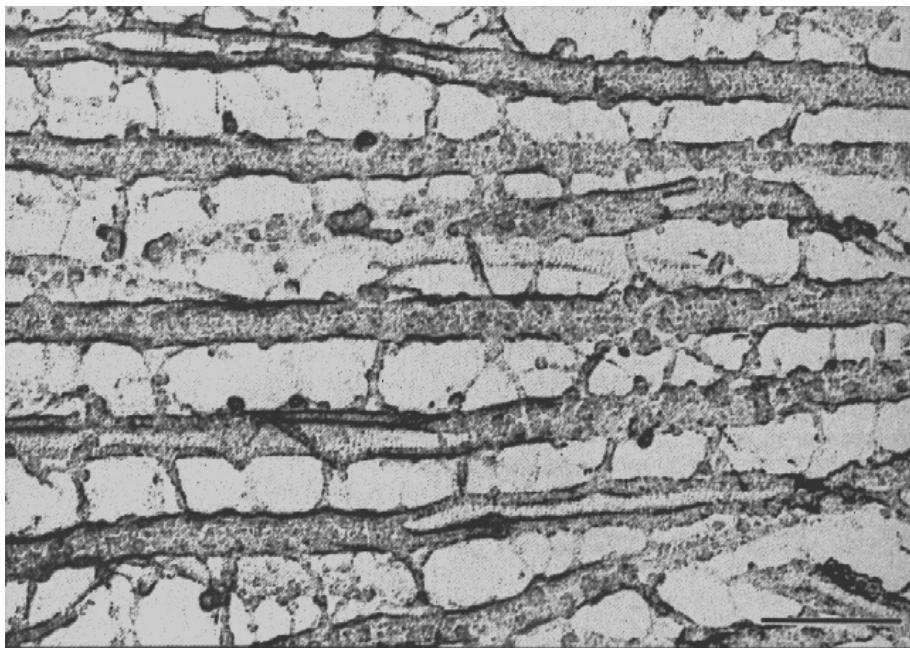


Figure 64. Neurofilaments in a saponin treated axon. The space between adjacent neurofilaments varies in width between 20 and 50nm. "Cross linking" thin filaments (4-6 nm in diameter) connect adjacent neurofilaments, in Hirokawa, (1982) by kind permission of the Authors and Rockefeller University Press. See also Hirokawa, Glicksman and Willard (1984) and Hirokawa, (1991).

7. An appearance similar to thick and thin filaments was first noted in chloroplasts by Ernst, (1963, pages 75-78). The latter illustration also has a slight hint of cross bridges between the filaments. Particles were also seen in Descemet's membrane from the cornea (Jakus, 1956). The same appearances are also seen in chloroplasts (Heslop-Harrison, 1963) (Figure 65).

8. Vacuoles, sometimes lined with mitochondria, are often seen between muscle fibres in micrographs, but they are not seen in living muscle. Therefore, they are almost certainly dehydration artifacts.

9. If muscles are stretched so that the thick and thin filaments no longer overlap, then they should not contract when stimulated, but they do.

10. Huxley HE, (1963), Slayter and Lowey, (1967) and Walker, Knight and Trinick, (1985) separated myosin molecules, some of which were furnished with two heads, such two-headed molecules do not appear in electron micrographs of whole muscle (op.cit.).

11. The sarcoplasmic reticulum and the T-tube system are seen by electron microscopy to have diameters of 0.4 - 2.8 μm . (Huxley and Niedergerke, 1954; Peachey and Huxley, 1962), and thus should be within the resolution of the light microscope.

Smooth muscle

In form, visceral muscle is very different from voluntary muscle. It is not attached by ligaments or tendons. Visceral muscle aggregates in tubes, sheets or spirals. It is controlled by the fine plexuses of nerves of Auerbach and Meissner, which permeate the longitudinal and circular muscles of the intestine. Single fibres are spindle shaped, 50 - 400 μm long, and 1.5 - 8 μm in diameter. They have central nuclei. Their maximal lengths in relaxation are about 5 times those during maximal contractions, compared with 1.5 - 2 times in voluntary muscles.

On electron microscopy, filaments with granules whose dimensions are

intermediate between thick and thin, are seen. They have diameters of about 10 nm (Ashton, Somlyo and Somlyo, 1975). These filaments are not believed to be involved in contraction, since, after they had been extracted from *Taenia coli* muscle with Triton X-100 and collagenase, the muscle still contracted following the addition of ATP (Small and Sobieszek, 1977). Thin filaments of 3 - 8 nm in diameter predominate in muscle (Somlyo et al, 1987), and they are also decorated with large numbers of circular ‘caveolae’, whose diameters are between 30 and 50 nm (Gabella, 1981). Cross bridges have been seen between the different filaments (Somlyo et al, 1977a,b). A reflection of 14.3 nm, corresponding to that seen in voluntary muscle (Huxley HE, 1972), has been reported in guinea pig *Taenia coli* (Lowy, Poulsom and Vibert, 1970). There are no Z-lines, A bands or I bands, in visceral muscle.

Reasons given for which thick filaments were sometimes not seen in smooth muscle by electron microscopy

Smooth muscle myosin had a different solubility than striated muscle myosin
 They were labile and broken down by fixatives
 The muscle had not been ‘equilibrated’ in the cold
 The shape, number and sizes of filaments depended upon ‘pre-treatment’
 The thick filaments appeared ‘best’, when the muscles were stretched to 1.5 x length
 The appearances depended on the fixative used for electron microscopy
 The appearances depended on the pH during glycerination
 The appearances depended on the concentration of ions during incubation and fixation
 The appearances depended upon the temperatures at the time of incubation and fixation

Table 83. Nearly all these reasons were quoted from authors cited by Shoenberg and Needham, (1976) and Squire, (1981, pages 443-461). Huddart, (1975, page 77) listed 5 vertebrate preparations, whose filaments had diameters of 7-17 nm. *An alternative view is that the filaments are not seen because they are not present in unfixed preparations.*

A number of reasons was given for which thick filaments could not be seen in smooth muscle, and these are summarised in Table 83. The whole Table indicates the empirical nature of procedures for electron microscopy to show up the thick filaments, Choi, (1962) Devine and Somlyo, (1971). Demonstration of their existence enables the sliding filament hypothesis to be extended from skeletal muscle to include visceral muscle (please see Chapter 36). What other structures or artifacts might be revealed by changing the conditions or reagents of preparation?

The structure of visceral muscle will be discussed later, but a few points are

worth making.

(a) The absence of a Z-line means that the contracting fibres would have no structure against which to contract.

(b) The width of the circular and longitudinal fibres seen by histological procedures are well within the resolution of the light microscope, say 200 - 250 nm, so they are much wider than the 10 - 20 nm filaments seen both in electron micrographs, and in extracted actin and myosin. However, when one looks in electron micrographs of whole sections, one sees the filaments as discreet spaced entities, which are not bunched closely enough together to be within the resolution of the light microscope.

(c) As in voluntary muscle, one does not see oblique views of any of the filaments in electron micrographs of relatively large pieces of muscle.

(d) There are considerable shrinkage artifacts between fibres in most histological and electron microscopic preparations in many voluntary, visceral and cardiac muscles (see, for example, Jones, Somlyo, Somlyo and Rice, 1973; Gabella, 1981; Squire, 1981; Pollack, 1990). They are not seen in fresh muscle.

(e) If the ratio of the maximal to minimal length of the fibres of visceral muscle is 4 - 5: 1, it is difficult to know where the excess elongate filaments would go when the muscle contracts.

(f) As with voluntary and cardiac muscle, one can not measure dimensions on dehydrated and shrunken tissues.

(g) The caveolae appear as admirably uniform circles in electron micrographs, presumably representing uniform spheres. Of course, in sections, they should appear in a normally distributed profile of diameters. Their uniformity indicates that they, also, originate after the sections had been cut. Their numbers and diameters do not change during contraction or stretch (Gabella and Blundell, 1978). The simplest explanation for the caveolae is that they are bubbles of gas previously dissolved in tissue, which came out of solution as a consequence of rapid freezing and dehydration.

One can dissect out single fibres from circular and longitudinal fresh visceral muscles and they do appear in all orientations on histological sections. The fact that the filaments do not, is best explained by the suggestion that the contents of sarcomeres are liquid in life, and the filaments appear after the sections have been cut, that is to say, during the sojourn of the sections within the chamber of the electron

microscope. It is not at all surprising that one may extract myosin or actin and view it with the electron microscope, and it looks like the filaments in a section of muscle. The fact that the extracted filaments do not appear in all orientations can be attributed to the fact that the liquid extract is deposited and dries out - as crystals would - in the two dimensions of the microscope stage.

Cardiac muscle

Cardiac muscle usually appears to have vacuoles between the fibres (Huddart, 1975, pages 49-63). Stained fibres show anastomoses. The sarcomeres have a range of appearances from that similar to those of voluntary muscle to rather unstructured fibre bundles. In vertebrates, the sarcomere lengths vary from 1.5 - 3 um, and the cell diameters from 3 - 80 μm . Most appear to have sarcoplasmic reticulum, and some to have T systems. This must be interpreted as meaning that the T system is not necessary for contraction of cardiac muscle (*Ibid*, page 52). All cardiac cells have mitochondria, located between muscle fibres. The nuclei are usually within the muscle fibres.

Artifacts

A summary of the artifacts in muscle seen by electron microscopy, and the reasons for which they are artifacts is given (Table 84).

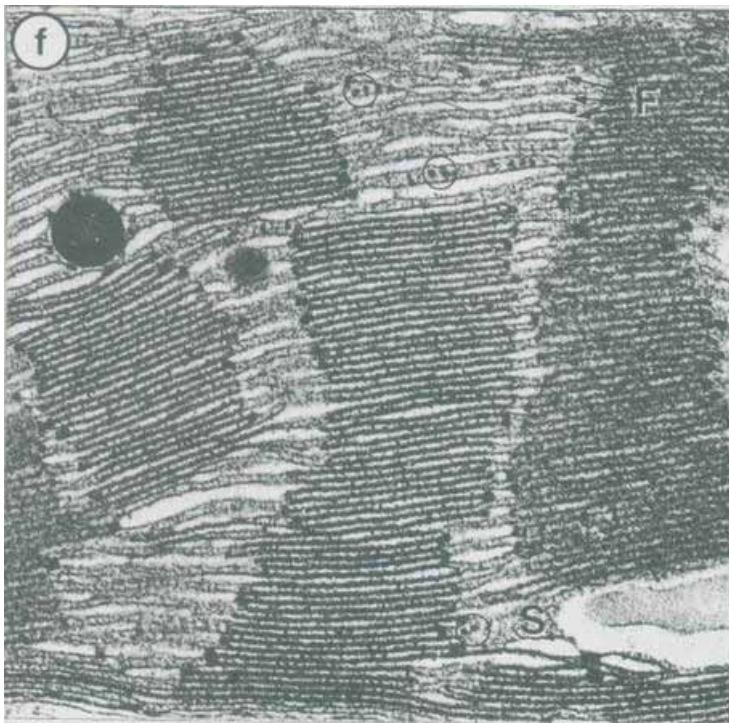


Figure 65. Electron micrograph of maize chloroplasts showing a similar appearance to 'thick' and 'thin' muscle filaments, from Gunning and Steer, (1975) reproduced by kind permission of Hodder, Headline Ltd. Magnification is x 75,000.

Artifacts of muscle under the electron microscope

<i>Structures</i>	<i>Why they are artifacts</i>	<i>Nature of the artifacts</i>
Very thin filaments going from one Z-line to the next.	They all appear to have constant widths and diameters. They are nearly always seen in perfect longitudinal or transverse section, never obliquely	Two-dimensional precipitates of myoplasm, which is a liquid in life; cf electron micrographs or low angle diffraction, of gelatine, glycerol, aminoacids and salt (Frey-Wyssling, 1953; Luyet, Tanner and Rapatz, 1962; Luyet, 1966)
Thin filaments		
Intermediate filaments		
Thick filaments		
Cell membrane around muscle fibres	not seen in electron micrographs	seen in diagrams
Spaces between muscle fibres	not seen in unfixed fresh muscle	shrinkage artifacts
Cross bridges	rarely seen in transverse sections of voluntary or visceral muscle, but seen in longitudinal sections	when two adjacent linear precipitates appear, particles collect between them

<i>Structures</i>	<i>Why they are artifacts</i>	<i>Nature of the artifacts</i>
Sarcoplasmic reticulum	only by electron microscopy. Never seen obliquely, rarely longitudinally	shrinkage artifacts are lined by capillaries, tubules or unidentifiable circles
T-tubules	do not become wavy during contraction	shrinkage artifacts
Caveolae	not seen by light microscopy although within its resolution Too constant in diameter	bubbles

Table 84. *Geometry can not be ignored.*

Chapter 37

The Anatomy of Cells

One may classify cells according to their general forms. Firstly, one has the syncytia, in which several nuclei share cytoplasm, without a membrane around each cell. These occur in a remarkable large number of tissues (Table 25). Secondly, there are the elongated cells, such as muscles, fibroblasts, neurons and sperm. Thirdly, there are many cells, which are spherical, cubical or columnar, such as immature red cells, white cells, epithelia, endothelia, glandular cells, germ cells, zygotes, etc. Despite the variety of shapes, the latter resemble the ‘generalised cell’ most closely. Those that excrete fluids or particles have granular cytoplasm. Cells which secrete, such as plasma cells and salivary gland cells, are rich in endoplasmic reticulum.

In order to understand the anatomy of cells, it is useful to summarise artifacts, which have been seen when examining cells by light, and more especially, by electron microscopy (Table 85). Two points must be stressed in evaluating the reality in life of cell structures. Firstly, geometry must be respected. Secondly, the extracellular compartment, the cytoplasm, the mitochondrioplasm and the nucleoplasm are fluid in life.

Likely artifacts of microscopy of cells

<i>Artifact</i>	<i>Why it is an artifact</i>	<i>Possible origin</i>
Trilaminar appearance of cell, nuclei and mitochondrial membranes, but not necessarily the membranes themselves	the two laminae appear nearly always equidistant	explosion of embedded tissue beneath the metal deposit
The Golgi body	unfixed cells; appearance and dimensions too heterogeneous in different preparations; would prevent intracellular movements of particles	precipitate of cytoplasmic solutes with silver or heavy metal deposit, during dehydration
Endoplasmic reticulum	would prevent intracellular movements of particles; seen far too often on transverse section, never oblique sections away from the maximum diameter of the cells; not seen clearly by light microscopy, except in diagrams	as Golgi body
Uniporters, symporters and antiporters	not seen on electron micrographs	biochemical models

Artifact	Why it is an artifact	Possible origin
Transmembrane molecules	they are never seen by electron microscopy in whole tissues	an explanation for extracellular affecting intracellular events
Receptors and ion channels	with the exception of the sodium channel or nicotinic acetylcholine receptor - no one has claimed to have seen <i>any</i> other of the thousands believed to be present on the cell membrane, although it is claimed that many of them have been sequenced and their structures are known	it is <i>assumed</i> that transmitters, hormones, antibodies, drugs and toxins act by binding to receptors, nearly all on the cell surface
Peroxisomes	not seen by light microscopy	chemistry is of subcellular fraction only
Mitochondrial cristae and inner membranes	they nearly always appear in perfect transverse section, irrespective of the orientation of the whole mitochondria	the mitochondrial plasma is a fluid in life, and crystallizes in the plane of the section during dehydration and electron bombardment
Stress fibres	no proof that they result from stress; unnecessary hypothesis	dehydration artifact
Gap junctions, intermediate junctions, tight junctions and desmosomes	only seen in one orientation; one can not know whether the shrinkage during electron microscopy will increase or decrease apparent distances between membranes	heavy metal deposit

Table 85. Structures, seen by electron microscopy, which are probably artifacts.
Only the Golgi body is also seen by light microscopy.

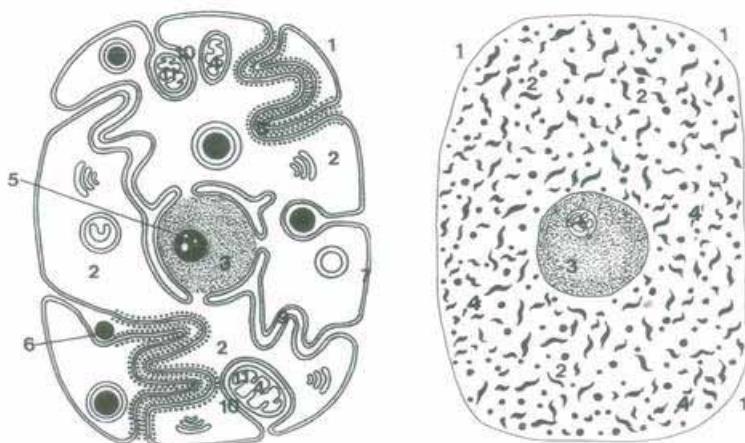


Figure 66. The generalised cell, as currently believed, *left*, by electron microscopy

with trilaminar membranes, (Robertson, 1959), *right*, as seen by light microscopy. 1, extracellular space; 2, cytoplasm, 3, nucleoplasm; 4, mitochondria; 5, the nucleolus; 6, lysosomes; 7, membrane wall; 8, ribosomes; 9, cisternae; 10, invagination of cell membrane; 11, mitochondrial cristae. The Golgi body should be added to the *left* diagram.

However, when shorn of artifacts, the structure of the cell reverts to what was believed in the early 1890's (Figure 66), when Golgi (1898) decorated the cytoplasm with his body. Strangely, the nucleolonema of Estable and Sotelo, (1951) was also seen as a skein-like structure in the nucleolus, although the electron microscopists see it as an unstructured blob.

Compartments in cells

<i>Compartments</i>	<i>Comments</i>
<i>Light</i>	
Extracellular	fluid in life; limited by cell membranes
Cytoplasm	fluid in life; contains most of the solutes and substances suspended in the cells; particles, often unidentifiable,
Mitochondrioplasm	fluid in life; its chemical composition is unknown and may be unknowable
Nucleoplasm	fluid in life; nucleolus is moving within it; translucent
Nucleolus	contains moving nucleolonema
Pars amorpha	translucent; no apparent structure
<i>Electron microscopy</i>	
Between two layers of unit membrane	the trilaminar appearance is an artifact
Cisternae of the endoplasmic reticulum	the reticulum does not exist, and the cisternae are spaces between the cytoplasmic precipitate
Golgi lamellae	the Golgi body does not exist, and the lamellae are spaces between the cytoplasmic precipitate
Space between outer and inner mitochondrial membrane	an artifact, as that in the unit membrane
Space between the nuclear membranes	there is one, not two, nuclear membrane layers

Table 86. The upper compartments can be seen in living cells by light microscopy, while the lower ones require heavy metals to be deposited on dead tissues, and can only be seen by electron microscopy, often in subcellular fractions.

There is much literature on ‘compartmentation’, - that is, that different biochemical reactions occurring only within particular organelles. A list of the fluid compartments believed currently by cytologists is given (Table 86). One must distinguish between the states of ions and molecules (Table 8), the phases outside and within the cells (Table 7), and the compartments (Table 86). Metabolic maps indicate hundreds of enzymes reactions, (Greenberg, 1967-1975; Salway, 1999), and there are, of course, many non-enzymic reactions in cells. The current consensus recognises about 10 compartments, while the light microscopist sees 5 (Table 86). It is thus clear that whichever view one takes, the cell compartments are heavily overcrowded. The overcrowding is even more severe for unicellular organisms, which have only one compartment to house virtually all chemical reactions in the living cells. Thus the argument that compartmentation is a necessary condition for oxidative phosphorylation to occur only in mitochondria, or protein synthesis only in ribosomes, loses some of its force.

With these reservations, one may conclude that the cell on the right (Figure 66) describes the anatomy of the generalised living cell more accurately than that on the left.

Section C

The Biology of The Living Cell

Chapter 38

The Properties of Living Cells

It is useful to summarise the properties of living cells which are generally agreed. They should be regarded as the minimal number of essential properties (Table 87).

Properties of living cells

Cells are translucent
They arise by division of a parent cell
They take up oxygen, and release carbon dioxide
They have nuclei and cytoplasm, and most have cell membranes and mitochondria
Particles in the extracellular compartment, cytoplasm, nucleus and nucleolus, move
Cells require substrate to live
The membranes around the cell, the nucleus and the mitochondria are semipermeable
The extracellular compartment, the cytoplasm, and the nucleoplasm, are chemically different, have different total chemical activities, and there are potential differences between them
The cytoplasm has a low viscosity
The extracellular compartment has a high Na^+ and low K^+ activity, while the cytoplasm, has a low Na^+ and high K^+ activity
There are enzyme activities in cells, not necessarily equally distributed in all compartments
Anabolism and catabolism occur in cells
Water is the major component of all animal cells
Some cells can take in and expel macromolecules and particles
Changes in one compartment can affect the chemistry of a neighbouring compartment
Cells grow
Cells react to their environments
Some multiply
Cells resist their own destruction
Cells die

Table 87.

Chapter 39

Water

Water comprises the largest single constituent of the body, but its exact proportion in living organism can not be known, because of the difficulties in measuring it. All measurements involve a number of important but not always warrantable assumptions (please see Chapter 5). Furthermore, the concentration of water in the whole body is not the same as that in each constituent organ, tissue, cell and organelle. There are several important textbooks on the properties of water, its relationship to DNA and protein, cryobiology, and regulation of intracellular movements (Harned and Owen, 1958; Klotz, 1962; Eisenberg and Kautzmann, 1969; Mentré, 1995; Ball, 1999 and Mentré, 2001). I am grateful to Professor Pascal Mentré, of the Institute de Biologie Physico-Chimique, Paris, for the extremely useful discussions on this topic, although she does not share my views about many of the elements of it.

Many of the studies of water in biological systems have involved purification, evaporation, extraction, freezing, dehydration, measurements of conductivity and electron microscopy. These procedures change the following properties of water: its temperature, activity, concentration, rate of diffusion, viscosity, location, osmotic pressure, pH, heat conductivity and heat capacity. Therefore one needs, perforce, to examine *empirically* the properties of water in living cells.

The following types and components of water many be recognised in living cells:

- (i) ‘pure’ water;
- (ii) isotopes of water, including deuteriated and tritiated water;
- (iii) H^+ , OH^- and other dissociated ions;
- (iv) water believed to form shells around such ions as K^+ , Na^+ , Ca^{2+} , Cl^- , etc;
- (v) water associated with DNA, ATP, proteins and other macromolecules (Franks and Mathias, 1982; Ling, 1992; Westhoff, 1993; Nakasako, 2001; Cserelmy, 2001);
- (vi) water adherent to any chemicals or particles in tissues, due to its solution, charge, chemical affinity or Van der Waals forces. Of course, solubility is relative; the solubility of any substance in water or vice versa, can be measured. They range from considerable solubility and ionisation to total insolubility;
- (vii) water at the surface of skin and the respiratory tract, in contact with moist air.

This is rarely in equilibrium with its environment;

(viii) bulk water in the extracellular fluid, in the cytoplasm, and in the nucleoplasm, not adjacent to membranes;

(ix) water adherent to both sides of membranes.

The various kinds of water in cells must be considered to be in dynamic equilibria. The quantity of water in each compartment determines the concentration of each of the soluble components within that compartment.

The earliest assumptions were that all water was free in the cellular compartments, but Ling, (1992) has brought abundant evidence that this is not so. Its very reactivity make it highly unlikely. It is a fact of physical chemistry that any soluble material added to water *diminishes* its chemical activity.

Tissue can be dehydrated by heating, by low pressure often with freezing, or by the addition of chemical reagents. Heating causes: evaporation of water; evaporation of other volatile substances; breakdown of macromolecules; denaturation of proteins; acceleration of reactions; increased rate of diffusion; changes of equilibria.

Low pressure also extracts volatile substances in addition to water. Dehydration is best done at room temperature or cooled, so that micro-organisms do not have time to grow in the tissue, before it is dehydrated. Freeze drying is slow.

Tissue may be dehydrated by such chemical reagents as ethanol, acetone glycerine or silica gel. Any dehydration: increases the concentrations of all solutes in each phase; eventually precipitates solutes; causes shrinkage of all phases; increases cytoplasmic and nucleoplasmic viscosity, depending upon their initial water contents; denatures proteins; kills the cells; inhibits microbial growth; stops intracellular movements.

From a chemical view point, in whatever states water exists in tissue, there will be a particular change in free energy in the tissue, when the water is extracted. Obviously, the minimum amount of that energy would be the same whichever method was used to extract the water, although very different quantities of energy may be applied, depending upon the method of dehydration used. The widespread belief that only water is lost from a tissue heated for 2 hours or more to constant weight, ignores all these other factors.

Chapter 40

Enzymes

Enzymes carry out metabolism, and synthesise all substances except water and ions, which are found in the diet. One would like to know the enzyme activity in particular phases within the whole organisms, but their properties have been studied either in crude homogenates of tissue, or after they have been extracted. In the latter circumstances, they have been studied in simplified systems, containing substrate and co-factors, or after they have been crystallised. The use of the simple system can tell one about their properties, such as the range of substrates upon which they can act, and the co-factors and pHs, which will increase or decrease their activities. They can not give accurate information about the enzyme activity in a particular organelle, because one does not know the effects of preparation procedures (please see Chapter 15).

An enzyme, which has been purified, can be crystallised and sequenced, and the assumption is always made that the chemistry of this crystal in 100% concentration is usually the same as it is in the dilute, precise, but changing, physicochemical states in the tissue. Enzymes can be studied in many different states (Table 9), and the ions, which are known to activate and inhibit them have been listed (Table 11), but many other factors also affect their activity (Table 89).

Variables determining a local enzyme activity

<i>Parameter</i>	<i>Comment</i>
(i) quantity of enzyme	not usually measured; may be unmeasurable
(ii) its rate of synthesis	unknown, and possibly unknowable
(iii) its rate of degradation	unknown
(iv) its stability <i>in situ</i>	not known
(v) quantity of substrate available <i>in vivo</i>	not known <i>in intact cell</i> ; is controlled <i>in vivo</i> and <i>in vitro</i>
(vi) rate of binding with substrate	can be measured in simple systems
(vii) degree of binding	can be measured in simple systems
(viii) the reversibility of binding	can be measured in simple systems
(ix) the presence of natural activators and inhibitors	soluble ones may diffuse during procedure; in experiments <i>in vitro</i> , those known are added in suitable concentrations
(x) the equilibria of the reactions	often controlled by concentrations of products

<i>Parameter</i>	<i>Comment</i>
(xi) the presence of feed-back mechanisms	due to hormones
(xii) the solubility of the products	which are different from the reactants
(xiii) the osmotic pressure exerted by the products	the products may exert different osmotic pressures from that of the reactants
(xiv) the rates of excretion of the substrates, co-factors and products of the reactions	these are not usually known individually, and may interact during excretion
(xv) the subsequent chemistry of the products	this is usually known, but not the rates of their reactions
(xvi) the local pH	may not be measurable
(xvii) the degree to which the reaction is open or closed	this is extremely difficult to determine and varies during a procedure
(xviii) the local free energy	this is difficult to measure except in simplified systems

Table 88. *Many of these variables are unknown in a particular phase of a whole tissue, since the isolation of the enzymes themselves alters these properties. The question of the relevance of findings in simple systems to whole systems in vivo is problematical.*

Although some enzymes are widely considered as markers for subcellular fractions, there are considerable problems with such generalisations. They can be seen in examining the early publications about enzyme localisation, (Mirsky and Osawa, 1961; Dixon and Webb, 1964). These are as follows. Firstly, the former authors talk about enzymes ‘mainly’ present in a particular fraction, that is, even under the optimal conditions for demonstrating the enzyme in the fractions under study, the same enzymes may also be found in other fractions. Secondly, there is a list of enzymes not present mainly in one fraction. Thirdly, there are very large and significant differences between different tissues and different species on the subcellular localisation of enzyme activities, using similar procedures to measure them. Fourthly, some enzyme activities are marked as ±, which means that the authors could not decide whether or not the enzyme activity was present in that fraction. Fifthly, the presence of an enzyme activity in a fraction in which it is not expected to be found, is labelled, ‘contamination’ and further agents and reagents are used until the ‘contaminant’ is removed. This should not be acceptable in procedures, such as all subcellular fractionations, in which each step, such as subjection to a physical agent or another chemical, is empirical. Biochemists should have to justify chemically all the steps, the agents and the concentrations used. In biochemistry, the

situation is worse than in cookery where recipes are often designed to *preserve* the volatile chemicals imparting smell and taste to dishes, while no such respect is accorded to the volatile chemical activities, enzymes, sera, growth factors, etc, in the biochemical procedures. It may be impossible to find out the location of enzymes by using such enormous amounts of energy and powerful reagents, as are employed in the separation of subcellular fractions. Sixthly, the latter considerations make likely the conclusion that the subcellular locations as currently understood are uniquely the consequences of the particular procedures used. This leads to the final consideration. Supposing that in the living intact cell in the healthy animal, the process defined as oxidative phosphorylation occurred in the nucleus and the cytoplasm, but not significantly in the mitochondria, the educationists would merely have to change the headings on the list of enzyme locations, and re-route the arrows on their diagrams. This would not necessarily look so neat in the examination scripts, but it may merit the badge of integrity.

The nearest one can approach the enzyme activities in living cells is to measure them in naturally occurring single cells, such as red blood cells, white blood cells, sperm, ova, or cells which can be separated using relatively little energy, (Table 32). An enzyme activity can be measured either using a microdivider, or a microscopic technique, such as a split beam spectrophotometry.

It would probably be fair to restate that it may be extremely difficult, or even impossible, to know the activities of more than very few enzymes in intact cells in living animals. Knowledge of what they do overall in metabolism and excretion may be the nearest one can reach towards this aim.

Chapter 41

Transmembrane Potential Differences and Pumps

It is unlikely that intracellular pipettes can measure transmembrane potential differences accurately, because it is difficult to know what contribution other sources within the recording system, the voltage clamp and the stimulating circuits, make to the voltage indicated on the oscilloscope (Chapter 16). However, potential differences have also been recorded from giant axons of cephalopods using much larger electrodes (Hodgkin, 1951; Huxley and Stämpfli, 1951; Eccles, 1953, pages 1 - 64). The significance of these measurements was enhanced, when it was shown in giant axons that the transmembrane potential difference was a function of the K^+ gradient, and the action potential was a function of the transient Na^+ gradient (Hodgkin and Huxley 1952a,b).

These generalisations were often made by substituting choline or Li^+ salts for Na^+ , Rb^+ for K^+ , and hepes buffer for several physiological cations. These substitutions imply that the choline, Li^+ , Rb^+ or hepes have no 'specific' actions themselves, other than replacing the other cations. Such assumptions seem to be very dangerous, especially as they are testable. However, the use of such substances is part of a much wider looseness of thinking, in which biologists - especially pharmacologists, - assume that enzyme and drug 'blockers', inhibitors, agonists and antagonists, have no effects other than those on the simple pure systems, to which they have turned their attention.

Although one measures and talks about the potential differences across the cell membrane, it may originate from: (a), the recording system; (b), between the extracellular compartment and the cell membrane; (c), across the membrane only; (d), between the membrane-cytoplasm interface; (e), the internal interface of the membrane and the bulk cytoplasm; (f), injury potentials caused by the micropipette; (g), the effect of the fluids contained in the micropipettes on any of (a) to (f). It would seem to be almost impossible to design experiments to distinguish between each of these possibilities, so the measurements with voltage micropipettes (including, ion sensitive micropipettes) must be regarded as qualitative.

Skou, (1957) showed that homogenised peripheral nerves contained an ATPase, which was activated by Na^+ and K^+ ions in concentrations within the physiological range. A similar enzyme was found in many other, mostly nervous,

tissues (Askari, 1974; Stein, 1988; Skou, Nørby, Maunsbach and Essman, 1988). Gardós, (1954) had shown that if red cells were placed in hypotonic solutions, the haemoglobin left them, and they appeared like ‘ghosts’; ATP could then enter them. Since then, ‘ghosts’ have been used as models of pure membranes. The Na^+ activated ATPase has been shown to be inhibited by 1-2.5 μM ouabain (Whittam, 1962; Wheeler and Whittam, 1962). It has also been shown that the injection of ATP into giant axons of squid increased the efflux of Na^+ (Caldwell et al, 1960). $\text{Na}^+ \text{K}^+$ ATPase has been purified and characterised (Jorgensen and Skou 1971).

The experiments on mammalian tissues, have produced results compatible with those from the cephalopod giant axons (for example, Coombs, Eccles and Fatt, 1955 a,b; Hillman and McIlwain, 1961; Hillman and Hyden, 1965), but not comprehensively enough to validate them yet. These have led to the following generalisations:

- (i) all cells have resting transmembrane potential differences, which are a function of the K^+ gradients between the extracellular space and the cytoplasm;
- (ii) the Na^+ ions are distributed opposite to the transmembrane potential difference. If energy is taken away from the system, the Na^+ ions diminish their gradient and the potential difference across the membrane disappears;
- (iii) the distribution of the Na^+ ion is due to a ‘pump’ situated in the cell membrane, which also keeps the cytoplasmic K^+ higher than the extracellular K^+ ;
- (iv) this pump is situated *within* the cell membrane and is a $\text{Na}^+ \text{K}^+$ ATPase which can be partially inhibited by ouabain and other metabolic inhibitors. Thus, incoming Na^+ activates an enzyme causing the release from ATP of energy, which itself expels the Na^+ by a feed-back mechanism;
- (v) whereas there is a potential difference across all cell membranes at rest, some cells, notably nerve and muscle cells, can be excited by electrical stimulation, high K^+ , hypoxia, and physical damage. In this circumstance, they produce a transient action potential difference. From the outside, their voltage changed from -70 to -100 mV to +20 to +50 mV. These transients are recorded by intracellular pipettes. As the action potential difference falls to 0 mV, the cells are ‘depolarised’ but they then ‘overshoot’ to positive voltages. After the action potential falls back to its negative value, it often hyperpolarises by -10 to -20 mV, which is interpreted as the membrane ‘pumping out’ the Na^+ ions, which have entered the cells during the action potentials. In mammals, the whole event takes about 1 msec. The amplitude of the

action potential difference is constant for a particular nerve, and there is a certain delay before a new action potential can be generated. The action potential is an ‘all-or-none’ phenomenon, with a threshold of stimulation.

(vi) the maximum amplitudes of the action potentials are a function of the logarithm of the Na^+ gradient across the cell membranes;

(vii) the rising phase of the action potential has been interpreted in giant axons as being due to an increased conductance to Na^+ ions and an inhibition of the Na^+ pump, and the falling phase due to an increase in K^+ conductance. When these two are added together, they produce a signal, an action potential (Hodgkin and Katz, 1949; Hodgkin, Huxley and Katz, 1952).

These experiments and the concepts arising from them merit a few comments, using the same headings:

(i) it is extremely likely that there is a potential difference between any two adjacent phases of the cell, because they each have different chemical compositions, and are likely to have different total chemical activities. It is very unlikely that one can know the activities *in vivo* because of the inescapable artifacts of intracellular pipettes and electrodes (Chapter 16.) Even if one knew the concentrations of all the chemical species at one time in such dynamic systems, their interactions are liable to alter their activities, and it is highly unlikely that they can be separated without altering them both significantly.

Furthermore, the elegant experiments carried out by Hodgkin, Huxley, Keynes, Stämpfli, Baker, Caldwell and others on cephalopod axons, by Whittam and his colleagues on red cell ghosts, and by Ussing and his school on frog skin, have generally been assumed to be applicable to all cells, including excitable ones, in all mammals. The giant axons are from cold blooded animals and their membranes are much thicker than the ‘unit membranes’. The red cell ‘ghost’ is devoid of haemoglobin, and therefore presumably of many other small molecules. The frog skin is widely used to show ‘active transport’, but it contains layers of many elongate cells and connective tissue, each cell having two long faces so it can not be used as a physiological model for a single cell membrane.

(ii) the findings that the gradients of Na^+ and K^+ and the potential differences across membranes all fall considerably, when the cells are deprived of oxygen or substrate, or if they are poisoned by metabolic inhibitors, show that the mechanisms generating them depend upon the ‘healthy’ metabolism of the cells. They do not

necessarily mean that the gradients and the potential differences are related, although this reasoning is often used. This is an argument from *post hoc* to *proper hoc*:

(iii) it is not at all surprising that if the Na^+ gradient is found experimentally to be distributed in a way opposite to that expected from the potential difference across the membrane, and that both of these change in the expected way in ‘adverse’ conditions (please see (ii) above), the concept of an energy-dependant ‘pump’ should be considered. Yet Ling, (1962; 1992; 2001) has shown in muscle and other tissues, that metabolic inhibition does *not* cause the Na^+ gradient to fall rapidly. He has also shown that the amount of energy to maintain such a ‘pump’ is well beyond the measured metabolism of cells. His experiments over many years are brilliant and quite unequivocal, and it is unfortunate that pumpers have shown little interest in them. Over the years, he has developed the ideas of Troshin, (1966). Ling, (1992) regards the mixture of chemicals, particularly ATP, of which the cytoplasm is composed as acting as a cation-exchanger, which prefers K^+ to Na^+ , and this preference varies with the precise chemistry of the cytoplasm at each moment.

If one adduces a pump to expel Na^+ from the cytoplasm to the extracellular compartment, because the Na^+ is distributed against the expectations of the resting membrane potential difference, then the other ions such as Ca^{2+} , phosphate, bicarbonate, etc. must each have their own pumps;

(iv) the idea that the Na^+ pump is located *within* the cell membrane (Skou, 1975) is extremely unlikely to be true (please see Chapter 20), for the following reasons:

(a) ATPase is such a large molecule (Maunsbach, Skriver and Jorgensen, 1979; Skou and Nørby, 1979; Sachs, 1999) that electron microscopists should have seen it in the membrane. They do not;

(b) the enzymes and precursors of ATPase should be present in the membrane, and all the metabolic pathways associated with them would be there, or close by. Would the Davson-Danielli, Robertson, or Singer-Nicolson models allow them enough space for all these large molecules?

(c) the belief that the Na^+ pump is in the membrane comes from the finding that Na^+ activated ATPase is located in the microsomal fraction (Wheeler and Whittam, 1962). Of course, this does not show that the ATPase was originally in the cell membrane. Nor does the finding that the ATPase was sensitive to Na^+ in physiological concentrations prove the hypothesis that

this is the mechanism in the intact cell; it may be unprovable.

(v-vii) the ionic theory, to explain resting and action potentials, has been very well worked out by the Cambridge School, and may well be right, but it does depend crucially on the assumptions that the ions used to substitute for Na^+ , K^+ , etc. have no effects other than to deprive the tissue of the latter ions.

Finally, Na^+ , K^+ activated ATPase is partially inhibited by ouabain (Schatzman, 1953). There is much literature on the effects of other substances on that part of the enzyme which is 'ouabain-sensitive'. If the inhibition were genuinely specific, either there are more than one inhibitory site, or increasing the concentration of the inhibitor should eventually produce complete inhibition. Those who study the ouabain sensitive part of the enzyme activity must assume that this proportion does not change with the variables of the experiments.

It should be added that in the many syncytia in the body, in which the individual cells lack their own personal cell membranes, (Table 25), it is quite likely that the syncytial cytoplasm has the same chemical activity as the extracellular compartment. Therefore no resting membrane potential difference would be recorded, when one penetrated the syncytial cytoplasm. The neuroglia would be a typical example of such a situation (please see Chapter 34).

A very interesting preparation of isolated cell membranes was devised by Hyden, Cupello and Palm (1987). They cut open neurons (Cummins and Hyden, 1962), washed out the cytoplasm, and spread the membrane over very fine holes in plastic. They perfused both sides of this preparation, and studied the rates of movements of ions and aminoacids across the holes. The problems here are that (i) they did not know how the isolation of the membranes affected their permeability, (ii) they could not know how well the membranes sealed the holes, (iii) the membranes would leak where dendrites were pulled off them; (iv) the effect of the very narrow plastic holes was difficult to assess.

Chapter 42
Homeostasis

The extracellular phases (Chapter 19) are perfused by fluids of rather similar chemical composition (Figure 12). Outside the cells are the following other extracellular fluids: air; bronchial secretions; alimentary fluids; perspiration (sensible and insensible); spermatic fluid; vaginal secretions; milk. All these are part of the homeostatic systems, which keeps the chemistry of the extracellular fluids remarkably constant. Sensory receptors, such as the chemoreceptors, sensitive to CO_2 , O_2 , pH and osmotic pressure, are located in the walls of blood vessels, and these act with the hormones.

The constancy of the chemistry of the extracellular fluid in healthy individuals means that the cell membranes make the cytoplasm even more constant. More important it means that in life, the transmembrane gradients are extremely constant. This may be interpreted in one of two ways. The feedback mechanisms, including digestion, excretion, detoxification and metabolism, are so accurately tuned in health, that they resist significant changes. However, there is a huge number and complexity of the individual reactions in metabolic pathways and cycles, so that any single reaction may be accelerated or decelerated by changes in free energy, presence of co-factors, arrival of substrate, or removal of product. Therefore, one should marvel at the efficiency and rapidity of feedback. For example, action potentials repolarise in a fraction of a second, the respiratory reflex takes less than half a second, reflex response times are fractions of a second, violinists and cricketers can tune their ears and eyes within 100s of milliseconds.

It would be reassuring to think that one could measure the rates of the feedback mechanisms accurately. This is true for such phenomena as the respiratory reflex, the sensory response or the action potential, but it is not usually true for the chemical reactions underlying them. Many of the latter are measured either in poikilotherms, in which reactions are known to be slower than in mammals, or in disrupted tissues, in which measurements of rates of reactions can not be considered to reflect the rates in vivo accurately (please see Chapter 15).

Chapter 43

Movements

Organisms, organs, cells, organelles, particles, molecules and ions, all move. Movements of cells may be classified as: movements of whole cells relative to each other; movements in and out of cells and organelles; movements within single organelles (please see Aspects of Cell Motility, 1968; Stebbings and Hyams, 1979; Warner, Satir and Gibbons, 1989; Inoué and Spring, 1997; Hyams and Lloyd, 1998).

Movements of cells can be studied by a large range of techniques, as follows: physicochemical experiments on simplified systems; colpoids of artificial mixtures of water, salts and oils; artificial (usually lipid) membranes; observations on living unicellular organisms, tissue culture, plant and fungal, cells; implanted ‘windows’ on webs of feet, brains, mesenteries and rabbits’ ears; microscopic observations on blood cells and sperm; injections into cells; ‘supravital’ stains; microspectroscopy; measurements after haemodialysis and peritoneal dialysis; use of radio isotopes; light microscopy; time lapse photography. Of all these procedures, observations on living cells are the most fruitful, but the nearest one can approach to movement of ions and small molecules in the intact animal is by examination of the clinical biochemistry of haemodialysis and peritoneal dialysis, when they are operating satisfactorily. *Obviously*, movements can not be studied in dead, fixed, frozen, denatured or dehydrated tissues; even studies before and after any apparent movements are highly problematic. This chapter should be read after reflection on Tables 8 and 44. Different types of movement are listed (Table 89).

Causes of movements

<i>Movement</i>	<i>Comments</i>
1. <i>Natural</i>	
Diffusion	caused by concentration gradients
Brownian movement	see below
Streaming	cause not known
Mitochondrial movement	cause not known
Convection	temperature differences in cells
Particle movements	believed to be due to molecular motors
Nuclear rotation	cause not known
Passive movements	caused by differences of pressure and body movements

Movement	Comments
Ionic movements	caused by potential differences
Cell division	caused by fertilisation or pricking
Free energy changes	caused by metabolism
Osmosis	caused by differences of osmotic pressure
Circulation	from heart, blood vessels, lymphatics, choroid plexus, ciliary body
Metabolism	changes the chemical activities in cells
Biosynthesis	changes the chemical activities in cells
Ciliary movements	found in upper respiratory tract and Fallopian tubes
Secretion and excretion	caused by precipitation and expulsion from cells
Filtration	in kidneys and choroid plexuses
Perspiration	involves evaporation of water
Fertilisation	swimming of sperm
Death	redistributes fluids and solutes; allows bacterial invasion
2. <i>Experimental</i>	
Fixation	denatures proteins; changes their solubilities
Dehydration	decreases the solubilities of solutes
Homogenisation	mixes natural compartments
Freezing	decreases the solubilities of solutes
Inhibitors	alter the equilibria of reactions

Table 89.

Malone, (1991), Wheatley, (1993) and Agutter, Malone and Wheatley, (2000) have argued powerfully that the derivation of the equations of diffusion from Fick's Law was wrong, partly because it was originally calculated for the dissipation of heat in solids, not the diffusion of ions in liquids. Their arguments are clearly worthy of examination in detail, but I am not sure whether they deny that diffusion occurs as an *ionic and molecular phenomenon*. That is to say, if one adds a soluble or particulate dye to one end of a bath, it will gradually colonise the whole bath, and one can measure the rate at which this occurs quite empirically. One can alter the temperature, the viscosity and the chemical contents of the bath, and this will alter the rate of diffusion of the dye as measured.

However, there are problems with the applicability of all such measurements. Most rates of diffusion are measured in aqueous conditions and at low concentrations. One can not know or calculate in a cell, what all the other constituents of the

cytoplasm or nucleoplasm will have on the rates of diffusion. As soon as other reactive species are added to any mixture, the effects of diffusion are affected; these can be measured rather than calculated. One does not know how the chemicals react with each other in the very chemically cramped conditions of a cellular organelle. The use of such terms as Reynolds numbers to measure viscosity in narrow tubes, or the designation of an activity coefficient, when ion activities are found to be different from their calculated concentrations, are both tacit admissions that simple models can not be applied to elucidate the conditions inside cells. Therefore, one must rely on measurements rather than on calculations, involving many assumptions, even when one is blessed with a computer.

It is widely believed that membranes can 'facilitate' the movements of ions, molecules or particles across membranes. For example, it is thought that 'carriers' assist the movement of ions across membranes and that the Golgi apparatus helps protein to reach the endoplasmic reticulum. It has already been argued that the addition of a macromolecular carrier can not assist the passage of an ion across a fine pore in a membrane, because it makes the ion bigger. The *only* way a membrane could 'facilitate' the passage of an ion, molecule or particle would be if the membrane contained within its wall the chemical machinery to react with the species, impart energy to it, and dissociate from it. All this would have to be done *more* rapidly than diffusion would have permitted, if no membrane were present. Otherwise, all membranes, even semi-permeable ones, especially if they contain pores, could only slow down ions, molecules or particles.

The many differences of chemistry on either side of a membrane may induce passage across it. For example, if the cytoplasm constitutes a chemical mixture, which has a greater affinity for K^+ than the extracellular mixture does, the K^+ will cross the cell membrane into the cytoplasm as quickly as its semi-permeability will permit. If the hydrostatic pressure in the circulation is greater than that in the cells, oxygen will diffuse along the pressure gradient in to the latter. These movements require no machinery in the cell membranes themselves although they occur across them.

It is worth bearing in mind that Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Cl^- , aminoacids, glucose, etc, which are believed to be carried through membranes, pass through dialysis tubing, which presumably, does not contain any carriers in its walls. One should only claim that carriers are present in biological membranes, if, either ion can not cross the

membranes without carriers, or if they move more quickly than they do across dialysis tubing. Unless that can be demonstrated, the concept of ‘carrier’ becomes redundant.

Furthermore, haemodialysis and peritoneal dialysis are used for exchanging Na^+ , K^+ , Cl^- , Ca^{2+} , Mg^{2+} , acetate, bicarbonate, lactate, glucose, urea, creatinine, aminoacids, inorganic phosphate and toxins, with the membranes of the kidneys of patients with renal failure (Geiger, 1931; Addis, 1950; Henderson et al, 1986; Bonomi and Berland, 1995; Palmer, 1999). This indicates that these ions do cross both dialysis membranes *and* intact renal tubules, although, of course, the permeability of the latter membranes may be affected by renal failure. The difficulties of some of the current terms are listed (Table 90).

Problematic terms used to describe movements

<i>Terms used</i>	<i>Why they are problematic</i>	<i>More accurate terms</i>
Transport	means minimally movement only	movement
Active transport	implies that energy must be supplied continuously; could be due to higher affinity of X for a particular phase	higher concentration in one phase; apparently against the electrochemical gradient
Exchange transport	implies that the ions exchange along the same route; is a consequence of Le Chatelier’s Principle	
Carrier transport	implies that a carrier is present, in the membrane and can ‘facilitate’ passage across the membrane	cells can become saturated with X; systems have finite volumes
Transmembrane transport	implies that a mechanism other than, or in addition to, diffusion, causes the movement	movement across the cell membrane
Phagocytosis and Pinocytosis	it is not known how widespread these processes are outside the reticulo-endothelial system, nor their mechanisms	
Exocytosis	this is a term used both to describe the phenomenon and imply the mechanism	leaving a cell
Endocytosis	this is a term used both to describe the phenomenon and imply the mechanism	entering a cell
Membrane transport enzymes	implies that the enzyme systems are present in the membrane in the living intact cells, because they are found in microsomal fractions	
Diapedesis	movement of blood cells across capillaries is observed, but its mechanism is not known	only observed in blood capillaries

<i>Terms used</i>	<i>Why they are problematic</i>	<i>More accurate terms</i>
Axonal transport	it has not been shown to be different from diffusion, occurring along axons	diffusion

Table 90. Difficulties with some terms used in connection with movement. *Most of these terms are descriptive and do not indicate mechanisms.*

So many variables can alter the movements across membranes, which are believed to be semi-permeable that is, ‘selectively’ permeable. The main places at which movements can occur are across pores in them. The rates of passage will depend upon the dimensions, incidence and charges on the pores. This presents a difficulty, namely that one measures the size of the pores by diameters of the hydrated or non-hydrated ions they permit to cross or prevent crossing, (please see Chapter 31). These pores are generally less than two orders smaller than the nuclear pores, and one to two orders larger than the measured diameters of ionic channels. However, one must be beware of the latter measurements, because, firstly, they are made on electron micrographs and, secondly, the visible pores are artifacts.

Further, the very confident statements about charges in channels and pores belie the misfortune that they are too small to be measured directly. However, it seems reasonable to accept that there are holes of less than 1 nm in diameter in dialysis tubing, black lipid membranes and cell membranes, across which diffusion may occur, while the rest of the membrane is impervious. All the other movements may be of the types listed in Tables 91 and 92, without the necessity for carrier transport, exchange transport or active transport. Transmembrane differences can determine the activities of ions and, therefore their movements.

A few more comments are apposite about the terms used in connection with movements (Table 91). The word ‘transport’ implies that there is some mechanism which moves or carries X, other than diffusion, Brownian movement or streaming.

Exchange transport is regarded as the encouragement of the flux of ions from the inside of cells by the increase of external ions, and is a criterion of ‘activated’ transport (Wilbrandt and Rosenberg, 1961). Of course, if a concentration increases in one phase, water would enter, and would change the equilibria across both sides of a membrane to which the ions were permeable.

The extent of phagocytosis outside the reticulo-endothelial system is simply not known, nor has pinocytosis been shown unequivocally in mammals, except in the

absorption of fat in the small intestine. The movement of particles in and out of cells (endocytosis and exocytosis) is seen in tissue cultures, in epithelia and in protista, but it is not known how widely they occur in the intact animal. How different are they from phagocytosis during bacterial infections?

Membrane transport enzymes, mainly ATPases, are believed to be housed *within* the cell membrane (please see Chapter 20). Diapedesis is a process in which blood cells cross the capillary walls to tissues, both ill and healthy. It can be seen by time lapse photography in the webs of frogs' feet and in windows in rabbits' ears. The blood cells are so large compared with any pores in the cell membranes that it is presumed either that they enter between cells, or that the membranes of the latter cells have sealing mechanisms.

In addition to classifying movements phenomenologically (Table 90), one may also classify them by their locations and destinations (Table 91).

Types of cell movements

<i>Parts of cell</i>	<i>Types of movement</i>
Passive movement of whole cell	due to changes in body posture, tissue pressure, circulation, inhalation, intra-abdominal pressure, muscle contraction
Whole cell movements	unicellular organisms; cells in growth and development; cells in tissue culture; fertilisation; meiosis; mitosis; shedding of epithelial cells
Extracellular movements	by cilia, flagella, sperms
Across the cell membrane	diffusion*, osmosis*, secretion, pinocytosis; phagocytosis; entry* and exit* of particles; diapedesis; entry of viruses, microbes, parasites and toxins
Intra-cytoplasmic	diffusion*; Brownian movement*, streaming*, binding*, convection*, mitochondrial movement; vacuolation*
Nuclear membrane	changes in shape; movements across the nuclear membrane; tissue pressure
Nucleolus	movement of the nucleolonema; change in shape of the nucleolus
Axonal transport	away from and towards the neuron cell body

Table 91. *Some of these movements* also occur in colpoids.*

A few comments are appropriate about this classification. The passive movements of whole cells due to their being surrounded by other cells which compress, mix, stir and heat them. These have often been ignored in considerations of 'transport' of small molecules. This is as important as the homogenisation, centrifugation, shaking, and warming, tissues during biochemical experiments and

haute cuisine.

Whole cells move in circulations, growth, fertilisation, cell division, etc. All of these phenomena have driving forces and mechanisms, which are not yet understood. The movements of cilia, flagella and sperms are generally believed to require energy, and are completely different than the movements of particles, ions or molecules. There are large academies of scholars studying the movements of the latter across cell membranes, and within the cytoplasm. It must be emphasised again that these phenomena can be examined by histological techniques, but the *rates* and *equilibria* can not be studied in disrupted or dead tissues.

There are movements across the nuclear membrane, of particles, molecules and ions, generally considered to pass through the nuclear pores. Alas, if the latter are artifacts, another route has to be postulated. The nucleolonema is also seen moving continuously in living tissue cultures.

The likely effects of metabolism on intracellular movements have been given little attention. However, it had two main kinds of effects. Firstly, it continuously changes the activities of many of the chemical species within the cells. Secondly, it changes the free energy available to reactions within the cells. After muscle contraction, a significant temperature rise is measurable. However, generally cellular metabolism occurs in too small locations to measure the effects of the many exothermic and endothermic reactions going on simultaneously within them.

Those movements which occur in colpoids have been marked with asterisks in Table 91, and it is evident that no biological explanation is needed for them. Their physical chemistry can be studied in simple, artificial, systems (Bütschli, 1894; Herrera, 1900; 1928; Bastian, 1913; Fox and Dose, 1977).

Movements believed to be caused by molecular motors

Spermatogenesis	Activation of hearing
Anaphase of meiosis and mitosis	Memory
Polarisation of eggs	Cell shape changes
DNA sliding, hopping and looping	Cell crawling and migration
Ribosomal movement along mRNA	Melanosome movement
Gastrulation	Anterograde and retrograde axonal transport
Left to right asymmetry	Movement of varicosities along axons and dendrites
Movement of subcellular organelles	Muscle contraction

Intracellular targeting of proteins	Flagellar movements, including axonemal and flagellar
Immune defence	Ciliary movements
Secretion of granules	Neuronal growth
Pinocytosis	Movement of transmitters to synapses
Phagocytosis	Growth of cones in tissue culture
Endocytosis	Rhodopsin' trafficking'
Exocytosis	Wound healing

Table 92. Movements are believed to involve actins, myosins, tropomyosins, F⁻, ATPases, kinesin, factor G, RNA polymerase. (Valleé, 1998). See also Scholey (1990).

Molecular motors

In recent years, many intracellular movements have been attributed to the existence of 'molecular motors', which are believed to propel intracellular organelles and particles around the cytoplasm. Some of these are listed (Table 92); (see, for example, Okabe and Hirokawa, 1990; Boyer, 1997; Bagshaw, 1997; Vale and Fletterick, 1997; Banting and Higgins, 2000). The idea that such motors could pull structures around probably arises from the following findings: (i) contractile proteins such as myosin, actin and tropomyosin have been detected in muscle in many animal cells, not all contractile (Tilney, 1975; Inoué and Stephens, 1975; Goldman, Pollard and Rosenbaum, 1976; Hitchcock, 1977). The proteins were found in unicellular organisms, cells in tissue culture, dividing cells, varicosities, extracted tubulin, cilia, flagella, microtubules, extruded axoplasm, etc. (Koenig et al, 1985; Koenig, 1986; Schnapp et al, 1986; Sheetz et al, 1987; Sammak and Borisy, 1988; Schroer et al, 1988; Amos and Amos, 1991; Kuznetsov, Langford and Weiss, 1992); (ii) when 2 mM ATP was added to actin filament complexes α_3 , β_3 and γ , they rotated anticlockwise (Noji et al, 1977; Adams, 1982). When 20 V current was applied to a weakly cross-linked polymer (PAMPS), it swelled in water to 45 times its original weight, and the gel moved forward at a rate of 4 mm / second (which is equivalent to 14.4 m / hour) (Osada, Okuzaki, and Hori, 1992). Earlier experiments had also shown that extracted actin and myosin shrank when ATP was added (Ernst, 1963 pages 336-337). Fluorescent techniques using 'specific' antibodies showed that there were networks of actin, myosin, tubulin, etc. in most cell bodies examined.

The belief that so many movements were driven by molecular motors needs

closer examination. Firstly, one had to assume or believe that the antibodies to the contractile proteins are specific to each one. If they were, the total number of fibres, filaments, cytoskeletal elements and contractile proteins, would be so crowded together, that relatively large particles simply could not dart around the cytoplasm in real time (please see Table 91). Secondly, the mitochondria, lysosomes, vesicles, varicosities and particles, would have to be attached to the molecular motors which were moving them. Such attachments are simply *not seen* by electron microscopy. Thirdly, the same filaments moving the structures would also have to be attached to the solid structures towards which they are moving, as the rope from a barge has to be attached to a horse or a man. Fourthly, if such a system were to have to move the particles in a particular direction, the ropes would have to be attached at the leading edge of the direction in which the particles were moving, and detached all the way round them, including the following edge. One awaits the demonstration of such attachments. The only circumstances in which possible attachments have been claimed to have been shown are the cross bridges in muscle and peripheral nerves (please see Chapters 36, 46). Fifthly, if the motors move particles in particular directions, the following conditions would have to apply: (a) there would have to be some mechanism within the particles moved, or the fluid in which they moved, to determine in which direction they were going to travel next; (b) another mechanism would have to make the particles move in that particular direction; (c) mechanisms (a) or (b) or another would have to determine which of the molecular motors attaches to particular parts of the particles moved (d) both the motors and the particles moved would have to have particular chemical confirmations or charges for them to attach; (e) there would have to be mechanisms instructing unused motors and part of particles to *detach* from each other, when the particles changed direction. It would be of interest to hear from motoring enthusiasts, who would disagree with the necessity of the mechanisms, or would propose verifiable hypotheses of how they might work.

Sixthly, the movements which Koenig, Sheetz, Schnapp, (*ibid*) and Cohn, Saxton and Lye, (1993), have shown in living cells (q.v.) are of relatively large spherical structures moving along, and adherent to, nerve processes. They appear completely different from the Brownian movement, streaming and mitochondrial, movements, visible in unicellular organs and tissue cultures. The latter have been mapped and appear random, when photographed.

Schroer and Sheetz, (1991a,b) gave the following reasons for believing that

intracellular movements of particles and chemical constituents do not move by diffusion. (a) particles in axons travel hundreds of millimetres, so the process would not be fast enough for the movements observed. However, tissue movements due to mechanical pressure, circulation, free energy, etc. would accelerate the movements seen in pure solutions. Even without these added stimuli, the majority of distances that would have to be moved by intracellular particles would be 1 - 30 μm , which would be achieved within a maximum of *minutes* by diffusion. They regarded movements of vesicles of less than 20 μm in diameter as 'slow,' at 2 μm per minute; (b) chromosomes are so large that they would move particularly slowly by diffusion. The suggestion will be made that during cell division, they do not move apart by being pulled by the spindles, or by diffusion; (c) they believed that movement from cell bodies to axons was a different process than moving in the opposite direction, and required different mechanisms, driven by two different motors. However, particles also move up and down a column of fluid randomly; (d) they pointed out that 'early' 'endocytic' and 'exocytotic' movements are not affected by such 'microtubule inhibitors', as colchicine, colcemide, nocodazole and taxol, which slow the movements of mitochondria. One must ask, what is a microtubule inhibitor? Does it cause them not to be stained, not to appear or not to exist? How can one inhibit a structure? Unfortunately, the answer to this problem must be equivocal. A substance, even in low concentration, added to a tissue, could make it move more rapidly or more slowly by (α) altering the viscosity of the cytoplasm, (β) altering its metabolism, (γ) altering its chemistry, (δ), changing the osmotic pressure. The substances added may have profound effects on the metabolism of the cells, in addition to, and perhaps causing, the changes ascribed to molecular motors.

Seventhly, the elegant finding that the addition of ATP causes F-ATPase to rotate usually in one direction (Noji et al, 1997), shows that such a system *can* move rather few molecules, but not necessarily in intact tissues. Nor can such systems necessary do external work. 'Motor' proteins such as dyneins, myosins, F-ATPase, kinesins, factor G and others are believed to be driven by ATP (Sheetz and Spudich, 1983; Banting and Higgins, 2000). It is useful to examine the brilliant experiments of Kinoshita, Yasuda and Noji, (2000), to see what assumption are implied in their execution. They prepared $\alpha_3\beta_3\gamma$ Escherichia coli subcomplexes. The $\alpha_3\beta_3$ cylinder was fixed at one end to a glass surface, and the α end by streptavidin to an actin

filament, which was labelled with a fluorochrome. When 0.6 μM ATP was added, the actin could be photographed under direct vision using a fluorescence microscope, rotating anticlockwise in a cycle, which took about half a second.

Their interpretation was that the ATP caused the motors to rotate. However, certain questions arise in respect of what the latter authors called the “currently popular but unproven view:”

- (i) why did they use $\alpha_3\beta_3\gamma$ complex from *E. coli*, in preference to a complex obtained from muscle or cilia?
- (ii) the $\alpha\beta\gamma$ protrudes in diagrams from the ‘inner mitochondrial membrane’ like church steeples, but the electron microscopists have never shown them sticking out of membranes in intact tissues. Why are they not seen? Indeed, references to molecular motors have fashionable diagrams, but rarely list ‘structure’ in their indices;
- (iii) have the authors considered the possibility that the energetic ultraviolet light, which induced fluorescence, could provide the energy for rotation? It has been shown that light makes the terminal bond ATP less stable (Hillman, 1966)?
- (iv) is it not necessary to test the effects of ATP on non contractile, as well as contractile proteins?
- (v) have control experiments been carried out adding ADP, AMP, K^+ , inorganic phosphate, and Ca^{2+} at low concentrations, to the motors?
- (vi) have the following possibilities been considered for the mode of action of ATP:
 - (a) the kinetic energy of the injection;
 - (b) the chemical reaction of ATP, ADP, AMP or phosphate with the streptavidin or the fluorochrome;
- (vii) according to the diagram of Kinoshita et al, (2000), the calibration makes the actin filament about 50 nm thick, yet the fluorescent microscope which shows it would have little more resolution than about 200 nm, even although fluorescence does apparently magnify structures. What is the explanation for this?
- (viii) could ATP have an electrostatic effect?
- (ix) there is no doubt that actin does work as it moves through the viscous

sarcoplasm, (Hunt, Gittes and Howard, 1984; Finer, Simmons and Spudich, 1984; Harada et al, 1987; Howard, 1994). Wang and Oster, (1998) have assessed the work done when the actin contracts, but, so far, they do not seem to have published the relationship between that and the work done when a whole muscle contracts. That is, how much external work can the system do, in addition to that necessary to move through a viscous solution?

- (x) the motor enthusiasts generally accept the existence of inner and outer mitochondrial membranes (Chapter 25), and the validity of the chemiosmotic hypothesis (Chapter 47). Therefore, it would be most interesting to hear their responses to the objections to the structures and hypothesis;
- (xi) the fact that contraction of muscle can be induced by high K^+ , low Ca^{2+} , hypoxia and electrical stimulation – all of which *lower* ATP – means that ATP probably provides energy for contraction.

With all these reservations in mind, one must wonder whether the concept of ‘molecular motors’ is necessary, if similar movements can be observed in the colpoids, and can be explained by recognised physico-chemical mechanisms.

Brownian movements, streaming and convection

It seems appropriate to deal with all these phenomena together, because: firstly, they also occur in colpoids; secondly, a wide variety of particles may move thus, including ground glass, pollen, gamboge, mastic, carbon black, bacteria, quartz, rubber solution, etc.; thirdly, there could not be enough energy in the biochemical reactions of the fluid phase of the particles for the movements to continue indefinitely; fourthly, they involve particles from about 250 nm up to about 4 μm in diameter (Perrin, 1910; Burton, 1916; Kamiya, 1962); fifthly, there is an enormous volume of experimental evidence about these phenomena.

Suggested causes of Brownian movement and streaming

- | | |
|--|--|
| 1. Incident light or heat | 2. Contamination with microbes |
| 3. Energy due to ambient temperature | 4. Evaporation of liquid |
| 5. Surface tension between particles and fluid | 6. Mechanical vibration, including sound |
| 7. Convection | 8. Electromagnetic energy |
| 9. Diffusion | 10. Magnetic forces between particles |
| 11. Bombardment by smaller molecules | 12. Gravity |
| 13. Gas absorbed by particles | 14. Cosmic rays |
| 15. Infusoria | |

Table 93. Most of these were suggested by Burton, (1916), Einstein, (1956) and Kamiya, (1962).

A summary of the many hypotheses proposed to explain these phenomena is given (Table 93), and a few comments about them are appropriate. All these phenomena can be seen indefinitely, if the particles listed above are placed in fluids, such as water, oil, ethanol, etc. Since there is no evidence of them dissolving in, or reacting with the solvents, the energy to move the particles could not originate in chemical reactions. Therefore the energy could only come from outside the particle-fluid system. The phenomena can only be seen under the microscope, when the particles are illuminated and heated. The particles and fluid each have different heat capacities and heat conductivities. The rate of streaming was diminished when the heat filter was interposed between the light source and the particle-fluid system (Burton, 1916).

Smoluchowski (1923) and Einstein (1956) were of the opinion that Brownian movement was caused by the uneven bombardment of one side of a particle by molecules and ions in the fluid. The particles have masses several orders larger than the molecules that move them, so that the latter either would have to be blessed with tremendous momenta, or there would have to be a very large number of them to push the relatively huge particles. If a large number of molecules on one side lost their randomness to drive the particles in one direction, why should not those on the other side of the particle moved lose their randomness and resist the motion of the first group? It seems that the random movements of very many particles is extremely unlikely to produce a substantial net movement in a particular direction. Otherwise, how could a liner dock in a harbour?

The sources of energy for these kinds movement are almost certainly, light and temperature in colpoids, (Wiener, 1863) plus metabolism in biological tissues (Table 94), because the specimens are viewed by microscopes at fluctuating temperatures, well above absolute zero. Surface tension differences, or charges on particles, are easier to postulate than to measure. The suggestion that gas on the surfaces of particles or dissolved in the fluid could move particles is very imaginative, but it cannot account for Brownian movement being immortal. The idea that the preparation could be contaminated by infusoria or microbes is also an interesting possibility, but they can not be seen, and contamination may be prevented. Evaporation of the fluid from the specimen, and subjection of mechanical or acoustic vibrations can also be prevented. The action of cosmic and radio waves seem likely to be unimportant, because they would transmit so little energy. The particles are not usually diamagnetic. Gravity would make the movements more directional than random.

One may conclude that the virtually unlimited energy required to move particles in colpoids and cells, almost certainly comes from the ambient temperature and the light source, used to view them (Regnault, 1858). This hypothesis could be tested, by: (a) repeating experiments showing that a greater intensity of light increases the number or velocities of particles moving; (b) that particle movement could depend on the energy associated with the wave length or colour used for illumination; (c) comparing the momenta of the fluid molecules with the inertia of the particles moved; (d) computing the amount of light energy likely to be absorbed by particles with the energy they dissipate when they move; (e) comparing the concentrations of fluids and particles causing different degrees of movement. Other important experiments on the phenomenon are reviewed in Burton, (1916, pages 51-91).

Whatever the outcomes of these experiments, it seems inescapable that the ambient temperature and illumination must be the source of Brownian movement, streaming and mitochondrial movement. Metabolism may also contribute energy for movement in living cells. Light induced movements must therefore be regarded as artifactual. Nevertheless, such movements are incompatible with the existence of a cytoskeleton.

Axonal transport

Ions, amino acids, catecholamines, organelles and particles, move down axons, and, presumably, fibres of fibroblasts and astrocytic processes. Weiss and Hiscoe (1948) showed that if peripheral nerves were tied, substances accumulated proximal to the tie, and the axon swelled. Whereas axonal flow was originally thought as only going from the cell body to the periphery, which was called anterograde, it was also observed subsequently to occur in the retrograde direction, (Lubinska and Niemerko, 1971; Bisby, 1986; Brady and Lasek, 1982, Morrell et al, 1982).

The phenomenon was named axoplasmic flow or axonal transport, and the latter term will be used here. During the latter half century, many different procedures have been used to examine it, including: injecting radioactive isotopes in the region or directly into the cell bodies; injecting fluorochromes similarly; histochemistry; electron microscopy; subcellular fractionation; examining the properties of axoplasm; direct microscopic vision of unfixed axons; observations in tissue culture; theoretical calculations. A number of preparations have been favoured, including peripheral nerves, optic nerves, hypoglossal nerves and dorsal root ganglia.

Whereas the axoplasm was originally considered to be a suspension (Leduc, 1928), the electron microscopists found it to contain endoplasmic reticulum, microtubules and microfilaments; they postulated that molecular motors pulled along the particles.

A few comments are appropriate about some of the methods listed, although many of these procedures have already been discussed earlier in connection with the cellular movements (Chapter 43). A procedure, such as histochemistry, electron microscopy, or fluorescence, should only be used properly if *either* the fixing or freezing of the tissue is so rapid, that the substance measured does not have time to move, *or* if the segments of axons in which the substance are to be measured have been separated before the substances have moved between them. It is not always appreciated that fixation, dehydration, freezing, precipitation, extraction, rinsing, etc, often move small ions and molecules. When one injects radioactive fluorescent substances into an eye or autonomic ganglion, there may be leakage (Grafstein and Forman, 1980), so that only a small proportion arrives in the cell bodies. Much of the rest is lost in circulation, and may subsequently arrive at the same site by a slower route. Examining the properties of axoplasm extruded from giant axons of cephalopods is a very useful technique, but one must be a little wary of regarding the

results of such experiments as being necessarily applicable to mammalian axons. The former are much bigger and their axoplasm has very different chemistry.

Watching particles in isolated unfixed axons is probably the best way of studying their dynamics (Smith, 1982). Tissue cultures are living, but cells are in very different environments than they are in intact parent tissues (Chapter 12). Calculations made on empirical measurements are very useful, but the structures are so small, that many of the physico-chemical parameters can not be measured with certainty.

Velocities of axonal transport

Tissue	Velocity (mm/day)	Authors
Rabbit retinal ganglion	1 - 2	Weiss and Hiscoe, (1948)
	3	Samuels et al, (1951)
Rabbit neuraxis	2-11	Koenig, (1958)
	4.5	Ochs, Dalrymple and Richards, (1962)
Rat and mice peripheral nerves	1	Droz and Leblond, (1963)
Rat sciatic nerve	1-3	Weiss, (1963)
	30-60	Lubinska, (1964)
Optic lobe of rabbit	2 - 151	Karlsson and Sjöstrand, (1971)
Mammalian ventral brain	230 - 410	Ochs, (1972)
Rabbit vagus and hypoglossal nerves	5 - 123	Fonnum, Frizell and Sjöstrand, (1973)
Rat sciatic nerve	1 - 4; 40	Grafstein and Forman, (1980)
Mammalian sciatic nerve	2-3	Dahlstrom et al, (1981)
Chick sciatic nerve	175 - 411	Couraud and Di Giamberardino, (1982)
Mammalian optic nerve	0.1 - 10, 50 - 400	Brady and Lasek, (1982)

Table 94. Some of many publications showing the rates of axonal transport. Many more are found in Weiss, (1963), Lubinska, (1964), Dahlström, (1971), Ochs and Smith, (1971), Ochs, (1975), Waxman, (1978), Weiss, (1982), Weiss and Gorio, (1982), Vale et al, (1985), Waxman, Kocsis and Stys, (1995); x mm per day is equivalent to approximately $2x/3 \mu\text{m}$ per minute.

One can now consider the large number of velocity measurements which have been made using these procedures (Table 94). These range from 0.1 to 12 mm/day, through 30/60 mm/day to 200 - 420 mm/day. These may be expressed as 0.7 to 3.7 $\mu\text{m}/\text{min}$, 28 - 40 $\mu\text{m}/\text{min}$ and 133 - 267 $\mu\text{m}/\text{min}$, respectively. This means that an ion, molecule or particle, moving 20 μm in the cytoplasm of any cell, would take from 4

seconds to five minutes, to reach anywhere in the cell. Bearing in mind the possibility that the 'slow' movements may represent recycling of the label of markers, it is probably only useful to look at 'rapid' movements, say from 30 mm to 420 mm/day. They probably represent the earliest arrivals of the substances moving by the shortest routes. Obviously, there is a very large range of velocities. This is not at all surprising, since different axons may be developing, myelinating or active; they display a wide range of diameters, lengths and temperatures. The variability in chemistry will cause a range of activities, viscosities, particles and degrees of metabolism, so it would be very surprising indeed, if particles moved along at uniform rates. What then is the point of studying axonal transport?

The recognition of the phenomenon arose out of studies on the regeneration of nerves following section by bullets and explosives. Powerful schools were set up by Young, Guttmann, Lubinska, Vrbova, Ochs and several others. At the same time, at the Cambridge School, Keynes, Hodgkin, Huxley, Baker and others, were examining the chemistry of nerve conduction in giant axons of cephalopods. The aim of the former group was to understand the chemistry of axonal flow with a view to encouraging cut nerves to join up more accurately and more frequently. Chemical and pharmacological measures to improve the efficiency of axonal regeneration have been singularly unsuccessful so far, but, the theory of conduction seems to have been solved, at least for giant axons of cephalopods. In the early 1960's, kittens and rats were fed with large doses of nerve growth factor, which gave rather equivocal results. In 1985 I suggested that recovery of cut nerves and, perhaps, spinal cords, could be accelerated by the *local* injection of nerve growth factors, steroids, fresh serum, ATP, glucose, etc but at the time I could not obtain funds to test this possibility.

The velocities of axonal transport of 1 - 400 mm/day (Table 94) are very slow compared with the metres/sec of the action potentials (Lapicque and Legendre, 1913; Erlanger and Gasser, 1937). Conduction may continue even when little axoplasm is present and (Baker, Hodgkin and Shaw, 1962a, b). These findings are usually interpreted by the general hypotheses that axonal transport is a physico chemical property mainly of axoplasm, while the action potential belongs to the membrane of the axon (Ochs, 1982; 1987).

This brings one to the simplest and, perhaps, the most important question about axonal transport. Is it more, the same, or less, than would be expected from the effects of diffusion, Brownian movement and convection? The coefficients of linear

diffusion of various substances in water are given in Table 94, and should be compared with the rates of axonal transport (Table 94). The former were measured in water, and diffusion coefficients in skinned frog muscles have been found to be about half of those in water (Kushmerick and Podolsky, 1969). Nevertheless, over the whole range diffusion coefficients in water were 21,600,000 times to 20,019 times faster than those found in axonal transport. The huge discrepancy in these rates mean that diffusion alone could easily account for axonal transport.

Diffusion in water

<i>Substance</i>	<i>Diffusion coefficient (cm²/sec)</i>	<i>Linear diffusion (mm/sec)</i>
KCl	1.9	95
NaCl	1.5	75
CaCl ₂	1.1	55
Glucose	0.7	35
Sucrose	0.5	25
Glycine	1.1	55
Alanine	0.9	45
Citric acid	0.7	35
Glycerol	0.8	40

Table 95. These values are calculated from the diffusion coefficients (mm/sec), given in the *CRC Handbook of Chemistry and Physics* (1987), page F - 47. Similar values are given in the International Critical Table of Numerical Data, Physics, Chemistry and Technology (1929).

The simplest view of axonal transport is to explain it as a mixture of diffusion, movement due to temperature, convection, movement due to pressure from surrounding tissue, and movement due to cell metabolism. The suggestive evidence is as follows:

- (i) it occurs in both directions;
- (ii) all the movements except those due to metabolism can be seen in colpoids;
- (iii) when an axon is cut, the axoplasm is seen to flow out of it mellifluously;
- (iv) axoplasmic transport stops when the tissue is fixed, probably, because the proteins are denatured and their viscosity increases;
- (v) particles can not pass along a constricted tube. The accumulation of fluid near a tie seems likely to increase the permeability of the axon, resulting in leakage of

proteins in tissue, causing the axon to swell;

(vi) colchicine could affect the viscosity of axoplasm by accelerating the sol-gel transformation, as it does during mitosis. It has a considerable affinity for extracted proteins (Lits, 1934; Dustin, 1963);

(vii) diffusion of ions and small molecules in aqueous solutions is much more rapid than axonal transport (Tables 94,95); it must occur in aqueous cytoplasm, unless the tissue temperature is reduced to absolute zero, or the viscosity becomes very high, or the axoplasmic chemicals precipitate. Magid, (1973) calculated that particles could not move by diffusion alone.

(viii) if microtubules, microfilaments and endoplasmic reticulum were not artifacts, they would represent an obstruction to axonal flow.

Chapter 44

Transmission, Modulation, the Vesicle Hypothesis and Conduction

In the days before the chemistry of living tissues was reinvented as molecular biology, a transmitter travelled across a neuromuscular junction to make a muscle contract, or across a synapse to excite another nerve, or to a gland to make it secrete. Acetylcholine and adrenalin were the two well known transmitters. Then it was found that some of the actions of acetylcholine could be ‘mimicked’ by muscarine - an extract from the poisonous mushroom, *Amanita* - , and others, by nicotine, an extract from the tobacco plant. Muscarine and nicotine were subsequently used as ligands to define the actions of acetylcholine, which is rather unstable. Adrenalin was found to be released with noradrenalin, and their actions were defined by binding to several α and the β receptors (Table 54). Since then, many receptors have been discovered, several of them blessed with families and treasure troves of agonists and antagonists. Drug companies prosper from their competitions. Vizi, (1984, page 3) remarked that, “The difficulties in defining a neurotransmitter increased exceptionally as an increasing number of new substances were proposed for carrying out the transmitter function.”

A transmitter may be described as a diffusible agent which excites nerve, muscle or secretory cells to activity. These criteria have been elaborated, (Hoffman, Lefkovitz and Taylor, 2003). Florey, (1967) defined the concept of a ‘modulator’ as “any compound of cellular and non-synaptic origin that affects the excitability of nerve cells and represents a normal link in the regulatory mechanisms that govern the performance of the nervous system.” Modulators could affect the quantity and time course of transmitter release; the reaction of the transmitter with one or more receptors; the time course and the degree of permeability of the synaptic membranes; the intracellular resistance and impedance. In summary, one may say that the modulator may affect any of the postulated steps in transmission - often unmeasurable and unprovable - but it is not a transmitter itself (Kacmarek and Levitan, 1987). A list, by no means comprehensive, of transmitters and modulators, is given (Table 96).

The criteria for transmitters and modulators have appeared in many publications, (see, for example, Paton, 1961; Werman, 1966; Hubbard, Llinas and Quastel, 1969; Hubbard, 1973; Barchas et al, 1978; Bowis and Bunn, 1995 and Rang et al, 2003). Theories of transmission depend upon the ability to distinguish between,

the action potentials in the presynaptic region during orthodromic passage of excitation, and the postsynaptic excitatory and inhibitory potentials appearing as partial depolarisation or hyperpolarisation in the postsynaptic cell or the muscle. The excitatory and inhibitory potentials are of opposite polarisation (Eccles, 1957; 1964). It is usually assumed that the signals originate at the anatomical synapses and neuromuscular junctions, respectively. In addition to the localisation by the use of intracellular pipettes, the pharmacologists have separate criteria for distinguishing between drugs acting presynaptically and postsynaptically, while the physiologists, anatomists and biophysicists all believe that they are talking about events unfolding at the same sites. The electron microscopists identify the presynaptic regions by the presence of synaptic vesicles and synaptic thickenings. This gives a situation in which a region must be presynaptic, because the vesicles are seen there, and they are seen wherever an axon, dendrite, neuroglial fibre or astrocyte appears to be in contact (see, for example, Pappas and Purpura, 1972; Peters, Palay and Webster, 1998). Thus every part of a neuron or neuroglial cell which is adjacent to another is regarded as a synapse. If synaptic vesicles and anatomical synapses are artifacts (please see pages 233-242), this throws into doubt the current beliefs in chemical transmission (please see below).

Non synaptic transmission has been reviewed (Agnati and Fuxe, 2000; Agnati et al, 2000; Nicholson, 2000).

Another criterion to distinguish pre- and post-synaptic regions is the separation of fractions believed to consist mainly of each of these parts of the neurons (Bradford, 1986), but the very different conditions in which each of them is fractionated make it likely that they will exhibit different chemistry. Another criterion is to 'block' or 'destroy' post synaptic axons, or 'poison' them. One then assumes that the resulting properties will only be those of the presynaptic regions, and that the agents destroying the structures or functions of the postsynaptic regions will have no significant effect on the presynaptic chemistry. Both of these assumptions seem to be rather dangerous. Furthermore, if a drug does not stop the 'release' of a transmitter but prevents it having an effect, such a drug is assumed to be acting postsynaptically. Synapses can be stained and viewed by the light microscope, but here one must assume that the staining does not cause shrinkage, and that the resolution of the light microscope is sufficient to visualise different parts of the synapses clearly. A good discussion of the localisation of effects on the synapses is given by Cooper, Bloom

and Roth, (2003, pages 85-89).

One may now turn attention to the criteria, which have been elaborated for defining transmitters and modulators. A list of transmitters and modulators has been drawn up from the literature (Table 96). It can be seen to contain a large range of hormones, aminoacids, catecholamines, proteins, growth factors and even gases.

Transmitters and modulators

<i>Transmitters</i>	
Adrenalin	Aspartate
Dopamine	Glutamate
Noradrenalin	Prostaglandins
Tyramine	Corticosteroids
Octopamine	Oestrogens
Phenylethylamine	Testosterone
Phenylethanolamine	Thyroid hormone
Dimethoxyphenylethylamine	Bombesin
Tetrahydroisoquinolines	Cholecystokinin
Serotonin	β -endorphins
Melatonin	Tachykinins
Tryptamine	Gastrin
Dimethyltryptamine	Neurotensin
5 - Methoxytryptamine	Proctolin
5 - Methoxydimethyltryptamine	Prolactin
Tryptolines	Oxytocin
Adenosine	Substance P
Adenosine monophosphate	Somatostatin
Adenosine diphosphate	Angiotensin
ATP	Luteinising hormone releasing factor
Acetylcholine	Vasopressin
Carnosine	Vasoactive intestinal polypeptide
Histamine	Adrenocorticotrophic hormone
γ - aminobutyric acid	Sleep factor delta
γ - hydroxybutyrate	Purine
Glycine	Nitric oxide
Cysteine	Carbon monoxide
Taurine	Hydrogen sulphide
β - Alanine	

<i>Modulators</i>	
Corticotropin	β - endorphin
Growth hormone	Met - enkephalin
Lipotropin	Leu - enkephalin
α - melanocyte stimulating hormone	Kytorphin
Vasopressin	Corticotrophin releasing factor
Angiotensin	Luteinising hormone releasing factor
Calcitonin	Somatostatin
Glucagon	Thyrotrophin releasing factor
Insulin	Bombesin
Cholecystokinin	Bradykinin
Gastrin	Calcitonin gene releasing peptide
Motilin	Carnosine
Pancreatic polypeptide	Neurokinin
Secretin	Neuromedin
Substance P	Neuropeptide γ
Vasoactive intestinal polypeptide	Neurotensin
Dynorphin	Proctolin

Table 96. This list of transmitters, modulators and mediators is not comprehensive. *Some substances appear under both headings, which is rather curious.*

The earlier definition of a transmitter as any substance which excited a junctional tissue would apply to all the transmitters listed in table 96. Since the simple definition, several other criteria have been added. These may now be examined individually-

(i) they must be present presynaptically. This just means that they must be present, where they are believed to act. This is hardly a specific criterion, bearing in mind that substances are generally present at the sites they are believed to act. According to current views, synaptic clefts are less than 1 μm wide, so that when a substance is applied to a synapse experimentally it is difficult to know how far it diffuses;

(ii) precursors and synthetic enzymes must be present nearby. Some hormones and aminoacids are believed to be transmitters, and are carried in the blood stream to their sites of action;

(iii) stimulation of the afferent nerves causes the transmitters to be released, but many other substances, which are not generally called transmitters, are also changed locally by stimulation (Table 97);

Substances changed locally by stimulation

<i>Increased</i>	<i>Decreased</i>
CO_2	O_2
Na^+	K^+
Ca^{2+}	Glucose
NH_4^+	Phosphocreatine
Phosphate	Adenosine triphosphate
Adenosine diphosphate	Lipids
Adenosine monophosphate	
Adenosine	
Lactate	
Co-transmitters	
Second messengers	

Table 97. These have been shown in brain, peripheral nerves and homogenates.

Other agents which activate nerves or muscles

<i>Agents</i>	<i>Targets</i>
K^+	probably depolarise all cells
Lowered Ca^{2+}	probably act on all cells, including nerves
H^+	chemoreceptors
CO_2	chemoreceptors
Mg^{2+}	local to neurons
Mechanical damage	probably all cells, includes 'injury' potentials of nerves
Light	rods and cones
Mechanical displacement	hair cells
Sound	cochlear cells
Pressure	touch receptor
Smell	mitral cells in nose
Taste	gustatory cells in tongue

Agents	Targets
Pain	free endings in skin
Heat	directly stimulates nerves and muscles
Burns	applied to cortical surface
Cold	stimulates skin receptors
Al^{2+}	applied to cortical surface
Co^{2+}	applied to cortical surface
Penicillin	applied to cortical surface
Strychnine	applied to cortical surface
Electrical current	applied to nerve or muscle tissue of neurons or muscle
Hypoxia	

Table 98. All these agents have been found experimentally to cause firing of neurons, or stimulation of muscle. (For earlier references see Dusser de Barenne, 1916; Dusser de Barenne et al, 1942; Burns, 1958). *They are not considered to be transmitters, mediators, modulators, agonists, antagonist or ligands.*

(iv) application of the transmitter produces the same effect as stimulation. The effect is to generate action potentials, but these are caused by several other agents and reagents, which are not considered to be transmitters. There is no reason to believe that most of them act only on synapses (Table 98);

(v) there should be ‘specific’ presynaptic receptors. The receptors are generally separated and identified from subcellular fractions. The concept of ‘specificity’ is really an anthropomorphic construct;

(vi) interaction of the substance with its receptor should increase or decrease the permeability of the neuronal membrane, producing depolarising or hyperpolarising effects. Changes in permeability are believed to occur whenever cells fire, but one must ask whether these have been sought when neurones are excited by agents other than those listed as transmitters (Table 98);

(vii) ‘specific’ inactivating mechanisms are believed to stop interaction of the transmitter in physiological time. It is difficult to know what specific means in this sense. The idea that the excitation is inactivated comes from the finding that it lasts rather a short time, say, a millisecond. Therefore, it was assumed that there were inactivating mechanisms. However, many phenomena - such as lightning or the striking of a clock - have a short natural history, which does not require an

inactivating mechanism. The activation is often believed to be associated with autoreceptors (please see below);

(viii) postsynaptic activation or inactivation should both be effective, that is, the neurons can be excited before or after the synapse. Of course, one can activate a circuit anywhere along its route, for example, anywhere on an afferent or efferent fibre. This criterion only means that any particular point, which is part of a circuit, is accessible to intervention.

The characteristics of modulators or mediators, may be summarised as follows:

(i) they act across the synapse, rather than from the presynaptic region to a nerve, or the motor fibre to a muscle. Since, in life, signals are orthodromic, this distinction seems to lack power;

(ii) they must be present in physiological concentrations, as are all active naturally occurring chemical components of normal tissue;

(iii) they must have access to the sites of action in these concentrations. Obviously, if they act, they must have access to the sites of action;

(iv) change of the modulators affects neuronal activity. This means that they increase or decrease their activities, as do agonists or antagonists, but so, of course, do transmitters;

(v) direct application of the modulator has the same effect as increasing the endogenous concentration of the transmitter, so does application of transmitters;

(vi) the modulators should have ‘specific’ sites of action, that is, they should activate or inhibit the effects of other transmitters or modulators in low concentrations. This implies that they act at the same sites;

(vii) inactivating mechanisms should affect the time course of the changes in concentration of the modulator. This means that the modulation gradually weakens, so that it is assumed that inactivation is involved;

(viii) the effects of increasing endogenous concentrations are increased by exogenous administration.

There is a widespread belief that there are ‘autoreceptors’ in the presynaptic region. Their properties have been summarised by Starke, Göthert and Kilbringer, (1989). Autoreceptors are believed to be activated by the transmitters which arrive to increase or decrease the activities of the receptors and themselves; secondly, antagonists have opposite effect to the transmitters; thirdly, agonists compete with

antagonists. All these effects are seen in synaptosomes (Brock, 1995).

One of the early classic demonstrations of autoreceptors was that of Wessler et al (1986). They incubated rat phrenic nerves and diaphragms with 1 μM [^3H] choline. The preparation was then superfused with 10 μM hemicholinium to inhibit the uptake of choline. The nicotine antagonist 1-1 - dimethyl - 4 - phenylpiperazinium - (DMPP) - (10 μM or 30 μM), or the neuromuscular blocker, tubocurarine (1 μM), was added. The phrenic nerve was stimulated with 100 pulses for 4 seconds and the radioactivity from acetylcholine was counted in the superfusate. It was found that electrical stimulation of the phrenic nerve in the presence of Ca^{2+} increased the outflow of acetylcholine, compared with that of the control, while tubocurarine decreased it; it antagonised the effect of DMPP. The authors concluded "that the terminals of the phrenic nerve possesses nicotine autoreceptors, that the receptor activation by DMPP facilitates action potential-evoked release of acetylcholine, and that receptor blockade by the antagonist tubocurarine causes an inhibition, which reveals a normal auto-facilitation by endogenous acetylcholine" (Starke et al, 1989).

The latter authors expressed the following reservations about the experiments: changes in outflow may reflect changes of release rather than retention in the tissue. (It is difficult to distinguish between these two processes experimentally in real time); it is not certain how similar are the properties of radioactively labelled exogenous compounds to those of the endogenous substances; the (subcellular) preparations added should be devoid of neuron cell bodies, to rule out the involvement of somadendritic receptors, (Is it possible to make a cell-free preparation devoid of broken somas and dendrites?); the endogenous transmitters are present near the receptors at unknown and varying concentrations; an exogenous agonist can exert its effect as long as the autoreceptors are not maximally activated by endogenous transmitters (there is no way of measuring them or their effects independently); the agonist effect declines as the concentration of transmitter released increases; an antagonist can only exert its opposite effect as long as the autoreceptors are activated by endogenous transmitter; it is difficult to know whether a decrease in the effect of an agonist is due to competition for an autoreceptor, or near-maximal autoreceptor activation by endogenous transmitter; it is difficult to determine agonist or antagonist affinities or potencies. "At autoreceptors, the endogenous transmitter is an additional 'rival' since both the potencies of agonists and antagonist are underestimated" (Starke et al, 1989).

These experiments have further problems, some not new; (a) the incubation of brain slices and the preparation of synaptosomes, almost certainly change the chemistry, the rates of binding, and the affinity of both the agonists and antagonists; (b) many of the agonists, antagonists, activators, blockers and ligands are not normal tissue constituents; (c) the claim that an autoreceptor can inhibit or activate the transmitter which engendered it, means that one can interpret any change in any such experiment by the suggestion that an autoreceptor is present; (d) as far as I can find in the literature, no electron microscopist has claimed to see and identify the relatively large autoreceptors. If they can not be seen as macromolecules they are probably chemical reactions which may be too fleeting or labile to detect.

In summary, one may say that the interpretations of experiments claiming to show autoreceptors are not susceptible to verification. Furthermore, it is not at all evident why a transmitter would cause the release, or a modulator modify, the synthesis, binding, storage, release or effect of another substance, which would enhance or oppose its activity. Why should a transmitter arriving at a synapse or a neuromuscular junction give out two conflicting signals? It would be much simpler to suppose that it excited or inhibited, and the subsequent diminution of its action would be due to its diffusion away, to binding to other local molecules, to its lability, or, perhaps, to local enzymes breaking it down. None of these possible mechanisms require ‘activation’, ‘blocking’, ‘release’, ‘agonism’, (partial or complete), ‘antagonism’, (partial or complete), ‘autoreceptors’, or the manipulation of unnatural reagents. The mechanisms suggested here can be tested much more easily than the hypothesis of autoreceptors.

One may now return to more general considerations about the criteria for transmitters and modulators, (Table 96). There are a very large number of them including aminoacids, catecholamines, hormones, etc. There are by no means always located or synthesized at junctional tissues, so their presence in such regions can not be considered to be a strong criterion. The application of transmitters, perhaps modified by modulators, ‘liberates’ endogenous transmitters, but so do also several other compounds (Table 98). There is no suggestion that these involve synapses, transmitters, modulators or autoreceptors, and they have not been regarded as acting on receptors.

There is another empirical way of viewing the phenomenon of transmission. Living cells react to changes in their environments, and nerve, muscle and glands, are

metastable, and are in dynamic equilibria. A vast number of naturally occurring substances and conditions, and many drugs, act at low concentrations to disturb the natural dynamic equilibria. Thus, cells are excited for brief periods; but, shortly afterwards, they are restored to their previous resting states by powerful homeostatic mechanisms. There is no inherent problem about naming some of the naturally occurring agents transmitters, but the original definition seemed too simple, so that it was elaborated by a number of further rather tenuous criteria.

The response of excitable cells depends upon the following variables: the frequency, amplitude, duration and shape of the afferent signal; the chemistry of the excitant; any co-transmitters released; the relative quantities of the excitant and the co-transmitters; whether there is any reaction between the transmitters, co-transmitters, other excitants and autoreceptors; the quantity of any of the latter substances 'present' in the region; the dynamics of the mechanisms which may release them from 'storage'; the chemistry of the axoplasm, of the afferent fibres and neurons; the permeability of the neuron membrane; the excitability of the presynaptic neuron. Most of these properties can not be measured in real time or rapidly enough, in the very small neurons under discussion. However, these considerations do make it extremely difficult to interpret simple dose-response curves, since the responses depend on interaction of so many different variables. Furthermore, equations about reactions, enzyme activities, drug binding, are usually worked out on simple one-step reactions, and their application to the complex, minute and dynamic properties of living cells must be questioned.

Chemical transmission and the vesicle hypothesis

Elsewhere, I have examined the vesicle hypothesis in extenso, (Hillman, 1985a). Electrical transmission was previously proposed (Eccles, 1937). It was succeeded by the chemical hypothesis which was summarised by Katz, (1969), and he was awarded the Nobel Prize for it in 1970. The main elements of the vesicle hypothesis of chemical transmission are:

- (i) spherical or oval vesicles about 50 nm in diameter are found in the presynaptic regions;
- (ii) they each contain an equal quantity of excitatory or inhibitory transmitter;
- (iii) at rest, only a small number of vesicles strike the inner membrane of the resting presynaptic membrane (Figure 67);

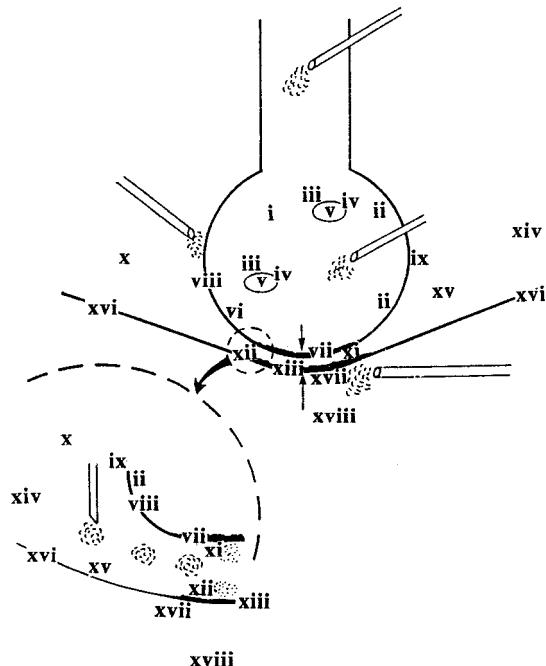


Figure 67. A diagram to show different sites around the synapse. These are numbered i - xviii. The inset is to show how a transmitter applied extracellularly would reach the membrane of the distal neuron, *before* it diffused laterally to the synaptic cleft. The phases are as follows; (i) presynaptic cytoplasm; (ii) adherent to presynaptic membrane away from presynaptic thickening; (iii) free inside the synapse; (iv) synaptic vesicle membrane; (v) inside synaptic vesicle; (vi) inside presynaptic thickening; (vii) presynaptic thickening; (viii) presynaptic membrane other than presynaptic thickening; (ix) adherent to presynaptic knob extracellularly in the synaptic cleft; (x) extracellular away from the synaptic cleft; (xi) synaptic cleft aspect of presynaptic thickening; (xii) synaptic cleft aspect of postsynaptic thickening; (xiii) postsynaptic thickening; (xiv) neuron soma away from synapse; (xv) adherent to non-postsynaptic area of distal soma; (xvi) distal soma membrane outside postsynaptic thickening; (xvii) adherent to intracellular surface of postsynaptic thickening; (xviii) free in cytoplasm of distal soma.

(iv) when the vesicles hit the presynaptic membrane, they liberate 'packets' of transmitter;

(v) the transmitter is released through the presynaptic thickenings;

- (vi) when the afferent nerve is stimulated, more of the vesicles release transmitter than at rest;
- (vii) it diffuses across the synaptic cleft to reach the postsynaptic membrane;
- (viii) this causes the postsynaptic membrane to depolarise (see Koelle, 1961);
- (vx) this initiates an action potential in the postsynaptic neuron;
- (x) some synapses are excitatory and some are inhibitory;
- (xi) different transmitters are involved in excitation and inhibition.

One may examine the assumptions inherent in this widely accepted hypothesis; (asterisks* indicate unprovable and unfalsifiable assumptions).

(a) the presynaptic vesicles exist in living synapses. It has been shown that synapses and the vesicles are artifacts (see Chapter 34)*

(b) synapses and motor nerve fibres are connected to dendrites (please see Chapter 34);

(c) it is assumed that the apparent uniformity of diameters of vesicles is due to the fact that they each contain the same quantity of transmitter. Transmitter has been detected in fractions believed to consist of vesicles, and the vesicles so isolated do appear to be very uniform in diameter (Whittaker, Michaelson and Kirkland, 1964);*

(d) transmitters are found in fractions identified as containing synaptic vesicles, but since their molecules are too small to be seen by electron microscopy, it must be an unprovable assumption that they are located within the vesicles. They could be adherent to the outside, or even in the liquid phase of the fraction;*

(e) one of the most fundamental weaknesses of the vesicle hypothesis is the assumption that vesicles are bombarding the presynaptic membranes. The bombardment is assumed to occur in living synaptic knobs, yet the vesicles can only be seen in fixed, stained, that is, dead tissue. Thus this assumption can not be proved or disproved;*

(f) the idea that the vesicles discharge the transmitter both at rest, and more as a consequence of stimulation, is believed to be shown by the ‘depletion’ of the number of vesicles after stimulation. However, Ceccarelli and Hurlbutt in a review of the literature as far back as 1980 showed that in some preparations the number of vesicles decreased, in others it remained constant, and, even some, it rose;*

(g) whereas it is believed that the synaptic vesicles strike presynaptic thickenings, (vi, Figure 67), the latter represent only a small proportion of the total area of the inner surface of the synaptic knob, so that the vast majority of vesicles

would hit other areas of the surface of that membrane (iii, Figure 67). Are the latter vesicles bounced back? Do they release their transmitters into the extracellular compartment? Can they distinguish between the non-synaptic part of the knob, and the presynaptic thickenings? Or is the former impermeable to the transmitter released, while the latter is permeable? None of these questions can be answered experimentally, so another part of the hypothesis remains bedevilled with uncertainty:*

(h) it is assumed that both the presynaptic and the postsynaptic membranes have channels in them, both for the individual transmitters, and for Na^+ , K^+ , Cl^- and Ca^{2+} . Such channels are not seen (Couteaux, 1958). Although it is claimed that the muscarine Na^+ acetylcholine channel has been visualised and isolated (Figure 17), the literature does not seem to feature micrographs of such channels in the thickening. One must also wonder about the ‘wisdom’ of thickening the two membranes in the region in which they are believed to be most permeable. Furthermore, one has no way of knowing whether the increase in permeability during transmission is confined to the synaptic thickenings or occur all around the presynaptic knobs and the postsynaptic cell membrane;*

(i) when an afferent neuron is stimulated the number of vesicles hitting the presynaptic membrane is supposed to increase; that is, energy is transferred from the membrane of the afferent nerve to the synaptic vesicles. Since the membrane of the axons, dendrites and synapses is continuous, one would expect this excitability to bypass the *contents* of the synaptic knob, travelling along its membrane*;

(j) the histochemists and immunocytochemists find that enzymes that break down the transmitters, for example, acetylcholinesterase for acetylcholine, and catecholamine-o-methyl transferase for adrenalin, are present at their highest concentrations in the synaptic clefts (Silver, 1974). If the latter localisations are correct - which seems to me to be rather unlikely – (Chapter 8), one must postulate that the transmitters diffuse through media, which make them very unstable. *What is left of their activities* must then bind to postsynaptic receptors, and *then* initiate their activities. Viewing the matter perhaps a little naively, it would seem, *prima facie*, that it is very unlikely that a natural mechanism would *diminish* its activity, before it had acted, especially if one believes that its transmission may well be thwarted by autoreceptors of its own making, which may inhibit or activate it. It is generally believed that the finding that the concentration of a transmitter is increased in the

region where synapses are found, means that they are released into the synaptic cleft. The observations simply do not have the microscopic resolution for this assumption. Even if one had shown rigorously that they are found at highest concentrations in the clefts, it would not indicate whether they came from the presynaptic regions themselves (vi, Figure 67), or any other part of the membranes of the synaptic knobs;*

(k) it is usually assumed that the synaptic delay, say, up to approximately one msec, is due to the necessity of the signal to activate the mechanism of chemical transmission. An alternative hypothesis will be put forward - the conduction hypothesis, by which a signal in the dendrites of one neuron is conducted through the neuroglia; this is also the compartment extracellular to the dendrites, cell bodies, and axons, of neighbouring neurons;*

(l) the assumption is entertained that particular synapses are excitatory or inhibitory. One can stimulate a pre-synaptic fibre and stimulate or inhibit the firing of a postsynaptic fibre, but one can not localise whether this occurs in the presynaptic fibre, the presynaptic membrane, the synaptic cleft, the postsynaptic cell body, the dendrites or the neuroglia;*

(m) the assumption that miniature end plate potentials in living synaptic knobs are produced by the release of equal packets of transmitters, probably arises from the observations that both the voltages from living cells and the vesicles seen in micrographs are small and of uniform size.* This assumption can neither be proved nor disproved, so that it forms a very weak link in the chain of assumptions making up the chemical hypothesis. It must be assumed further that the miniature potentials propagate around the synaptic membranes, although experimentally they are detected at equal amplitude by the recording micropipettes. Why are they not recorded as having different amplitudes, depending upon their distance from the electrode?*

(n) it is widely believed that after stimulation, the number of vesicles decreases, although this is not a universal finding (see (f) above). The vesicles are believed to contain the transmitters, not to be composed of them. The transmitters themselves can not be seen by electron microscopy. Thus, when the vesicles discharge their transmitters, there must be simultaneous and related mechanisms, which refill the vesicles after they have been 'depleted';*

(o) one of the lines of evidence for the chemical hypothesis consists of counting the number of vesicles seen by electron microscopy, but the number staining

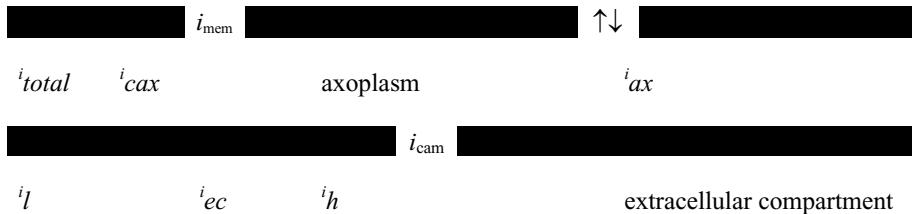
depends upon the Mg^{2+} and Ca^{2+} concentrations used during the staining procedure. This would mean that the number of vesicles counted would not necessarily be the same as those in the intact animals;*

(p) ligands are often employed to study the ‘specific’ properties of transmitters,* as they are believed to be more robust and detectable, but these, of course, are not the transmitters themselves (please see pages 127 to 133);

(q) dendritic spines are seen in many Golgi - stained preparations, (Figure 51) and they are generally assumed to be postsynaptic. Corresponding presynaptic fibres are simply not seen. This means *either* that the presynaptic fibres do not stain,* while the postsynaptic dendritic spines do, *or* the spines are not postsynaptic. It would be quite amazing if the former were the case. However, as far as I can find, no explanations have been offered in the literature, as to why the presynaptic fibres should not stain. It seems fairly likely that there are no presynaptic fibres, and the spines are, in fact granules on dendrites, which can also be seen in unfixed brains (Hillman and Jarman, 1991, their figures 52-57; Segal, 2002). When stained by the Golgi procedure, the granules could well appear as spines (Chughtai, Hillman and Jarman, 1988).

(r) it is said that vesicles can ‘recycle’ back from the synaptic clefts back into the synapses (Matteoli et al, 1992; 1993). Until now, there was absolutely no way of deciding in which direction such vesicles have moved, whether they are outside a synapses, on its membrane, or within it, since one is looking at dead, stained and embedded sections. Any particle originally present anywhere near the synapse, may move during the preparation for electron microscopy. However, recently, quantum dot fluorescence microscopy has improved the resolution of the light microscope to one or a few molecules (Michalet et al, 2005). Therefore, it should be possible to test the chemical hypothesis directly. This will be an important task for the early 21st century.

Assumptions* (c), (d), (e), (f), (g), (h), (i), (j), (k), (l), (m), (n) and (o) are neither provable nor falsifiable. These are such central assumptions to the vesicle hypothesis and to chemical transmission in general, that both hypotheses overall seem to be highly unwarrantable.



Figures 68. The locations of the different currents flowing down an axon. Similar diagrams could be drawn for the nerve cell body, the dendrite or the muscle cell.

Consider the case of a non-myelinated fibre, ensheathed by a membrane. For present purposes, the same considerations apply to a cell body or a dendrite.

The total current passing along the nerve fibre will consist of the following elements:

- (i) current travelling along axoplasm, i_{ax} ;
- (ii) current passing through the membranes, i_{mem} ;
- (iii) capacitance current of the axoplasm, i_{cax} ;
- (iv) capacitance current of the axonal membrane, i_{cam} ;
- (v) leak current across membrane, i_l ;
- (vi) current induced in the extracellular compartment, i_{ec} ;
- (vii) current converted into heat, i_h .

That is, the total $I = i_{ax} + i_{mem} + i_{cax} + i_{cam} + i_l + i_{ec} + i_h$, according to Kirchhoff's Law (Figure 68). Some of these currents can be measured individually.

One must then pose the question about which of the transmitters, modulators or other substances (Tables 96, 98) are likely to change in the intact animal sufficiently to excite cells. Regrettably there is no general answer to this question. Physiological electrical excitation and, probably, hypoxia, are the only signals which can be regarded as normal stimuli in this situation. All the others have been shown in vivo or in vitro to act on the excitability of cells. Nevertheless, one does not know in the intact animal to what extent homeostatic mechanisms actually prevent significant changes in tissue concentrations, either before or after the changed concentrations have had their effects on tissue. One can conceive of either possibility. For example, the mechanisms controlling the K^+ concentration around a neuron might prevent it changing beyond the range of 4-6 mM in the extracellular fluid, so that it would have

very little effect on the excitability of the neuron. Or, the homostatic mechanisms might not be activated until the extracellular K^+ concentration had risen high enough to fire the neurons; after excitation, the feedback mechanisms might then restore the K^+ concentration in the plasma to unexciting concentrations. Of course, simple tissues *in vitro* as opposed to perfused organs in intact animals do not enjoy the luxury of homeostatic mechanisms.

The Goldman Equation, (1943)

$$E_r = \frac{RT}{F} \log_e \frac{P_k(K)_i + P_{Na}(Na)_i + P_{Cl}(Cl)_i}{P_k(K)_o + P_{Na}(Na)_o + P_{Cl}(Cl)_i}$$

was regarded as defining the conditions for the resting potential, where P_k , P_{Na} and P_{Cl} were permeability constants for (K) (Na) and (Cl), within and without the membranes. The potential differences across the membranes were measured and the concentrations were changed, and they were in approximate agreement experimentally. However, this raises the question as to whether the other ions on both sides of the membrane (Fig 12) have *no* chemical activity or their channel activity do not contribute to the potential difference across the membrane.

The Cambridge School gave an account of the action potential (Hodgkin, 1951; Hodgkin and Huxley, 1952 a, b; Hodgkin, Huxley and Katz, 1952; Hodgkin and Keynes, 1955; Hodgkin, 1964). They worked on squid, crab and cuttlefish giant axons, and concluded that the rising phase of the action potential was due to an increase in the permeability to Na^+ , and arrest of the Na^+ pump. The falling phase was due to a slightly delayed increase in permeability to K^+ . The hyperpolarisation after the action potential was postulated as being due to the ‘excess’ Na^+ which had accumulated in the axon, being pumped out after the action potential propagated itself without decrement orthodromically, in the intact animal. The cell membrane acted as a K^+ electrode, while the cell was at rest, and acted as a Na^+ electrode very briefly during an action potential.

Although it seems rather strange that the action potential should originate from the axonal membrane, the important experiments of Baker, Hodgkin and Shaw, (1962a,b), showed that the axoplasm could be replaced by a high potassium ion solution and the axons would still fire. Furthermore, it has been possible to make artificial ‘black’ membranes, which are excitable, (Mueller et al, 1962; Mueller and Rudin, 1968). Thus, it is clear that the resting and action potential differences require

both appropriate ionic gradients and semi-permeable membranes.

It is now appropriate to formulate a new hypothesis about transmission, bearing in mind the reservations expressed about the chemical hypothesis. Consider two neurons whose cell bodies are adjacent in the brain (or spinal cord), the following findings will be the basis of the new hypothesis – the broadcast hypothesis;

- (i) the neurons and their processes are surrounded by a much larger volume of neuroglia, which is a jelly-like fluid, rich in mitochondria, or by extracellular fluid (Hillman, 1986, pages 201-204);
- (ii) the membranes of the whole neurons have high resistances, impedances and dielectric constants;
- (iii) the cytoplasm in the cell body and its processes, the neuroglial syncytium and the extracellular compartments, have low resistances, impedances and dielectric constants;
- (iv) electric signals can be conducted through body tissues, without passing through junctions, for example, in recording peripheral nerve activity, electromyographs or electrocardiographs;
- (v) Kirchhoff's Law is obeyed in respect of current (Figure 68);
- (vi) the cytoplasm of the cell bodies, and the cytoplasm of the axons and dendrites is of very similar composition in a particular neuron.

Before proceeding to put forward a hypothesis of transmission, it seems useful to examine the popular view that resting and action potential differences across the membrane are generated in the membrane itself. It has already been argued that the oft-repeated experiment that those voltages are *measured* across the cell membranes does not mean that they were *generated* there. By analogy no one would suggest that the current measured in an insulated wire means that it was generated in the insulation.

The biggest problem is at the beginning. As Colin Blakemore of Oxford University has often pointed out, no one knows the most vital piece of information - that is how an intention or desire is translated into an electrical signal originating in the motor cortex, which passes information down the radial and medial nerves of my right hand, which causes me to write. At the moment, nuclear magnetic resonance scanning, and positron electron tomography, of the brain, are elucidating those areas, in which the flow of blood and the local chemistry changes, when one thinks about carrying out a particular action, and before the motor system has indeed initiated it.

However, the blood flow and chemical effects, are themselves consequences of the translation of intention into chemistry, which is still such a mystery. Furthermore, current method of scanning can localise the region of the nervous system involved, but do not have the resolution to localise the neurons.

Incidentally, it is always assumed that only the neurons are involved in intention, thinking, learning, memory, etc. Experimentally, these appear to be the properties of the whole nervous systems in the intact animals, including the neuroglia, ependymal cells and blood vessels. It would seem imprudent to assume that these faculties reside only in neurons, until it has been shown that neurons alone can fire in the absence of other elements.

The hypothesis for both conduction and transmission consists of the following elements.

A. The differences of electrochemical potential across both the resting membrane and the active membrane are generated by the chemistry of mainly the cytoplasm and the axoplasm, possibly modified by the chemistry of the cell membrane, and the extracellular fluid.

B. The action potential may be initiated by, or be a consequence of, sudden changes in the permeability of the cell membranes. It means that the cell membrane - previously a slightly leaky insulator - permits the passage of a transient signal as a consequence of becoming briefly much more permeable to ions and other soluble substances, which can now cross it more rapidly.

C. The change of permeability which initiates the action potential could be caused by currents from: changes in cellular metabolism; conduction along the axoplasm; conduction along the axonal membrane; events occurring in the extracellular compartment. It is widely considered to be triggered by a wave of depolarisation from the adjacent sector of the excited membrane. It is impossible to know for certain, but since the impedance and resistance of the cell membrane is much higher than that of the axoplasm, according to Kirchhoff's Law, a much larger proportion of the current will travel by the latter than the former route. Therefore, it is more likely that the current travelling along the axoplasm, rather than that along the membrane, initiates the increase in permeability. It seems very unlikely that events in the extracellular compartment could be the source of sufficient current to increase the permeability of the membrane, since such current would be dissipated in the low resistance in the extracellular fluid.

D. The property of junctional tissues is that they are more sensitive than other parts of the neuron-neuron, neuron-muscle, or neuron-glandular interface, so that the neurons can be depolarised or hyperpolarised at these junctions by lower concentrations of transmitters, mediators and drugs or weaker agents applied at these regions than elsewhere on the motor nerve.

E. The simple fact that transmitters, hormones, agonists and antagonists, act in very low concentrations makes it likely that they react with (or, perhaps, bind to) a very small proportion of tissue, although the binding may be too short lasting or weak to be identified and extracted. The action of the substances mentioned above is usually regarded as binding at one or several 'specific' sites, but, of course, they could react with any active chemical constituents of the tissue, including proteins, enzymes, substrates, co-factors, etc, etc. These may be present in very small quantities, but occur in cycles, pathways and shunts. Of course, the transmitters, modulators and the ligands could act on several reactions. One simply should not continue to believe that the location of a ligand after a long chemical or histological procedure is necessarily the same as it was in the cells in the intact animal (please see Chapters 8 and 9).

F. When the signal arrives at the tip of a process such as a dendrite, whether it be located loose in the glial syncytium, or near another neuron cell body, muscle or gland, it will be broadcast in all directions through the conductive extracellular compartment. Here, it will decrement, and some of it will be lost through low resistant pathways. Any sensitive membranes in the region will be activated by the electrical signals from the membranes of the nearest cells. That is, one nerve cell broadcasts to its neighbours.

G. The synaptic delay measured currently, for example, would not necessarily be due to the release of transmitters, their binding, and the generation of postsynaptic potentials, but due *either*, to the excitation of the one cell membrane, its conduction to the next one, and the generation of the postsynaptic potential, *or* due to the charge of the membrane of one neuron, inducing a charge on another excitable membrane close by.

Thus, transmission can be regarded as a type of conduction by the route: (intention) - neurons - axons - dendrites - extra cellular compartment - dendrites of other neurons - their cell bodies - axons - dendrites - extracellular compartment (da capo) - another nerve, muscle or gland. The sensitivity of each of the elements to excitation or inhibition is determined by its local chemistry, including the presence at

low concentrations of transmitters, modulators, drugs and other agents. This still leaves open the question, which may or may not be possible to answer, and may not have one generalised answer. That is. Is homeostasis powerful enough to prevent transmitters and mediators, and other reagents (Tables 97, 99) changing sufficiently so that they rarely play a physiological role, or does it only return these to a narrow range of concentrations after they have acted?

Dale, (1935) put forward the ‘Principle’ that each synapse or neuromuscular junction could produce only one transmitter. Of course, these transmitters are extremely small, compared with the dimensions of the regions, in which they are detected experimentally. There is no realistic way of finding out which transmitter was released from a particular junction. The subcellular fraction of synaptosomes cannot answer this question either (Chapter 7). In recent years, histochemical studies have eroded the chemical transmitter hypothesis badly (Brownstein et al, 1974; Hökfelt et al, 1980; 1986; Cuello, 1982). The idea that a single transmitter is released is extremely unlikely, because of the wide variety of changes shown to result from stimulation (Table 97)

Finally, is the phenomenon of transmission more than a statement that living excitable tissues are in unstable dynamic equilibria, from which they can be displaced easily, although they recover rapidly?

Chapter 45

Signals and Signalling

Signals may be defined as substances or agents acting on cell membranes, nuclei and junctions, and even on cells from which they originate (autocrine actions). The *signals* are transduced in *to effects* (Holmes, 1990; Gomperts, Kramer and Tatham, 2002). For earlier reviews please see Nahorski, (1989) and Hancock, (1997). They include transmitters, hormones, cytokines, growth factors, interferons, electrical activity, light and odours (Table 99). Although many signals have been detected *in vitro*, it is generally believed that the same reactions occur in living intact animals (De Laat, Bluemink and Mummery (1989).

Signals

<i>Transmitters</i>	
Acetylcholine	Adrenocorticotrophic hormone
Adrenalin	Oestradiol
Noradrenalin	Progesterone
Histamine	Oxytocin
γ - aminobutyric acid	Vasopressin
Dopamine	16 Prostaglandins
Serotonin	Testosterone
Peptides	Bradykinin
Aminoacids	Neurotensin
Nitrous oxide	α – ecdysone
<i>Hormones</i>	Polypeptides
Insulin	<i>Growth factors</i>
Glucagon	Epidermal growth factor
Thyroxine	Platelet - derived growth factor
Tetraiodothyronine	Granulocyte colony stimulating factor
Thyroid stimulating hormone	Insulin - like growth factor
Parathormone	Nerve growth factor
Calcitonin	Retinoic acid
Growth hormone	
Follicle stimulating hormone	<i>Cytokines</i>
Luteinising hormone	Interleukin 1 α
Luteinising releasing hormone	Interleukin 1 β

<i>Other chemicals</i>	14 Interleukins 2
Oxygen	Tumour necrosis factor
Glucose	
Fatty acids	<i>Interferons</i>
Adenosine	α / β
Adenosine triphosphate	1FN - γ
Rhodopsin	<i>Other signals</i>
Pheromones	Transmembrane
Arachidonic acid metabolites	Action potentials
Toxins	Heat shock
	Osmotic shock
<i>Extracellular signal proteins</i>	Light
TNF - α	Smell
Fas L	Antigens
TRAIL	Bacteria
Apo- 3 L	Viruses

Table 99. Many of these are in Burgoyne and Petersen, (1992) and Bradshaw and Dennis, (2004). Most act in concentrations of 10^{-8} M.

Steps in signalling

<i>Step</i>	<i>Number of compounds</i>
Signals or first messengers arrive at sites	90-120
Signals bind to G - protein coupled receptors	1200 in human (Shöneberg, 2002)
G proteins are transduced	16-20
G proteins regulate effectors	not known (Simon, Strathmann and Gautam, 1991)
Intracellular second and third messengers are released	more than 13 (Table 102)
'Primary' protein kinases, phosphatases and other directly responsive proteins are activated	10's to 100's
'Secondary' protein kinases and phosphatases from regulatory pathways are activated	100's to 1000's
Enzymes and genes are regulated	several thousands

Table 100. These steps are indicated by Berridge and Irvine, (1984), Birnbaumer, Mattera, Yatani et al, (1990), Kendrew, (1994) and Birnbaumer, (1994). Please note that there are many less signals and G proteins than enzymes and genes regulated by them.

Signals, or first messengers, are believed to proceed as indicated (Table 100). They bind receptors on the outside of cell membranes; (De Mello, 1987; Iyengar and Birnbaumer, 1990). These receptors are bound to G-protein coupled receptors (Gilman, 1987). The latter consists of 22-24 aminoacids forming hydrophobic α helices; which are within the cell membranes and transduce the signals, or open ion channels within the membranes. The open ion channels or protein regulated effectors within the cell membrane activate second messengers (Table 101). The latter act on protein kinases, ion-activated enzymes, DNA and RNA (Birnbaumer, Mattera, Yatani et al, 1990; Barritt, 1992). These effect glycogen and lipid metabolism, the citric acid cycle and the genetics of the cell. The cell membrane is considered to be impermeable to most macromolecular enzyme signals. The signals may be amplified.

All these steps are believed to occur, for example, when acetylcholine slows a heart, when insulin acts on the islets of Langerhans, or when a nerve is excited. The steps mentioned in the last paragraphs are believed to occur within the millisecond of a synaptic potential or the minutes of glandular secretion, or the days of a genetic response.

Second messengers

<i>Messenger</i>	<i>Target proteins</i>
Cyclic AMP	protein kinases, ion channels
Cyclic GMP	protein kinases, ion channels
Ca^{2+}	Ca^{2+} and calmodulin - activated enzyme
Inositol triphosphate	Ca^{2+} channels in endoplasmic reticulum
Na^+ ions in excitable cells	ion channels; Na^+ activated enzymes
K^+ ions	ion channels, K^+ activated enzymes
H^+	H^+ activated enzymes
Cl^-	ion channels
Fructose 2, 6 biphosphate	enzymes in intermediate metabolism
Nitric oxide	cytoplasmic guanylate cyclases
Diacylglycerol	Ca^{2+} and phospholipid dependent protein kinases
Free fatty acids	H^+ channels in mitochondria of brown adipose tissue
Arachidonic acid metabolites	extracellular domain of membrane receptors

Table 101. Some of the many second messengers and their targets (see Dunkley and Ralston, 1992).

Barritt, (1992, page 55) summarises the different receptor sub-groups: 1. The receptor is an ion channel itself. 2. The cytoplasmic part of the receptor protein has its own protein-tyrosine kinase activity, which spans the membrane. 3. The cytoplasmic domain of the receptor couples with a protein tyrosine kinase, which does not span the membrane and has no extracellular domain. 5. The cytoplasmic domain of the receptor has guanylate cyclase activity. 6. The receptor protein couples with an enzyme or ion channel through a GTP-binding protein. 7. The receptor protein couples with an integral membrane protein. 8. The second and third messengers act (Table 100).

One may view the adenylate cyclase amplification cascade as studied in liver cells as an example of the complexity of the pathway, and the connection between a signal and the metabolism of glucose. The adenylate cyclase causes the cyclic ATP to cyclise AMP and phosphate. The cyclic AMP activates an inactive protein kinase.

In the presence of ATP, the protein kinase activates an inactive phosphorylase kinase to an active kinase plus ADP. ATP is also dephosphorylated to activate phosphorylase. The latter phosphorylates glycogen in the presence of phosphate to give glycogen 1-phosphate, which breaks down to glucose, which is also phosphorylated. This is the beginning of glycolysis, which feeds into the citric acid cycle.

Before examining the concept of signalling, it is useful to comment on some of the procedures used routinely to gather evidence for the concept.

(i) the use of homogenisation or full subcellular fractionation implies that homogenisation does not affect the enzyme activities, their location, the affinity of the ligands and tissues for each other, or any other reactions which they trigger. This has been examined in detail (Chapters 7 and 15);

(ii) slices of brain, liver, kidney etc. are subject to hypoxia and exchange with the incubating media, including water. It can not be assumed that the materials exchanging between the tissue and the medium do not affect the affinity for ligands, transmitters, hormones, etc, (Chapter 13).

(iii) ligands are used instead of transmitters, hormones, drugs, etc to examine the binding by the tissue of the latter substances. Their use is based mainly on the property that the ligands compete with them.

(iv) immunofluorescence has its own litany of assumptions (Chapter 9). In respect of signalling, the dangerous ones are that the activities and fluorescence of

none of the antibodies, fluorochromes, activators, inhibitors or quenchers used are displaced, enhanced or diminished, significantly, during the immunocytochemical procedures. However, the multiplicity of reagents rained on the issue makes these assumptions highly doubtful. There seems to be no escape from doing the relevant control experiments;

(v) confocal microscopy is an excellent modern method of achieving high resolution, but is sometimes carried out on tissue prepared for histology or electron microscopy, rather than on unspoilt cells;

(vi) purification of polypeptides is a complicated process, and the recovery of their activities is not always examined. As with high energy procedures for separation, such as mass spectrometry, electrophoresis and gas-liquid chromatography, it is often difficult to know whether an increased number of peaks represents a *separation* of different fundamental components, or breakdown products of a natural macromolecule. Also here, one must insist on the importance of recovery calibrations (Chapter 4);

(vii) crystallography and low angle X-ray diffraction are used for examining the properties of pure crystals. However, *in vivo*, the nucleotides, proteins and polypeptides are present in very low concentrations, often chemically bound to small molecules including water. So far, there have been insufficient experiments on the relationship between the properties of pure crystals of, say, DNA, RNA or ATP and the same molecules in intact cells in living animals, or colpoids imitating them chemically;

(viii) antisense RNA has also been used in plotting metabolic pathways. It has its own quota of certainties and uncertainties (for review, see Phillips, 2000);

(ix) G-proteins and G-protein coupled receptors have been cloned, isolated and their sequencers determined (see, for example, Dhanasekaram and Gutkind, 2001; Pangalos and Davies, 2002). Even folded, their diameters are much larger than the thickness of the cell membrane, so, like so many other receptors, they should be seen by electron microscopy. So, of course, should their extracellular, transmembrane and intracellular domains.

(x) electromicroscopy should not be used, because during the procedure tissues lose many of their contents, which were soluble in water and organic solvents.

(xi) the further fundamental difficulty is that measurements of binding, transport, enzyme activities, take minutes to hours, although the reactions are believed

to occur in fractions of seconds. Thus, it is very difficult to know the sequence of reactions depicted in diagrams. One does not know, and, perhaps, one can not know, the order of rapid simultaneous chemical reactions. It is difficult to know whether they represent the glories of chemical knowledge, or demonstrations of the skills of graphic artist. This is not a trivial point, when one realises that the majority of biochemical, as opposed to physiological, experiments do not demonstrate changes in real time.

Reasons for which the signalling concept is questionable are, as follows.

- a. Although the minority view is taken here that the electron microscope is not necessarily a valuable tool in examining cell structure, nevertheless, it is a proper question to ask electron microscopists again why they see what they claim to be receptors, G-proteins and transmembrane molecules, in ‘purified’ fractions, but they are *never* seen in sections of whole tissue?
- b. The transmembrane molecules are believed to pass the excitation across the cell membrane, but there are several other mechanisms, which permit passage of excitation, without the transmembrane molecules or channels (Table 45);
- c. There are said to be about 100 different signals, of which about 70 are listed in Tables 101 and 102. Many of the transmitters, hormones and drugs have ‘families’ of receptors, multiplying the effective number of signals several times. If there are only 16-20 G proteins, each would have to react to a large number of different signals. How can each pass on a ‘specific’ message from one particular signal. Secondly, how can the G-protein instruct a particular enzyme or gene?
- d. The existence of ionic channels in the cell membrane has been questioned (please see Chapter 21);
- e. The idea that a signal from the environment, or the extracellular compartment of cells, can influence genes in cells looks remarkably like Lamarckianism;
- f. A list of diseases in which G-proteins are believed to be ‘involved’ is given (Table 102). The diseases have such a range of aetiologies that it seems likely that any derangements of the G-proteins detected are *consequences* of the diseases, rather than causes of them.

Diseases in which G-proteins are believed to be involved

Cholera	Pseudohypoparathyroidism
Ischaemic heart disease	Allbright's hereditary osteodystrophy
Cardiomyopathy	Ovarian tumours
Congestive heart failure	Leukaemia
Hypertension	Lymphoma
Asthma	Alcoholism
Pituitary tumours	Cataracts
Hypothyroidism	Diabetes mellitus
Hyperthyroidism	Adrenal dysfunction

Table 102. The data are mainly drawn from Milligan, Wakelam and Kay, (1990); Milligan and Wakelam, (1992); Milligan, (1992).

g. The implications of the use of the overall term 'signals' is that each of them acts by similar mechanisms. In addition to the transmitters listed in Table 96, one must ask if other non transmitters (Table 98) are also liable to initiate all the steps of the signalling pathway (Table 99). This includes hormones, growth factors, cytokines, other agents, etc. In most cases, the mechanisms of action of each of these have been elucidated in great detail during the 20th century. They are very different for each category. What then does the term 'signal' add to the comprehension of biochemistry, physiology and pharmacology?

It would seem that one can consider signals as one did transmitters. Living cells are in metastable dynamic equilibria. The equilibria of the most sensitive cells, the excitable cells, are easily upset by substances called transmitters, modulators and signals. The equilibria of most other cells can also be influenced by signals, both chemical and physical. This seems to be a banal statement, and maybe, the concept of a signal is also rather banal.

Chapter 46
Muscle Contraction

There have been a large number of publications on muscle contraction, both before and after the currently accepted mechanism - the sliding filament hypothesis - was proposed in the 1950's (see, for example, Huxley and Hanson, 1955; 1957; 1959; Huxley, HE, 1963, 1964; Needham, 1971; Pollack, 1990). Its main elements are as follows: (i) electron microscopy showed that a muscle consists of bundles of thin filaments connected to the Z-lines, and between them thick filaments overlap the thin ones (Figure 63). The overlap looks darker and is named the A band. The regions between the A bands are called the I bands; (ii) the A bands consist mainly of myosin, and the I bands mainly actin; (iii) the myosin acts as a weak ATPase, activated by actin in the presence of Mg^{2+} . The ATPase is believed to be present in the heads of the myosin molecules, and the hydrolysis of ATP provides energy for the contraction (Engelhardt and Ljublimova, 1939; Engelhardt, Ljublimova and Meitina, 1941; Yanagida, 1985); (iv) fine cross-bridges are believed to join the thick and thin filaments, where they overlap; (v) when excitability comes from the neuromuscular junction, it is distributed to each muscle fibre; (vi) a system of T-tubules, which permeate the whole of the muscle, distributes excitability to the thick and thin filaments from the Z line, (Huxley and Taylor, 1958); (vii) muscle contracts only in the presence of Ca^{2+} and Mg^{2+} ; (viii) it is observed by electron microscopy that during isometric contraction, the A bands usually remain the same width, while the I band narrows. The H bands in the middle of the A bands also narrow and disappear, when the thin filaments overlap; (ix) the sliding filament hypothesis proposes that, during contraction, the thin filaments are pulled in the direction of the long axis of the muscle towards the thick filaments by the cross-bridges between them. This may obliterate the H-bands; (x) since the cross-bridges are nm in length, and the muscle contracts by mms, it has been postulated that the attachment and detachment of the cross-bridges between the thick and thin filaments must be cyclical, taking place many times during each contraction of a whole muscle (Huxley HE, 1957).

The sliding filament hypothesis is believed to be true not only for voluntary muscle, also for smooth and cardiac muscle (Shoenberg and Needham, 1976; Gabella, 1981; Stephens, 1984).

Unfortunately, the hypothesis has a number of difficulties:

- (a) it has been shown that the thick and thin filaments do not seem to appear in enough orientations to be three dimensional (Chapter 36), so that what appear as filaments are probably dried sarcoplasm deposited after the sections have been cut;
- (b) cross-bridges are randomly orientated, not longest at rest, and shortest during maximum contraction as they should be (Figure 63). This observation was made originally by Huxley HE, (1957), but was not regarded as threatening the hypothesis. In smooth muscle, Somlyo AV et al, (1977a,b) also found the cross bridges always at right angles;
- (c) ‘cross-bridges’ occur also in peripheral nerves, (Figure 64), which are not at all contractile;
- (d) when the muscles are stretched beyond the overlap of filaments, they should not be able to contract, but in many preparations, they can contract well beyond the overlap (Pollack, 1990, page 19);
- (e) the same author (pages 10-15) reviewed publication between 1954 and 1989 in which the widths of the A bands were measured after contraction. In vertebrates, 5 publications showed no change, 4 showed shortening by 5-15%, 12 showed 15-40%, and 5 showed shortening by more than 40%. He pointed out that these findings altogether hardly justified the generalisation that A bands do not shorten during contraction;
- (f) the concepts of ‘isometric’ and ‘isotonic’ contraction are contradictions translated into Greek. If the muscle were really isometric, that is, at the same length, it could not be contracting. Similarly, if it were isotonic, that is, at the same tension, there would be no force for it to contract. Isometric contraction actually means that the muscle is prevented from contracting macroscopically by being held firmly at both ends, so that it can not slip, but, on electron microscopy, the sarcomeres have shortened. The missing element here is the effect of the preparation for electron microscopy on the length of the A and I bands. However, the contraction of living muscles was examined by phase contrast and interference microscopy (Hanson and Huxley AE, 1953; 1955; Huxley AF and Niedergerke, 1954). These authors reported “substantially” similar results to those by electron microscopy, but the latter authors remarked rather cryptically, in respect of isometric twitches, that “No change in the widths of the bands could be detected, except that when slight shortening of the region of the fibre in the field of view took place, the changes were similar to those in

isotonic shortening of the same extent". Although care was taken to compare the changes seen in living muscle by light microscopy with observations on dead muscle by electron microscopy, one has to ask, whether preparation for electron microscopy would not change the absolute and relative lengths of the filament and sarcomeres, and how much subsequent change of length would occur during preparation after sections were removed from the devices keeping them isometric;

(g) although it is widely accepted that attachments of cross-bridges cycle during a single contraction of a whole muscle, (Huxley AF, 1973; Podolsky and Nolan, 1973), there seem to be no electron micrographs showing the location of the bridges when the filaments do not overlap;

(h) in order for cycling to occur, there would have to be a mechanism, presumably originating from the neuromuscular junction, instructing and co-ordinating different stretches of cross-bridges in single and adjacent muscle fibres, so that most of them contract effectively to cause useful contraction of the whole muscle;

(i) it is widely taught that the contraction of an individual sarcomere is an all-or-none process, so that greater tension is generated by 'recruitment' of more fibres, rather than a single fibre responding proportionately to the size of the stimulus. When one observes a living frog muscle, one sees directly that, except for the subthreshold and supramaximal stimuli, the larger the stimulus, the more the whole muscle contracts;

(j) the stimulus originates from the neuromuscular junction and spreads to both ends of the muscle. If it is submaximal, it would invade the fibres, proximal to the neuromuscular junction before spreading distally. Submaximal stimuli would stimulate proximal *before* distal fibres. Therefore, one would expect these stimuli to cause 'crimping' around the neuromuscular junctions. This is not seen. The alternative seems to be that submaximal stimuli produce graded responses in individual muscle fibres;

(k) it is widely believed that there is no work done during isometric contraction, but this is not so. Work is done during the contraction, before the fibres shrink, and also to support their massive degree of contraction to maintain the same length. This is well known to every sheriff who has used his powerful shoulders against the unyielding door of an outlaw's residence;

(l) a thick filament is attached by a cross-bridge to a thin filament (Figure 63). At rest, the cross-bridge should be at maximal length. At this point, the longitudinal

component of the contraction should be maximal, while the transverse component should be minimal. When the muscle is fully contracted, the longitudinal component of the force must be minimal, and the transverse component maximal. This means that the force of contraction should be maximum at the beginning and minimal during the height of contraction. This is simply not seen. The maximal force of contraction is found experimentally to occur at the height of contraction. When the lengths of sarcomeres were held constant by servo-mechanisms, it was found that the shortest lengths generated the maximum tension, the middle lengths (often approximating to the likely lengths in the living intact animals) exhibited sub-maximum tension, and at the greatest lengths, decreasing tensions were generated (Gordon, Huxley and Julian, 1966a,b). When single frog fibres were used, the shorter the sarcomeres, the greater the tension they generated (Ter Keurs, Iwazumi and Pollack, 1978). That is to say, the greatest force was generated, when the longitudinal component of the force of contraction of the cross-bridges would be least;

(m) furthermore, the transverse component of the contractile force seems to be rather lost. It should be minimal when the muscle was contracted maximally. Therefore, it should pull the filaments *together* transversely during maximal contraction. The result of this should be that the whole muscle should be thinnest, when it is maximally contracted. In practice, it bulges. This is normally explained by the idea that the membranes and connective tissues around the fibres, the fasciculae and the whole muscle, render them effectively isovolumic, so that if the muscle contracts longitudinally, it *must* expand transversely. However, in class demonstrations, frog muscle bulges as it contracts, like that of a famous sailorman;

(n) the framers of the sliding filament hypothesis are aware of the problem of the transverse component of muscle contraction. They draw the cross-bridges as either myosin molecules with the globular heads, or with triangular rollers at the edges. Many show the molecules to have hinges, and the heads are sometimes doubled. (Huxley, HE, 1972; Huxley HE and Kress, 1985; Toyoshima, Toyoshima and Spudich, 1989; Vale and Milligan, 2000). However, the structures involving two myosin heads and a hinge are simply not seen in electron micrographs. The knobs believed to be myosin heads were seen only in separated myosin fractions (Zobel and Carlsson, 1963; Huxley, HE, 1963; Slayter and Lowey, 1967; Lowy and Small, 1970; Rice et al, 1970; Walker and Trinick, 1986) but regrettably, not in electron micrographs of whole muscles. Heuser, (1987) explained that the myosin heads could

not be seen because of their proximity to the thin filaments. It seems possible that the idea of a hinge might have been designed to circumvent the problem of the transverse component, but it does not.

(o) Hill, (1938, 1950) proposed that there is a series elastic component in muscle. This could reside anywhere in the muscle. In the intact animal, skeletal muscle is always attached to a ligament or tendon, both of which seem to be less elastic than the muscle itself. Until experiments are carried out to examine the mechanical properties of the former and the latter separately - if that is possible - most measurements should be considered to be derived from these elements in unknown proportions;

(p) several other agents, in addition to motor stimulation may cause muscle to contract. These include artificial electrical stimulation, tissue damage, heating, freezing, high K^+ , ATP, low Ca^{2+} , Ba^{2+} , fixatives, dehydrating agents and death. Prima facie, it looks rather unlikely that all these agents act by liberating transmitters which travel across neuromuscular junctions, as has been proposed for 'classical' transmission;

(q) 10% glycerol has been used with the intention of extracting the cell membrane, to demonstrate contraction in glycerinated preparations, and consequently that the contraction does not require a cell membrane. However, if glycerol really extracted cell membranes, it is highly unlikely that it would be used as a cryoprotective agent for deep-freezing sperm, ova and red cells (Polge, 1957; Smith AU, 1961);

(r) although the sliding filament hypothesis is widely accepted, there have been many other theories of contraction since the 19th century. These have included: accumulation of lactic acid; movement of water; sol-gel transformation; changes in the molecular structure of contractile proteins; reaction of actomyosin with ATP and electrostatic reactions between filaments (Szent-Gyorgyi, 1945; 1947, pages 38-44; Straub and Feuer, 1950; Ernst, 1963, pages 328-369; Iwazumi, 1970; Needham, 1971, pages 127-189; Heumann, 1973; Noble and Pollack, 1977; Straub, 1980; Huxley AF, 1980, pages 1-36; Pollack, 1990, pages 9-37; Pollack, 2001). The literature on this subject is extensive but all modern views depend upon the existence of thick and thin filaments, and usually cross bridges. Iwazumi's and Pollack's publications deserve careful examination.

If one rejects the sliding filament hypothesis, it would be useful to propose an

alternative, which, in my view, would have to contain the following elements: the living muscle has A and I bands, but no thick or thin filaments; there are no cross-bridges; electron microscopy of dead tissues can not yield information about contraction in living muscles; the motor fibres direct excitation to the muscle by conduction; muscle fibres do not act by an all-or-none mechanism; the most sensitive region of the sarcomere is either the I band or, possibly, the Z line; muscle can be stimulated by electric current, acetylcholine, ATP, high K^+ and Ba^{2+} ; denervated muscle becomes hypersensitive; a number of drugs in low concentrations can paralyse muscles. At the moment, it is perhaps, premature to put forward an alternative to the sliding filament hypothesis, but it would be useful to list a number of experiments, whose results might lead to a more satisfactory hypothesis. One would seek to:

- (i) show a range of orientations of thick and thin filaments by electron microscopy;
- (ii) show a range of angles of cross bridges;
- (iii) show cross bridges adjacent to the thick filaments, when the thick and thin filaments do not overlap during relaxation;
- (iv) look for T tubules and sarcoplasmic reticulum by light microscopy, since their dimensions by electron microscopy indicate that they could be resolved by light microscopy (Huxley and Niedergerke, 1954,);
- (v) show micrographs of myelinated nerve fibres losing their myelination before joining the neuromuscular junctions;
- (vi) examine the effects of preparation for light and electron microscopy on the absolute and relative lengths of whole muscles, tendons, A bands and I bands;
- (vii) show the cell membrane around muscle fibres by electron microscopy;
- (viii) show the connections of the thin filaments to the tendons and ligaments by electron microscopy;
- (ix) establish if individual fibres in living tissues, change their volumes, when they contract;
- (x) demonstrate 'cycling' of cross-bridges during contraction;
- (xi) examine whether there are filaments which go all the way from one Z line to the next;
- (xii) compare the mechanical properties of whole muscles, tendons, ligaments, muscle without tendons, and muscle sheaths;
- (xiii) determine the intracellular viscosity in the sarcoplasm on a range of

species using magnetic particles, Brownian movement and electron spin resonance (see Heilbrunn, 1958);

(xiv) use confocal, video enhanced, dark ground, interference, phase contrast and anopteral, microscopy to examine whether Brownian movement can be seen in living muscle fibres;

(xv) see whether muscles bulge after or before they contract;

(xvi) measure during relaxation and contraction heat capacity, heat conduction, resistance and impedance, in unfixed tendons, muscles, sheaths and extracellular fluid;

(xvii) examine in carefully isolated single muscle fibres whether the contraction is an all-or-none phenomenon;

(xviii) calculate the current density with currents used for stimulating muscle fibres, at the point of stimulation;

(xix) find out which of the many biochemical changes occurring before and during muscle contraction by electrical stimulation, also occur when muscle is stimulated by ATP, high K⁺, heat or dying;

(xx) try to find the anatomical basis for the recruitment of fibres during increasing muscle stimulation;

(xxi) see if addition of acetylcholine, ATP or K⁺ can increase the force of contraction of a muscle in 'maximum' tetanus from electrical stimulation;

(xxii) see if electrical stimulation causes contraction of: non-muscular tissues, such as liver, brain, kidney, collagen and mitochondria, chloroplast and synaptosomal fractions;

(xxiii) examine whether guanosine triphosphate, inosine triphosphate and arginine phosphate, cause mammalian muscles to contract;

(xxiv) extract sarcoplasm from mammalian muscle using micropipettes, as has been done with axoplasm from cephalopods, and fluids from parts of nephrons, and examine its chemical composition;

(xxv) carry out further experiments passing small electric currents through pure and mixed solutions of, actin, myosin, meromyosin, tropomyosin, troponin, paramyosin, α -actinin, β -actinin, haemoglobin, myoglobin, casein, gelatine, immunoglobulins, and other macromolecules, to see what they do to their chemical and physical properties (Mandern, 1967; Ebashi and Nonomura, 1973);

(xxvi) myosin and actin filaments have been shown to move when ATP is added (Buchthal et al, 1947; Yanagida et al, 1984; Toyoshima et al, 1987). These important observations should be extended to the other substances mentioned in the latter paragraph;

(xxvii) examine the chemical compositions of the extracts from muscle of 10% glycerol, and of the mixtures for removing myosin, and actin, to find out what other active chemical substances have also been extracted from muscle;

(xxviii) if nanotechnology can produce microscopes which can examine very small structures of a few molecules in size, it should be able to demonstrate that cross-bridges, thick and thin filaments, exist as structures in living muscle.

This list of proposed experiments may be regarded as an indication of the considerable effort which probably should be made before one can devise an alternative theory of contraction.

Chapter 47
The Chemiosmotic Hypothesis

This hypothesis proposes that a proton electrochemical potential couples ATP synthesis to the electron transfer chains across the membranes of mitochondria, chloroplasts and microbes. It was put forward by Mitchell in 1961, and has been elaborated since (Mitchell, 1961; 1966; 1968; Greville, 1969; Mitchell, 1979; Nicholls, 1982). Strong criticisms and alternative hypothesis have been put forward by Williams, (1961; 1978; 1979; 1983) Ling, (1981), Malpress, (1984) Robinson, (1984), Wainio, (1985) and Malpress and McCallum, (1990). The structure of mitochondria (Chapter 29), and the methods by which mitochondrial fractions are prepared, have already been reviewed (Chapters 7, 25).

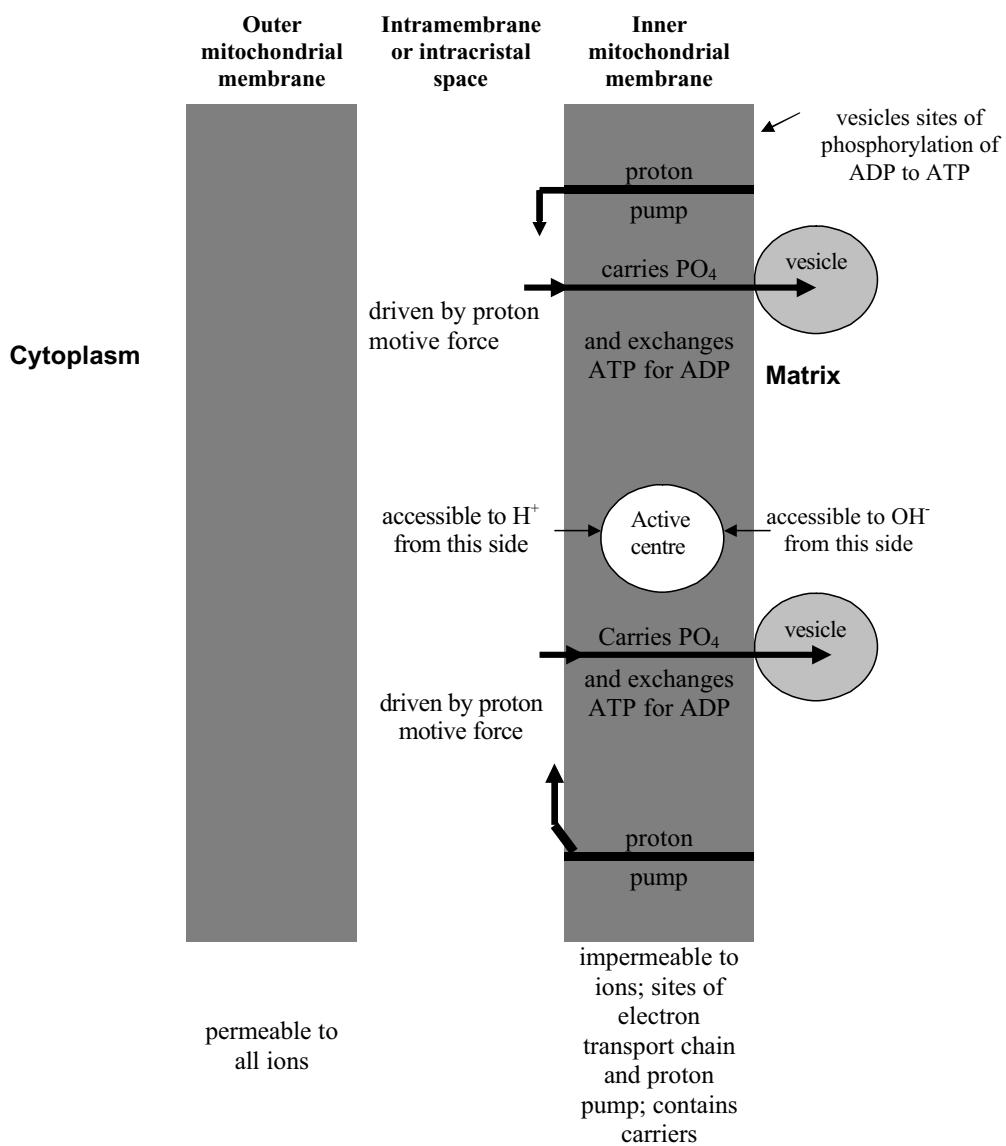


Figure 69. The chemiosmotic ‘pump’, couples the proton motive force to ATP. *In this monograph, the matrix is called the mitochondrioplasm, because Mitchell, like others, believes that there are cristae in living mitochondria, whereas it is concluded here that they are artifacts (please see Chapter 25).*

The hypothesis may be summarised, as follows, (Figure 69).

1. The mitochondria have outer membranes, which are permeable to all ions.

The outer membranes enclose spaces separating them from the inner mitochondrial membranes. The latter are thrown into folds, called cristae, which act as shelves

within the matrix of the mitochondria.

2. The inner mitochondrial membranes are believed to contain the enzymes, substrates and co-factors for the respiratory chain, as well as an enzyme ATPase, which is identical with ATP synthetase. The inner mitochondrial membranes are impermeable to Na^+ , K^+ , Cl^- , NAD^+ , NADH , NADP^+ , NADPH , AMP, CMP, CDP, GDP, CTP, CoA, Acetyl CoA, all of which stay in the matrix.

3. There are 'active centres' in the inner membranes. These are accessible to OH^- from the matrix, and H^+ from the cytoplasm. The inner membranes are impermeable to water. The electrochemical activity of the water at the active centres is given by the product of the H^+ in the cytoplasm, and the OH^- in the mitochondrial matrix.

4. High energy electrons are transferred along the electron carrier systems; they 'pump' 12 protons for every 2 electrons from the matrix into the intramembrane spaces. This creates a gradient of protons from the lower concentration in the matrix to the higher in the intramembrane spaces.

5. The proton gradient, plus the transmembrane potential, provide the driving force for H^+ and phosphate ions to move into the matrix and to phosphorylate ADP to ATP.

The biochemistry of these processes has been worked out on mitochondrial fractions by the use of 'specific inhibitors' (believed to block single enzyme reactions), classical electron microscopy, the use of micropipettes for penetrating single mitochondria, polarimetry, pH electrodes, studies on photosynthesis, and biophysical calculations.

Before considering the hypothesis in detail, it is appropriate to reiterate that biology is interested in the properties of the mitochondria in the living intact animal, and, also of those of mitochondrial fractions and subfractions, only in so far as they reflect those in life in the whole, normally functioning animal.

The first way to analyse the hypothesis is to examine the assumptions upon which it is based, not only those identified by Mitchell and those who disagree with him, but also other assumptions which do not seem to have been recognised hitherto. The assumptions are as follows:

(a) the redox potential of the mitochondria in a fraction is the same as that in the mitochondria in the intact living animal. This assumption is very unlikely to be warranted, because, say, the liver or brain will have been subjected to hypoxia,

pressure, hypertonic solutions, and dilution during fractionation. Other critics of the hypothesis have ignored this important assumption. It is not clear that it could be tested directly, although it could be in model systems;

(b) fractionation does not relocate ATPase, ATP synthetase, ATP, or co-factors;

(c) ATPase is the same enzyme as ATP-synthetase. These activities have been shown to occur in different locations (Cross, 1981; Wainio, 1985);

(d) the mitochondria have inner and outer membranes with a space between them. The cristae which are regarded as folds in their inner membranes, have already been shown to be artefacts (please see Chapter 29). The mitochondria are said to be stripped of their outer membranes, when digitonin is added to mitochondrial fractions. Electron micrographs of digitonised mitochondria *without* outer membranes are difficult to find. Digitonin is a cardiac glycoside, which is believed to ‘uncouple’ oxidative phosphorylation. Furthermore, electron microscopy always shows any single membrane as two lines - the ‘unit’ membrane of JD Robertson - because the heavy metal stains deposit on both sides of a single membrane (Chapter 9).

(e) ATPase is present in the mitochondrial membranes. Apparently, its activity is only found if compounds, such as 2,4 dinitrophenol, dicoumarol and carbonylcyanid p-trifluoromethylphenylhydrazine, are added to the preparation. These are regarded as ‘uncouplers’ of oxidative phosphorylation, and, of course, are not present in mitochondria in healthy intact cells (Ling, 1981);

(f) the inner mitochondrial membrane is impermeable to H^+ , OH^- and other ions. It is very unlikely that the permeability of the membranes in the subfraction measured after all the manipulations of fractionation have the same values as those in the mitochondria in the intact living cells. One must also ask what is the meaning of permeability measured in cell fragments. Permeability can only have meaning in a continuous unbroken membrane, since any measurement made on fragments would be ‘short-circuited’ by the conductive medium in which it was made. It is an unprovable and undisprovable assumption that this membrane is impermeable to these ions:

(g) the assumption that the membranes contain ‘specific’ carrier systems, which permit the entry and exit of metabolites, in response to the proton motive force across the membranes, would be warranted if the carriers had been demonstrated experimentally;

(h) that one can measure the potential difference across mitochondrial

membranes (Zamzani, Metivier and Kroemer, 2000). The mitochondria have already been fractionated, which involves pressure, shear, dehydration and centrifugation, while in an aqueous vehicle. It can not be assumed that such treatment would leave the membrane potential undiminished. Furthermore, it is difficult to measure the transmembrane potentials of small objects (Chapter 16). The diameter of the mitochondrion after preparation for electron microscopy is about 0.5 μm , but often they are 1 μm . Studies of membrane potentials of cells which are fairly uniform in dimensions, such as frog muscle or cat spinal motor neurons, showed that the larger the diameters of the tips of the micro-pipettes, the smaller the voltage which would be recorded. This was interpreted as meaning that large tips would make larger holes in the membranes, and some of the cytoplasm would leak out (Woodbury, Hecht and Christopherson, 1951; Haapanen, Kolmodin and Skoglund, 1958). Thus, it seems extremely unlikely that mitochondria would not be damaged by such relatively large micropipettes, and so the values measured would be less than they would be if the mitochondria had not been penetrated (Lieberman and Skulachev, 1970). Potential differences across mitochondrial membranes can also be calculated by measuring substances in the whole mitochondria and in the incubating fluid. However, here one must assume that during preparation the substances do not adhere to the outside of the mitochondria, or exchange with the preparation fluids from the inside.

Mitchell and Moyle, (1969) calculated that the potential difference across the mitochondrial membrane to drive the protons would have to be about 200 mV. Tupper and Tedeschi, (1969) found voltages in the giant chromosomes of fruit flies of 10 - 20 mV, of polarity opposite to that predicted by the chemiosmotic hypothesis. Mice mitochondria enlarged by cuprazone were found to have membrane potentials of 15 mV, also of opposite polarity than expected (Maloff, Scordilis, Reynolds and Tedeschi, 1978).

All measurements with micropipettes seem to record one direct current voltage on penetration. If there were outer and inner membrane, one would expect to see one voltage as the tip advanced from the incubation medium through the outer membrane to the intramembrane space, and a second as it advanced through the inner mitochondrial membrane. This is not seen. According to the chemiosmotic hypothesis, the voltage measured should only be that across the inner mitochondrial membrane. The hypothesis also requires the outer membrane to be permeable to all ions, but this can not be examined without the addition of digitonin. This itself is

very likely to change the permeability membrane, as it is used as a 'permeabiliser'.

(j), (k), there are 'active centres' which are permeable to OH^- from the matrix side and H^+ from the cytoplasm. The existence of the active centres does not seem to have been demonstrated by electron microscopy, and neither Mitchell nor his followers seems to have prepared a subcellular fraction of these bodies. There seem to me to be no way whatsoever of showing experimentally that either or any particular face, or even the whole 'active centre' is permeable to H^+ or OH^- . Therefore, this part of the hypothesis is untestable;

(l) 'translocation' channels for ATP are believed to be located in the inner mitochondrial membrane, but unfortunately, they are not seen there by electron microscopy;

(m) vesicles are believed to be present on the matrix side of the inner mitochondrial membrane, and to contain F_1ATPase . These vesicles are large enough to be seen by electron microscopy after the mitochondrial fraction has been treated with (or 'disrupted' by) sonication, trypsin or urea, but are not seen in 'intact' mitochondria.

The detailed criticisms by Williams, Ling, Malpress and Wainio (*ibid*) merit careful consideration, but these authors all agree that oxidative phosphorylation takes place in mitochondria, that the mitochondrial membranes consist of inner and outer components, that one can measure or calculate the potential differences across two mitochondrial membranes, and that inhibitors diminish only single enzyme activities *in vivo* or *in vitro*. It would seem that there are so many serious uncertainties about the chemiosmotic hypothesis that it must be regarded as doubtful. If one does not accept the reservations above (a) to (m), the views of its antagonists (q.v.) offer real alternatives. If one does, it has to be accepted that a large number of control experiments need to be carried out, before deciding upon the validity of the hypothesis.

Chapter 48
Free Radicals

Free radicals are chemical species, which possess unpaired electrons, and can have an independent existence. Their life spans are from 10^{-13} seconds to one second. They had been recognised as a phenomenon for many years, but the interest of biologists was aroused by a seminal paper of Gershman, Gilbert, Nye, Dwyer and Fenn, (1954). They drew attention to the similarities between the effects of oxygen poisoning with those of ionizing radiation. This resulted in a huge expansion of interest in the measurements of free radicals and their identification, study of their roles in metabolism, disease and toxicity, and attempts to counteract their effects in disease by the use of anti-oxidants.

A list of free radicals found in mammalian tissues is given (Table 103). They can be classified chemically (Table 104). Their short lives mean that they are often detected and measured by their effects on other substances (Table 105). The multiplicity of methods of measurement indicates that they constitute a very heterogeneous group. However, a large number of normal substances, systems and phenomena, are also believed to be affected by them (Table 106).

Free radicals active in mammals

Hydrogen	Chlorine
Hydroperoxy	Carbon dioxide radical
Hydroxyl	Cysteine
Superoxide	Nicotinic acid dinucleotide
Ozone	Thiyl
Nitric oxide	Perthiyl
Nitrogen peroxide	α – tocopherol
Alkoxy	Iron
Peroxy	Copper

Table 103. These radicals are listed in Waters, (1973), Hay, (1974), Ement and Michelson, (1982), Das and Essman, (1990), Halliwell and Gutteridge, (1999), Wiseman et al, (2000), Chieuh, (2000) and Droke, (2000).

Classification of radical reactions

<i>Unimolecular reactions</i>
radical fragmentations
radical rearrangements
radical cyclizations
<i>Biomolecular reactions between two radicals</i>
combinations
disproportionations
<i>Biomolecular reactions between radicals and molecules</i>
additions
abstractions
substitutions
transfers
<i>Electron transfer reactions of radicals</i>

Table 104. These data are from Nonhebel and Walton, (1974, pages 6 - 9), and Hay, (1974, pages 11 - 31). See also Fossey, Lefort and Sorba, (1995).

Substances and procedures used to detect free radicals

Aldehydes, including malondialdehyde	Hydroxyl radical detection
Anti-oxidants	Iron and iron complexes
Ascorbate	Lipid peroxides
Chlorinating species	Nitric oxides
Conjugated dienes	Peroxynitrites
Cyclooxygenase initiation	Proteins
DNA	Quinoid compounds
Dimethyl sulphoxide	'Scavengers'
Electron spin resonance	Singlet oxygen
Radical trapping	Sulphur-centred free radicals
Ethylene hydroperoxide decomposition	Superoxides
Finger printing	Thiobarbiturates
Free radical initiators	Tocopherol radicals
Hydrogen peroxide	

Table 105. Please see Packer and Glazer, (1990), Holley and Cheeseman, (1993), Halliwell and Gutteridge, (1999) and Wiseman et al, (2000). *Some categories overlap.*

Substances, systems and phenomena, believed to be affected by free radicals

<i>A. Substances</i>	<i>B. Systems</i>
Membrane lipids	Glycolysis
Proteins	Citric acid cycle
DNA	Electron transport chain
RNA	Hexose monophosphate shunt
Glyceral 3 phosphate dehydrogenase	Renin angiotensin system
Pyruvate dehydrogenase	Lungs
Fumarase	Liver
Aconitase	Microsomes
α -ketoglutarate dehydrogenase	Cytochrome P 450 systems
Superoxidase dismutase	Mitochondria
Steroids	Nitric oxide system
Cholesterol	Microglia
Fatty acids	Dopamine neurons
Prostaglandins	Long term potentiation in hippocampus
Glutathione peroxidase	
Leukotrienes	<i>C. Phenomena</i>
Caspases	Apoptosis
Urate	Signals
Porphyrinogens	Triggers
Thiols	Messengers
Toxins	
Drugs	
Xenobiotics	

Table 106. The data for this table are derived from Crastes de Paulet, Douste-Blazy and Paoletti, (1990), Sies, (1991), Féher et al, (1993), Halliwell and Gutteridge, (1999), Wiseman et al, (2000), Chieuh, (2000), Droke, (2002). *It can be seen that free radicals are believed to affect very many systems in normal metabolism.*

A few conditions are believed to be caused by a high oxygen concentration, defined as more than 105 mm Hg. This is approximately the partial pressure of oxygen in saturated arterial blood. Retrolental fibroplasia, which may cause retinal degeneration in neonatal babies, is believed to be due to excessive exposure to oxygen (Silverman, 1980). Hyperbaric oxygen can cause convulsions in rats, mountaineers and deep sea divers (Balentine, 1982; West, 2000). Of course, these are quite

unnatural circumstances, and it is difficult to distinguish between the effects of high pressure and those of oxygen toxicity in these conditions.

In the 1960's and 1970's, hyperbaric oxygen was used in the treatment of heart failure, respiratory failure, cancer, intermittent claudication and multiple sclerosis. This seems logical if the partial pressure of oxygen in the inspired air or in the blood were low. However, in both cancer and multiple sclerosis, the partial pressure of oxygen in the blood is usually normal, that is near 100% saturation, so that increased pressure could only increase the concentration of oxygen, *dissolved* in the blood, which at atmospheric pressure is only about 0.3 ml in 100 mls of blood. At 3 atmospheres this would only be increased to 0.9 ml, compared with approximately 19 ml associated with haemoglobin. Thus, increasing the pressure of inspired oxygen does not have a large effect on the concentration in the blood, so that hyperbaric oxygen is unlikely to have a significant effect in the presence of normal arterial pO_2 . High concentrations of oxygen have been shown to diminish succinic dehydrogenase and cytochrome oxidase in vitro (Stadie and Haugaard, 1945); but it is difficult to know the relevance of these findings to intact animals.

Diseases and conditions in which free radicals are reported to be involved

Acute respiratory disease syndrome	Cystic fibrosis
Ageing	Cytochromic P450 diseases
Alcoholism	Diabetic mellitus
Alzheimer's disease	Drug effects
Amyotrophic lateral sclerosis	Dupuytren's contracture
Atherosclerosis	Exercise induced oxidant damage
Autoimmune diseases	Fanconi's anaemia
Autoxidation	Freezing injury
Behcet's disease	Gastric ulcers
Car exhaust pollution	Hyperbaric toxicity
Carbon tetrachloride toxicity	Immersion injury
Cerebral injury	Inflammation
Chemical carcinogenesis	Ionising radiation
Intestinal injury	Polydermatomyositis
Ischaemic heart disease	Preterm infant disease
Keshan disease	Regional ileitis
Kidney failure	Reperfusion injury

Limb ischaemia	Retrosternal fibroplasia
Lipofuscinosis	Rheumatoid arthritis
Liver damage	Shock related cell injury
Liver regeneration	Sickle cell anaemia
Liver transplant complications	Sjögren's disease
Malignant diseases	Spanish 'cooking oil' syndrome
Multiple sclerosis	Systemic lupus erythematosus
Mutagenicity	Ulcerative colitis
Parkinson's disease	Viral diseases
Periodontal disease	

Table 107. These come from the same sources as Table 106. *Note their diversity at aetiologies.*

In addition to the normal chemistry believed to be associated with changes in free radicals, there are also a large number of conditions and diseases, in which free radicals are reported to be 'involved' (Table 107). One must draw the same conclusion from the list, as from others, that the variety of aetiologies of these conditions is such that it is extremely unlikely that derangements of the free radicals *cause* the diseases, it is much more likely that they are the *consequences* of the diseases. Cheeseman and Slater (1993, pages 481-493) noted that "it is essential that the role of free radicals in the causation of disorders, and their production as a consequence of disorders, be clearly distinguished. To do this, it is necessary to examine the time course of free radical production." Unfortunately, this is difficult when the life span of the radicals is so short, and the measurements take so much more time.

One of the expectations of the view that free radicals cause diseases, is that antioxidants such as ascorbic acid, would be effective against many of these diseases, (Krimsky, 1992). They have not been so, and Halliwell and Gutteridge, (1984) concluded that the radicals probably increased as a consequence of 'tissue damage', rather than a cause of it. Perhaps, it would be uncharitable to seek a precise definition of 'tissue damage'.

One must ignore the evidence of changes of free radicals in normal metabolism (Table 106), if one wishes to claim that as free radicals they have a special, or, indeed, any, role in the genesis of disease. This does not mean to say that study of the individual reactions in which the free radicals change is not useful, but it

does throw doubt on the idea that free radicals have an important 'particular' or 'general' role in either normal metabolism, or in disease. Nor will the continued study of anti-oxidants and 'scavengers' necessarily add to understanding of the diseases, or to the design of drugs against them.

Chapter 49

Inflammation

This may be defined as a syndrome resulting from the subjection of parts of the body, without killing the animal, to such agents as, mechanical damage, infection, corrosive substances, allergens, freezing, burning, ultraviolet or ionizing radiation, (Hurley, 1983, pages 1 - 3). The syndrome was described more than 2000 years ago, as redness, swelling, heat, pain and loss of function.

Cohnheim (1893) found that inflammation caused the arterioles of frogs' tongues to dilate, and increased their blood supply ten times. The heat is believed to be due to: increased blood flow; increased metabolism of the surrounding tissue, particularly muscles; release of pyrogens; and friction of the adjacent muscles. The pain is believed to be due to pressure on stretch receptors, and stimulation of pain receptors in the region of the inflammation. Subsequently, it was shown that bradykinin was liberated locally.

The loss of function includes such phenomena as skin no longer acting as heat insulation, lungs no longer able to exchange gases, muscles no longer able to contract, followed by invasion of local bacteria. Acutely, a patient has increased number of white cells, and local swelling. Plasma proteins leak through increasingly permeable capillaries into tissues (Starling, 1896; Lewis, 1927). Values were assigned for the osmotic pressure exerted by the surrounding tissues and the plasma proteins, and the hydrostatic pressure arising from the pumping by the heart. Starling (*ibid*) put forward the currently accepted model that damaged capillaries allow plasma proteins to 'leak' into the tissues where they increase the osmotic pressure which causes local swelling.

Fluid is extravasated from the regions of the inflammation. It contains many 'mediators' including bradykinin, histamine, kallikrein, complement, and many other substances (Table 109). These individual substances, when isolated, have been shown to: increase cellular permeability; lyse cells; coat bacteria with opsonins; cause antigens to react with antibodies; lyse infectious agents; and induce phagocytosis. Some of the exudate, dead white cells and organisms, is carried to local lymph nodes. There, it may be trapped and phagocytosed, or pass into the blood causing bacteraemia, septicaemia or toxæmia. Cells which are engulfed are believed to enter vacuoles or 'phagosomes' in cells, according to De Duve, (1984, page 55). These

phagosomes are joined to lysosomes.

Compounds involved in, and steps of, inflammation

<i>System</i>	<i>Effects</i>
Production of platelet activating factor	releases serotonin from platelets
Release of serotonin and histamine	increases vascular permeability
<i>Kinin system</i>	
Hageman factor XII	binds to negatively charged blood vessels to produce Hageman factor XIIa; clots blood
Hageman factor XIIa, plus prekallikrein	produces kallikrein in plasma; feeds back to Hageman factor XII
Kallikrein in plasma, tissues and secretions	cleaves kininogen; activates fibrinolysis; activates CLq in complement system
<i>Thrombus formation</i>	
Platelets, plus thrombin plus plasma	produce fibrinogen, then fibrin breaks down to peptides
<i>Fibrinolytic system</i>	
Plasminogen activated by plasminogen activators, urokinase, streptokinase, trypsin, splits	produces plasmin, cleaves C3; vasodilates; activates complement;
macrophages, plasminogen activators activates kinin system	Hageman factor XIIa subunits;
<i>Complement systems</i>	
'Classical' pathway of 22 components, mostly Cs, cascade (Figure 70)	releases histamine; causes chemotaxis; opsonises receptors on neutrophils, macrophages and eosinophils; increases membrane permeability of target cells; vasodilation; lysis of 'target' cells
Alternative pathway involving 10 new components is activated by causes lipopolysaccharides, endotoxins, aggregated IgM or IgG antibodies, antigen with IgA complexes or plasmin; inactive properdin is activated to properdin, which in the presence of factor D cleaves factor B to generate C3b. B, and converts C3 to C3a and C3b, which can feed into the classical pathway.	produces opsonins on neutrophils, eosinophils and macrophages; causes chemotaxis of all granulocytes; lysis of 'target cells'
<i>Eicosanoids</i>	
Cyclooxygenase generates PG _{1₂} , PGF _{2_a} PGE ₂ , PGD ₂ and thromboxane A ₂	produce vasodilation; increase vascular permeability; hyperalgesia; fever; smooth muscle contraction; bronchoconstriction; vasoconstriction and platelet aggregation

<i>System</i>	<i>Effects</i>
Arachidonic acid is acted upon by 5 hydroperoxyeicosatetraenoic acid (5HPETE) and converts it to LTA ₄ , or LTC ₄ , LTD ₄ , & LTE ₄	chemotaxis; vasoconstriction; bronchospasm; increased vascular permeability
<i>Platelet activation factor</i>	
Is generated by inflammatory cells, endothelial cells and injured tissue cells	causes aggregation of platelets; release of serotonin; vasodilation; increase in vascularity; enhances arachidonic acid metabolism
<i>Nitric oxide system</i>	
Endothelial cells produce nitric oxide	relaxes smooth muscle
<i>Neurogenic inflammation</i>	
Nerve fibres produce calcitonin gene-related peptides and substance P	causes vasodilatation; increases vascular permeability
<i>Cytokines</i>	
20-30 different ones (Goldsby, et al, 2000, pages 308-312)	change in vasomotor tone, permeability of blood vessels; chemotaxis; antibody secretion; growth and differentiation
<i>Others</i>	
Calcitonin gene related peptides	vasodilation
Factor P	increased vascular permeability
O ₂ free radicals	increased vascular permeability

Table 108. These systems and compounds are all believed to be involved in acute inflammation (please see Clark, 1986, pages 160-176; 415-500, Rocha e Silva and Garcia Lerne, 1972, Trowbridge and Emling, 1997, pages 19-39 and Roitt, Brostoff and Male, 2001, page 62). Most of them are believed to be produced by white blood cells, macrophages, plasma, or endothelial cells. No less than 80 different substances listed here are regarded as mediators. Several cause vasodilatation, clotting and complement. The substances are produced in the blood, bone marrow, liver, adrenal cortex and brain (Goldsby, Kindt and Osborne, 2000, page 386).

Hurley, (1983, page 102) summarised the evidence for endogenous mediators:

- (i) the similarity of the responses of mediators to a wide variety of causes of inflammation;
- (ii) some of the responses of inflammation are immediate and some are delayed;
- (iii) there is a latent period between a injury and an inflammatory response;
- (iv) injured tissues contain endogenous factors which act on blood vessels in a similar way, as they do in the localities of injuries.

Many of the white cells die and mix with the extravasate and dead pieces of tissue. These may constitute a liquid pus. If it causes a local reaction of fibrous tissue, a cyst may be formed. If there is no barrier the infection may invade the blood vessels. The cyst may contain either live or dead organisms. If an infection should be located near a duct, the intestine or the skin, it may erupt to the surface and a sinus may result. Occasionally, inflammation obstructs blood vessels.

Inflammation may be local (Table 109) or spread throughout the body (Table 110). There is a wide range of causes, effects and treatments. The treatments vary from removing allergens, administering antipyretic drugs, application of heat by poultices, immobilisation of affected areas, use of antibiotics, application of steroids, administration of local anaesthetics, etc. There are many inflammatory diseases (Table 111).

Local causes of inflammation and their treatment

<i>Cause</i>	<i>Treatment</i>
Laceration	zinc oxide; calamine lotion; dressing
Local infection	heat; cold; dressing; antibiotic
Minor bites	poultices; anti-histamines; local anaesthetics
Hay fever	anti-histamines
Asthma	theophylline; adrenalin
Venous obstruction	remove obstruction or parasite; thrombolysis
Gangrene	systemic antibiotics; debridement; excision of tissue
Mechanical damage	symptomatic; immobility

Table 109.

General inflammation and its treatment

<i>Cause</i>	<i>Treatment or inhibitors</i>
Antigens	antibodies
Infections and wounds	antibiotics; debridement
Toxins	antitoxins; stimulators of excretion
Fever	antipyretics; antibiotics
Fungi	antifungals
Parasites	anti-parasitic drugs
Viruses	antiviral drugs; lysozymes; interferons
Insect bites	carrionides

<i>Cause</i>	<i>Treatment or inhibitors</i>
Scorpion bites	local anaesthetic
Urticaria	anti-histamines; calamine lotion
Arthritides	ibuprofen; steroids; aspirin; paracetamol; indomethacin;
Radiation	sodium iodide; fluid replacement
Irritation by particles	withdraw particles
Burns, strong acids, alkalis	local washing, cooling and dressings
Angioneurotic oedema	psychotherapy
Anaphylaxis	adrenalin
Histamine	anti-histamines
Bradykinin	symptomatic
Prostaglandins	non-steroid analgesics
Complement	pharmacological inhibitors
Mast cells	
Allergies	avoid allergies, anti-histamines; adrenalin

Table 110. Please note the variety of causes, effects and treatments of inflammation.

Inflammatory diseases

Abscesses	In	Hepatitis	In
Acne	In	Herpes zoster	In
Allergies	Im	Ileitis	Im
Allograft rejection	Im	Influenza	In
Anaphylaxis	Im	Lymphangitis	In
Apoptoses	Im	Mastitis	In
Appendicitis	In	Meningitis	In
Arteritis	NK	Nephritis	In
Arthritis	Im	Orchitis	In
Asthma	Im	Osteomyelitis	In
Atherosclerosis	NK	Osteoporosis	NK
Atopic dermatitis	Im	Pancreatitis	In
Autoimmune liver disease	Im	Pleurisy	In
Blepharitis	In	Pneumonia	In
Boils	In	Pneumonic plague	In
Botulism	In	Psoriasis	NK
Bubonic plague	In	Rejection of transplant	In
Cancer metastasis	NK	Rhinitis	Im, In

Cholecystitis	In	Salpingitis	In
Common cold	In	Scarlet fever	In
Conjunctivitis	In	Septicaemia	In
Connective tissue disease	Im	Sinusitis	In, Im
Dermatitis	C, In, M	Sjögren's disease	Im
Eczema	Im	Small pox	In
Encephalitis	In	Synovitis	M, In
Endocarditis	In	Syphilis	In
Fasciitis	In	Systemic lupus erythematosus	Im
Free radical diseases	FR	Thermal injury	C
Gastritis	M, In	Thyroiditis	Im
Gastroenteritis	In	Tonsillitis	In
Glossitis	M, In	Vaginitis	In
Gonorrhoea	In	Vasculitis	Im
Hay fever	Im		

Table 111. Some of these are from Ward, (1983), Kay, (1987), Whicher and Evans, (1992), Morgan and Marshall, (1999), and Gordon-Letts and Morgan, (2000). In = infection; Im = immunological; M = mechanical; C = chemical or physical; FR = free radical; NK = not known.

The listing of the causes, effects and treatments of all these diseases raises several fundamental questions. The first and most important arises when one examines Table 111. Is one to suppose that 80 different mediators are released, a large number of tissue reactions occur (some of them several times), some of the reactions are opposite to those caused by other mediators, whenever a sensitive child swallows peanuts, a patient is allergic to penicillin, a limb swells up from a bee sting, or a patient suffers from an attack of asthma. Is one to suppose that the whole repertoire of reactions listed in Table 108 is performed within seconds of the patient being inflicted by any of the conditions listed in Tables 109 to 111? Each of the 'mediators' and enzyme systems must be synthesised, controlled by local small molecules, ions and co-factors, and broken down by other enzymes. Furthermore, inflammation and inflammatory diseases have such a wide variety of aetiologies and empirical treatments, that it seems extremely unlikely that they would each trigger the same series of chemical reactions listed in Table 108.

The latter take a few seconds to occur, but, unfortunately, the experiments to demonstrate them take minutes to hours, so one can not know in intact animals in which order the reactions occur, nor their rates. Finally, in attempting to elucidate the properties of living cells one has the same problems as those which bedevil so much of experimental biochemistry, namely the procedures of subcellular fractionation (chapter 7), and the problems inherent in histochemistry and in electron microscopy (chapter 8,9).

In summary, one may say that the concept of inflammation, described as a clinical syndrome so long ago, is still valid. The extensive experimental studies during the late 19th and the whole of the 20th century mainly in vitro, show many of the possible chemical and pathological changes, which may occur in inflammation of various kinds, but they do not give a clear picture of what particular chemical reactions may be generalised, nor how necessary each of them is to the overall reactions, nor whether it is more useful to study each kind of inflammation, or regard it as a unifying phenomenon.

Chapter 50

Phagocytosis

Phagocytosis is the engulfment of solid particles, such as bacteria, tissue debris and dead blood cells, into the cytoplasm. It was first described in starfish larvae by Metchnikoff, and subsequently seen in most mammalian tissues, usually *in vitro* (Metchnikoff, 1892). Pinocytosis is a similar phenomenon, in which droplets are ingested (Chapman-Andresen, 1977). Its main importance is that in mammals, fats are believed to be absorbed by this mechanism in the small intestine.

Phagocytosis is believed to be a property of structurally simple cells, phagocytes (Table 112). Particles are seen outside cells and in cytoplasm, and it is assumed that they have crossed the cell membranes. Of course, the event could not be seen happening by histology or electron microscopy. The following steps are believed to occur: (i) the phagocytes cause chemotaxis, that is, they produce agents which attract dead or foreign tissue; (ii) the particles adhere to the outsides of the phagocytes; (iii) they cross the cell membranes into the cytoplasm (endocytosis); (iv) vacuoles appear and the particles enter them; (v) the vacuoles are called 'phagosomes'; (vi) the phagosomes associate with lysosomes and the joint bodies are called 'phagolysosomes' (De Duve, 1984, pages 71 - 72); (vii) the particles are 'digested' in the vacuoles, or (viii) those that are not digested are expelled back into the extracellular compartment; this is called 'exocytosis', or 'defaecation' by De Duve, (*ibid*). In addition to what is believed to be a natural phenomenon, the entry of small particles of glass, iron filings, latex spheres and pollens have been studied experimentally. The cells can be injected, heated, cooled, subjected to magnetic fields and centrifuged, (Heilbrunn, 1956; Chambers and Chambers, 1961; McClung-Jones, 1967).

Phagocytes

<i>Professional</i>	<i>Non professional</i>	<i>Non phagocytic functions of phagocytes</i>
<i>Blood and bone marrow</i>	<i>Blood</i>	<i>Secret</i>
neutrophils	lymphocytes	hydrolases
eosinophils	platelets	colony stimulating factors
monocytes		transferrin
macrophages	<i>Mucous membranes</i>	erythropoietin
sinusoidal lining cells	epithelial cells	prostaglandins

<i>Professional</i>	<i>Non professional</i>	<i>Non phagocytic functions of phagocytes</i>
		T -cell stimulators
		B - cell stimulators
		Interferon
<i>Bone</i>	<i>Tissue</i>	<i>Compounds produced</i>
osteoclasts	mast cells	β -cell stimulation interferon complement compounds
<i>Peyer's patches</i>		<i>Extracellular killers of</i>
microphages		macrophages
<i>Connective tissue</i>		tumour
histiocytes		
macrophages		
monocytes		<i>Antigen presentation</i>
<i>Liver</i>		
Kupfer cells		
<i>Lung</i>		
alveolar macrophages		
pleural monocytes		
<i>Lymphatic system</i>		
free macrophages		
fixed macrophages		
monocytes		
<i>Spleen</i>		
free macrophages		
fixed macrophages		
sinusoidal lining cells		
<i>Thymus</i>		
free macrophages		
fixed macrophages		
monocytes		
<i>Inflammatory sites</i>		
macrophages		
epitheloid or multinucleate		
giant cells or Langerhans cells		

Table 112. *These kinds of cells mostly occur in the blood, lymphatic system, reticuloendothelial system, and connective tissue.* They are listed by Van Oss, Gillman and Neumann (1975), Van Oss, (1986) and Van Furth and Willenzee, (1979).

Parameters measured for phagocytosis

- Microscopic observations of particles such as carbon black, latex beads and bacteria extracellularly and in the cytoplasm
- Examination of stained sections, including histochemistry
- Autoradiography of particles
- Isotopic labelling of bacteria
- Flow cytometry
- Ligand binding of receptors
- Monoclonal anti-receptor antibodies
- Oxygen consumption
- Glycolysis
- Bacteriocidal activity of cells
- Culture of monocytes
- Surface tension of blood cells, bacteria and proteins
- Measurement of adhesion of cells or particles
- Observations on freezing fronts
- Sedimentation volume
- Lipid content
- Horse radish peroxidase assay
- Superoxide production
- Reduction of nitro-blue tetrazolium
- Assay of 'phagosome - lysosome' fusion

Table 113. These are listed by Di Sabato and Everse, (1986). *They are all believed to be measuring the same property.*

Direct examination by light microscopy of the phenomenon in living mesenteries, web and blood vessels, is probably the best technique (Krogh, 1929). It is assumed that observations in phagocytosis in tissue culture are relevant to movements in life (Chapter 12), although the latter do not contain capillaries.

Several different techniques are used to demonstrate phagocytosis experimentally (Table 113). These may be conceived either as techniques analysing one phenomenon, or several consequences of the one process, but the majority of these measurements can not be considered as exclusive methods to demonstrate the phenomenon of phagocytosis.

The biochemistry of phagocytosis has been studied extensively (Rossi and Patnarca, 1982).

A distinction was made between 'professional' and 'non-professional' phagocytes (Table 112). The 'main function' of the former is phagocytosis, while that of the latter have other 'main functions'. The former engulf and digest particles,

whereas the latter only ingest them. This distinction seems to me to be highly unprofessional.

Each step of phagocytosis can be examined more closely. Chemotaxis is regarded as being due to the release of a chemotactic agent. Yet, although agents may be released, particles could enter cells, if : the extracellular particles have an opposite charge to the cytoplasm; if there is a potential difference across the cell membrane; if some substance from the extracellular space crosses the cell membrane and precipitates any of the contents of the cytoplasm.

Although it is usually said that a particle has to adhere to the outside of a cell membrane to enter it, this is not at all necessary. Indeed, if it did so adhere, this would slow such passage. Of course, this does not mean that the mechanism of this is known. ‘Endocytosis’ is a neo-classical term to describe the entry, but it is not a mechanism. Particles could cross the membrane by: (a) the hydrostatic force in capillaries from the pumping of the heart or of the adjacent tissue. The membrane would have to be rather elastic and contain a self-sealing mechanism; (b) the particle entering the membrane would liberate one or several of the ‘mediators’ (Table 109), which would make sufficiently large holes in the cell membrane to enable the particles to pass through. However, iron filings, glass particles and latex balls would not contain such mediators; (c) it may be that in life, the cytoplasm, or the contents of the vacuoles do, in fact, break particles down to aminoacids, fatty acids, simple sugars and salts, to which the cell membrane is indeed permeable. On the other hand, in life, particles have been shown to enter the reticulo-endothelial system (Hoffman and Von Recklinghausen, 1867; Aschoff, 1924); (d) another possibility is that phagocytosis in vitro is different from that in vivo. In vitro, it may be that the cell membranes are more elastic and self-sealing than they are in the intact animal. Phagocytosis there could result from Brownian movements of the particles. In vivo, it is of interest that the reticulo-endothelial system consists of such tissues as lymph nodes, Kupfer cells and Peyer's patches (Table 112). Many of these are syncytia (Table 25). It should be noted that it is very likely that the spaces around stained neurons, the sinuses in lymph nodes and liver, and spaces in the Bowman's capsule are all shrinkage artifacts. In the original demonstration of the reticulo-endothelial system (*ibid*), particulate materials injected into veins ended up in sinuses, in connective tissues, and in lymph nodes - that is, in syncytia. They may have travelled from the plasma to the syncytia, without going through the cytoplasm of cells surrounded by cell membranes.

It is rather remarkable that the cytoplasm of lymphocytes, hepatocytes, histiocytes and many other phagocytes (Table 112) appears empty of granules, although mitochondria are often seen. The cytoplasm of the neutrophils, eosinophils and basophils in the blood does contain a few granules, but it is not crowded (Sandoz, 1952; Britton et al, 1969).

Vacuoles can be seen in living tissue cultures, and in gangrenous tissue. Dying cells in culture often show blebs, while the appearance in gangrene is believed to be due to the release of gas. However, the existence, dimensions or frequency, of these features can not be assessed, if tissue has been stained heated, rapidly frozen, or dehydrated, since gases evolve. It is not always appreciated that it is extremely difficult to distinguish by light or electron microscopy between, on the one hand, an *interface* between a vacuole and cytoplasm, and, on the other hand, the presence of a *cell membrane*. Both of them diffract light. Heavy metals deposit on both of them, and there may well be potential differences between the two phases on either side of them. Nevertheless, the vacuoles in the cytoplasm of unicellular organisms and cells in tissue culture seem to be immiscible - initially, at least - with the rest of the cytoplasm, and with the extracellular fluid.

Thus, in summary, one may pose the question whether phagocytosis as originally described by Metchnikoff may only occur in unicellular organisms, low metazoa and tissue culture. In intact mammals, particles may enter cell membranes by unknown mechanisms and be digested, or may remain extracellular until they are trapped in a syncytium. Presumably, undigested particles are coughed out, or excreted into the bile or faeces.

Chapter 51
Immunity and Autoimmunity

The philosophy of immunology is that animals live in a hostile world, and have ‘defence’ systems to prevent ‘foreign’ materials ‘damaging’ them. The immunological systems are part of a continuous ‘defence’ force, including inflammation, antibody production, and the reticulo-endothelial system. This is reflected in the overlap of these systems, and the broad field of study of immunology (Table 114). Their common properties include: location in the reticulo-endothelial system; presence of liver co-factors; involvement of white blood cells; presence of inflammation; fixation of complement; immune responses; cytokine involvement; presence of fluorescence; involvement of mediators; causation by antigens, allergens, toxins, drugs and foods; suppression by steroids.

There is a considerable literature on immunology, as well as several journals (see, for example, Burnet and Fenner, 1941; Burnet, 1959; Burnet, 1969, a,b; Burnet, 1972; Rose and Bigazzi, 1973; Golub, 1977; Barrett, 1978; Kimball, 1987; Lewis and McGee, 1992; Bock and Goode, 1998; Roitt, 1999; Kuby, 2000; Janeway et al, 2001). Those publications are profusely illustrated with colourful diagrams and flow charts. Electron micrographs of structures, which authors believe to be involved, are rather rare. They also tend to illustrate fractions more often than intact systems. A list of the tissues and cells believed to be part of the ‘defence’ systems of the body shows how ubiquitous they are (Table 115).

Subjects studied by immunologists

Diagnosis of disease	Immunosuppression
Vaccination and immunisation	Haemolysis
Blood groups	The complement system
Rhesus factor in blood	Autoimmune diseases
Inflammation	Immuno-deficiency diseases
Sensitivities to animals	Immunofluorescence
Drug allergies	Immunocytochemistry
Food allergies	Monoclonal antibodies
Tissue transplantation and rejection	Anaphylaxis
The immune response	Cancer immunology

Table 114. All these phenomena are believed to share common mechanisms.

‘Defence’ systems of the body

<i>Tissues</i>	<i>Cells</i>
Bone marrow	Haemopoietic cells B lymphocytes Megakaryocytes
Blood	T, B and Killer lymphocytes (Schmidt, 1990) Plasma cells Neutrophils Eosinophils Platelets Mast cells Monocytes Macrophages
Thymus	Thymocytes
Liver	Hepatocytes Küpfer cells
Spleen	Fixed and mobile macrophages
Skin	Langerhans cells
Tonsils and adenoids	Macrophages Lymphocytes
Brain	Microglia
Lung	Alveolar macrophages
Bone	Osteoclasts
Kidney	Mesangial cells
Connective tissue	Histiocytes
Peyer’s patches	Macrophages and lymphocytes
Reticulo endothelial system	Blood cells
Many tissues	Lymphocytes Dendritic cells (Lotze and Thomson, 1999) Connective tissue cells

Table 115. The appearances of these cells are remarkably similar. They are identified by the tissues in which they are found and their immunological properties.

It is useful to highlight some of the important steps in the development of immunology before summarising current views on the subject. Fortunately, an extensive literature facilitates the task (see, for example, Ehrlich, 1957; Barrett, 1978;

pages 3-21; Clark, 1986; Bibel, 1988; Silverstein, 1989; Brent, 1997; Mazumdar, 1989, 1995; Tansey, Willhoft and Christie, 1997; and Cruse and Lewis, 2005). This section concentrates on cellular aspects of immunology.

The idea of immunity goes back to Thucydides in the 6th century b.c.e., and the signs of inflammations probably originated from Celsus in the first century, a.d. (Silverstein, 1989, page 2). Jenner's vaccination of children against small pox was the first published account, although he probably heard of the practice in Turkey (Jenner, 1798). Pasteur, Chamberland and Roux, (1880) did the first control experiments in immunology, and Metchnikoff's demonstration of phagocytosis in starfish larvae has already been mentioned. He proposed the phagocytic theory of immunity. Ehrlich, (1901, reprinted in Himmelweit and Marquardt, 1957, pages 298-315) suggested that the body contained mechanisms, which suppressed its reaction against its own components - that is, it had a 'fear' of its own self-produced toxins. He called it the 'horror autotoxicus' (for discussion, see Silverstein, 1989, pages 160-189). This speculation proved to be the theoretical basis for autoimmunity. Buchner, (1889) discovered alexin, which was subsequently renamed 'complement'. Landsteiner, (1900) described the ABO system of blood groups, which made blood transfusion into a practical procedure. Portier and Richet, (1902) demonstrated anaphylaxis in guinea-pigs. Uhlenhuth, (1903) was the first to suggest that different tissues of the same animal could produce different antigens. Donath and Landsteiner, (1904) characterised paroxysmal cold haemoglobinuria, then believed to be an autoimmune disease, but now thought of as being due to 'cell stress'. Krusius, (1910) reported the autoantigenicity of lens protein.

Coons, Creech and Jones, (1941) introduced fluorescent antibodies. Medawar, (1944) proposed a theoretical basis for the immunology of transplant rejection, and Billingham, Brent and Medawar, (1953) described immunological tolerance. Burnet, (1957) put forward the idea that each lymphocyte 'expressed' preformed membrane receptors, to which antigens bind, causing the cell to proliferate in a clone, each element of which was similar to the parent cell - the 'clonal selection theory of antibody formation.' Roitt et al, (1956) showed that Hashimoto's disease was an autoimmune disease (Rose and Witebsky, 1960). See also Dopheide et al, (1969).

Miller, (1963) suggested that some lymphocytes (T lymphocytes) produced by the thymus had subsets (Gowans, 1965; Gowans and McGregor, 1965; Claman, Chaperon and Triplett, 1966) including helper T cells (Mitchell and Miller, 1968) and

suppressor T cells (Gershon and Kondo 1970). Antibodies may be produced synergically (Claman, Chaperon and Triplett, 1966).

Phagocytosis is considered to be the cellular part of the defence system while the antigen-antibody reaction is generally held to be the ‘humoral’ part of the body’s defences. The antigen causing the reaction may originate from outside the body, for example, toxins, drug, anaesthetic, or food, or from a molecular change within a tissue or organ; the latter causes it to create antibodies to itself. The latter are believed to cause a group of autoimmune diseases (Table 116). Whereas antigens were originally considered to be only single proteins, now it is claimed that there are many non-protein antigens, including polysaccharides, nucleotides, and drugs, (Table 117). Monoclonal antibodies are also purified, and are invaluable in diagnosing a range of diseases (Sikora and Smedley, 1984; Godin, 1986; Day, 1990; Epenetos, 1991; Bronson, Alexander, Anderson et al, 1995; Leong, Cooper and Leong, 2003).

Diseases believed to be autoimmune and their antigens

<i>Disease</i>	<i>Antigen</i>
Hashimoto's thyroiditis	thyroglobulin
Primary myxoedema	CA ₂ - colloid antigen cytoplasmic or cell surface
Thyrotoxicosis	probably cell surface
Myocardial infarction	heart tissue
Pernicious anaemia and gastric atrophy	intrinsic factor parietal cell microsomes
Diabetes mellitus	pancreatic β cells, glutamic acid decarboxylase
Addison's disease	cytoplasm of adrenal cells, 17 and 21 hydroxylases
Premature onset of menopause	cytoplasm steroid-producing cells
Male infertility	spermatozoa
Multiple sclerosis	myelin antigens
Goodpasture's syndrome	glomerular and lung basement membrane
Pemphigus vulgaris	desmosomes between prickle cells in dermis
Pemphigoid	basement membrane
Phagogenic uveitis	lens
Sympathetic ophthalmia	uvea
Evans syndrome	(not known)
Autoimmune haemolytic diseases	erythrocyte membranes
Idiopathic thrombocytopenic purpura	platelets
Primary biliary cirrhosis	mainly mitochondrial pyruvate dehydrogenase

Disease	Antigen
Active chronic hepatitis	hepatocytes
Rhesus haemolytic disease of the newborn	Rhesus factor
Autoimmune hepatitis	anti-liver, kidney microsomes
Autoimmune sclerosing cholangitis	anti extractable nuclear antigen and anti liver, kidney microsomes
Parathyroid disease	parathyroid tissue
Wegener's disease	cANCA
Polymyositis	muscle
Psoriasis vulgaris	streptococcal and skin antigens
Celiac disease	transglutaminase
Schizophrenia	
Syphilis	Wasserman antigen
Multiple myelomatosis	
Rheumatoid arthritis	altered IgG, connective tissue
Premature ovarian failure	
Narcolepsy	
Guillain-Barré syndrome	gangliosides
Juvenile chronic arthritis	IgM
Glomerulonephritis	myeloperoxidase
Nephrotic syndrome	quartan malaria
Adrenalitis	adrenal cortex
Vasculitis	cytokines
Acute lymphoblastic leukaemia	
Idiopathic haemochromatosis	
Behcet's syndrome	
Vitiligo	tyrosinase
Sjögren's syndrome	anti-Rho
Discoid lupus erythematosus	nuclei
Dermatomyositis	altered IgG
Scleroderma	nucleoli
Systemic lupus erythematosus	DNA, nucleoprotein, cytoplasmic soluble antibody, blood clotting factors, altered IgG, Wasserman antigen
Mixed connective tissue disease	nuclei and nucleoli
Mooren's ulcer	
Sympathetic ophthalmia	uvea

Disease	Antigen
Diffuse parenchymatous lung disease	
Antiphospholipid syndrome	phospholipids
Ulcerative colitis	colon lipopolysaccharide
Myasthenia gravis	acetylcholine receptors
Immune reconstitution inflammatory syndrome	antiviral therapy
Pancreatitis	
Autoimmune lymphoproliferative syndrome	anti- Rho
Graves' disease	thyroid stimulating hormone receptor
Polyarteritis nodosa	hepatitis B antigen in some cases

Table 116. The antigens are believed to come from the parent tissues. The data in this Table come mainly from Burnet, (1972), Rose and Mackay, (1985), Scholtz and Albert, (1990), Roitt and Delves, (1992), Playfair and Lydyard, (1995), Roitt, (1999), Kuby, (2000), and Roitt, Brostoff and Male, (2001). *The antigens may be derived from an organ, tissue, or a subcellular fraction.* Further diseases including immunodeficiencies, may be found in Gütterl, Seakins and Harkness, (1979).

Antigens

<i>Organs and tissues</i>	
Transplanted organs	Neurons
Thyroid	Astrocytes
Adrenal gland	Oligodendrocytes
Stomach	Nerve endings
Ovaries	Skin
Sperm	Colon
Islets of Langerhans	Joints
Blood vessels	Kidney
Skeletal muscle	Lens
Smooth muscle	Uvea
Heart muscle	Red Cells
Lungs	Neutrophils
<i>Subcellular organelles</i>	
Membrane	Food (Brostoff and Challacombe, 1978; Bultriss, 2002)
Mitochondria	Drugs

Nuclei	Dusts
Nucleoli	Industrial chemicals
	Bacteria
<i>Chemicals</i>	Rickettsia
Cell surface antigens	Viruses
DNA	Cell surface antigens
Proteins	Fungi
Polysaccharides	Pollens
Myelin	Worms
Amyloid	<i>Tumour antigens</i>
Leukotrienes	Histocompatibility antigens
Metals	Tumour specific transplantation antigens
	Embryonic antigens
	Differentiation antigens
	<i>Autoantigens (Table 116)</i>

Table 117. These are reported in the literature. The antigens are believed to be located on the outside of the membranes

Human diseases for which immunisation is available

Anthrax	Pneumonoccal meningitis
Antibody deficiency	Poliomyelitis
Botulism	Russian tick-bone encephalitis
Chicken pox	Small pox
Cholera	St Louis encephalitis
Diphtheria	Tetanus
Hepatitis A	Tuberculosis
Hepatitis B	Tularaemia
Influenza	Rocky mountain spotted fever
Japanese B encephalitis	Rota virus gastroenteritis
Measles	Rubella
Meningococcal meningitis	Typhoid
Mumps	Typhus
Neisserial meningitis	Undulant fever
Paratyphoid	Whooping cough
Plague	Yellow fever

Table 118. These diseases are mainly from Fulginiti, (1982) and Salisbury and Begg, (1996).

The entry of ‘foreign’ antigens originating from the environment, such as bacteria, toxins, viruses or mites, (Table 117) initiates an ‘immune’ response (Roitt et al, 1969). Many of the diseases can be prevented by vaccination or immunisation with specific antigens (Table 118). The body responds by producing antibodies which destroy antigens entering it. Antibodies may be injected during the active phases of some of these diseases. The immunity lasts for years in many of the conditions listed. It should be stressed that vaccinations and immunisations are *specific* for that particular disease, except in rare cases, such as the immunity from small pox conferred by cow pox. At any time, the body can be immunised against a large range of common diseases, (Table 118). They neither cause clinical disease, nor interfere with each other.

Many molecules involved in the immune systems are polypeptides, which are believed to be free in the cytoplasm, or bound to the cell membranes. They may be bound to chains of sugars, as glycoproteins, or fatty acids, as lipoproteins. The immune system is generally divided into an adaptive system, which reacts to the arrival of the foreign materials. Phagocytes and natural killer cells are the cells of the innate immune system, while activated lymphocytes producing antibodies are the adaptive immune system. The complex series of events (Table 119) is as follows: foreign material (‘non-self’) enters the body, or a tissue or organ is converted into an antigen in one of the many autoimmune diseases. The foreign material especially but not exclusively protein (Table 117) may enter by inhalation, ingestion, through the skin, or by injection. It binds to (or ‘recognises’) receptors of the innate immune system, activating phagocytes (Chapter 50) which engulf it. Or, it may be killed by natural killer cells, (Ortaldo and Herberman, 1984; Lotzova, 1991); the foreign material may affect B lymphocytes, T killer cells, T helper cells or T immune suppresser cells. The different cells can be separated by their dimensions, optical properties and fluorescence (Dabrowski and Dabrowski-Bernstein, 2000).

The immune response

1. Foreign material enters the body, or a normal tissue or organ is transformed into an autoantigen.
2. In the blood or reticulo-endothelial system it binds to ('recognises') receptors.
3. The innate system of phagocytes (Table 112) or natural killer cells engulf or destroy the foreign material.
4. Alternatively, the foreign material or autoantigen induces a response from the adoptive immune system, composed of B cells, T killer cells, T helper cell with surface immunoglobulins or T immune suppression cells.
5. The B cells become plasma cells which produce antibodies.
6. The B cells have receptors on their surface which combine with major histocompatibility complex; this binds degraded foreign antigens or peptides, and also B lymphocytes, to produce antibodies.
7. Specific antigen-antibody reactions occur.
8. At the same time cytokines are produced by many different cells, and these activate, modify or suppress the immune reaction.
9. The complement system is activated.

Table 119. A summary of the steps in an immune response.

All B cells are believed to have inherited antibodies to all possible antigens to which they might be exposed, and each particular population of B lymphocytes is believed to be covered by its own particular antigens, including ones to which it has never been exposed. It is widely believed that each lymphocyte produces clones (that is, it multiplies to produce exact replicas of itself), when it is activated (Burnet, 1959). The T lymphocytes are believed to be covered *either* by CD4 glycoproteins, which recognise the major histocompatibility complex (class II MHC), *or* by CD8 glycoproteins and Class I MHC. Thus, it is believed that the T lymphocytes have the mediaeval monarchical powers of inducing antibodies, killing cells or suppressing their immune responses. Presumably, there are regulators assigning the relative and absolute rates of these three reactions at any one time.

Some cytokines that modulate the immune response

Name	Source	Target cells / biological activities
Interleukin 1 (IL-1)	Virtually any cell including monocytes, macrophages and endothelium	Chemotactic for monocytes and neutrophils Induces IL-2 production Induces GM-CSF production Induces GM-CSF production responses Increases bone resorption by osteoclasts Promotes endothelial cells binding to leukocytes IL-1α induces fibroblast proliferation

Name	Source	Target cells / biological activities
Tumour necrosis factor - α	Monocytes, macrophages	Mimics many actions of 1L-1 Cytotoxic to some tumour cells
Tumour necrosis factor β	Activated CD $^{4+}$ T cells	CD $^{4+}$ induction of acute phase proteins Activation of phagocytic cells
Interleukin 2 (1L-2)	Activated T cells	Stimulates growth of activated T and B cells Activates and promotes growth of NK cells Activates monocytes
Interleukin 3 (1L-3)	Activated T cells mast cells	Supports growth of pluripotential hematopoietic stem cells Growth factor of mast cells
Interleukin 4 (1L-4)	CD4+ T cells mast cells, bone marrow stroma	Growth and differentiation factor for activated T and B cells Promotes the differentiation of Th2 cells Growth factor for mast cells Increases expression of HLA class 11 antigens on B cells
Interleukin 5 (1L-5)	CD4+ T cells, mast cells	Induces differentiation of activated B cells into antibody-producing cells
Interleukin 6 (1L-6)	CD4+ T cells, mast cells, fibroblasts, macrophages	Enhances maturation of activated T and β cells Stimulates growth of haemopoietic progenitor cells(including preosteoclasts) Inhibits growth of fibroblasts
Interleukin 7 (1L-7)	Bone marrow stromal cells	Proliferation pre- β cells, CD4+, CD8+ T cells, activated mature T cells
Interleukin 8 (1L-8)	Monocytes	Chemotaxis and activation of PMNs Chemotaxis for T cells and osteoclasts
Interleukin 12 (1L-12)	APCs, phagocytes	Potent inducer of cytokine production particularly INF- γ in T and NK cells Growth factor for preactivated T and NK cells Enhances cytotoxic activity in CTLs
Interferon- α	Leukocytes	Induces class 1 HLA
Interferon- β	Fibroblasts	Antiviral activity Induces NK-cell activity

Name	Source	Target cells / biological activities
Interferon- γ	Activated T cells	Activates macrophages Increases expression of class 11 molecules on macrophages and many other cells Activates endothelial cells Antiviral activity Induces NK-cell activity

Table 120. Abbreviations: CSF = granulocyte-monocyte colony-stimulating factor HLA= human leukocyte antigen (histocompatibility antigens coded for by MHC), from Trowbridge HO, and Emling RC, (1997). *Inflammation*, 5th edition, Quintessent Books, Carol Stream, Illinois, by kind permission of the Authors and Publishers. See also Meager, (1990). Please see also Oppenheim and Feldmann (2001).

Cytokines are polypeptides, produced by white blood cells, bone marrow cells and phagocytes, but are not hormones or transmitters (Table 120). They stimulate or inhibit the differentiation of many different cells of the immune system, and may act at concentrations as low as 10^{-15} M. They control haemopoiesis.

Cytokines are estimated by the effects of particular chemical mixtures on the growth and proliferation of cells in culture, (including clones) and by bioassay, radioautographic, immuno-assay and monoclonal antibody techniques. The group includes 18 interleukins, 3 interferons, leukaemia inhibiting factor B, and 2 tumour necrosis factors. They are believed to have receptors on their target cells, which include: lymphocytes, vascular endothelia, macrophages, hepatocytes, haemopoietic cells, mast cells, thymocytes, granulocytes, antigen-presenting cells, megakaryocytes, monocytes, embryo stem cells, tumour cells, Karposi sarcoma cells and epithelial cells (Goldsby, Kindt and Osborne 2002, pages 308-311). Obviously, the cytokines are believed to have effects on many of the cells, which produce them. Their effects are reported to include: growth, differentiation, protein synthesis, chemotaxis, inflammation, cell adhesion, interferon production, cytotoxicity, inhibition of viral replication, delayed hypersensitivity, inhibition of growth, phagocytosis and cachexia. The target cells have 5 types of receptor proteins, which have been sequenced. Each of their diameters exceeds the thickness of cell membranes.

Categories of substances constituting 'self' and 'non-self'

<i>Self</i>	<i>Non-self</i>
All body constituents in healthy persons	Vaccines and immunological products
	Allergens
	Bacteria
	Fungi
	Mycobacteria
	Viruses
	Incompatible organ transplants
	Incompatible tissue transplants
	Substances causing anaphylaxis
<i>Are they self or non-self?</i>	
Organs, tissues, cells, autoantigens <i>before</i> they induce antibodies	
Tissues from persons suffering autoimmune diseases, who do not have significant quantities of antibodies	
Grafts in patients who are treated with immunosuppressive drugs	
Foods or drugs to which human beings are not allergic	
Foods or drugs before or after a patient becomes allergic to them	
Allergens to which patients have been desensitised	
Commensals	
Symbionts	
Parasites	
Non-pathogenic organisms	
Compatible blood	

Table 121. *Some of the substances with which the body has contact, whose status can not be decided.*

Proteins and drugs which may be infused intravenously, and to which patients are occasionally sensitive

<i>A. Proteins</i>	
Albumin	Insulin
Casein	Interferons
Egg lecithin	Lenograstim
Factors VIIa, VIII, IX	Somatotrophin
Gelatin	Soya
Glucagon	Streptokinase
Heparin	Thrombin
Hyaluronidase	Vasopressin

Immunoglobulins	Vitamin K
<i>B. Antibiotics and antivirals</i>	
Acyclovir	Gentamycin
Amphotericin	Penicillin
Cephalosporin	Rifampicin
Chloramphenicol	Tetracycline
Clindamycin	Trimethoprim
Erythromycin	Vancomycin
Fusidic acid	

Table 122. The occasional sensitivities to these substances are indicated in pharmacopoeias. *Are they self or non-self before the tissues react to them, and when they are doing so?*

Classes of drugs sometimes causing allergy

<i>Anaesthetics and muscle relaxants</i>
general anaesthetics
muscle relaxants
local anaesthetics
<i>Analgesics/antirheumatics</i>
narcotic analgesics and antagonists
nonnarcotic analgesics/non-steroid anti-infammatories
antirheumatics
drugs against gout
miscellaneous
<i>Anticonvulsants</i>
hydantoins
succinimides
oxazolidines
barbiturates
miscellaneous
<i>Antihistamines, antiallergic agents and drugs affecting autonomic functions</i>
H ₁ -receptor antagonists
H ₂ -receptor antagonists
Xanthine derivatives
β-agonists
miscellaneous
<i>Antihypertensive agents</i>
adrenergic neuron blockers
centrally-acting antihypertensive drugs
peripherally -acting antihypertensive drugs
diuretics

angiotensin-I-converting enzyme inhibitors

Antithrombotic agents and blood products

anticoagulants

platelet aggregation inhibitors

thrombolytics

blood expanders

miscellaneous

Antiarrhythmics/cardiovascular agents

antiarrhythmics

other cardiac agents

Hormones and derivatives

corticosteroids

thyroid/antithyroid agents

sex hormone and antagonists

miscellaneous

Immunomodulators and vaccines

microbial derivatives

synthetic products

biotechnology products

vaccines

Immunosuppressants and antineoplastic agents

chemical immunosuppressants

monoclonal antibodies

antineoplastic agents

anti-microbial agents

sulphonamides

antibacterial chemotherapeutic agents

antimycotics

antituberculous agents

antileprotic agents

antiprotozoal agents

antiviral agents

miscellaneous

Psychopharmacological agents

neuroleptics

hypnotics/sedatives/anxiolytics

antidepressants

psychostimulants

antiparkinson drugs

antiepileptics

Steroids

testosterone

anabolic steroids

Miscellaneous

contrast media
vitamins and derivatives
drugs affecting lipid metabolism
dyes

Table 123. This list is drawn up from Dash and Jones, (1972), Descotes, (1990), British National Formulary, (2002). *It is assumed that the mechanisms of these 'allergies' are immunological.* See also De Week (1972).

'Foreign' materials are thought of as 'non-self' if they act as antigens, and it is believed that the body can distinguish between 'self' and non-self' (Table 121). Thus one has the following categories: (i) self; (ii) organs and tissues of 'self', which have not been transformed into autoantigens; (iii) foreign proteins and drugs (Tables 122, 123) and foods, to which animals may *sometimes* be 'sensitive'; (iv) bacteria, fungi, viruses and chemicals, to which animals are sensitive; (v) some foreign substances, including foods, drugs, bacteria, fungi, viruses, poisons and chemicals, to which animals do not show any immune response.

The complement system is another part of the defence of the body, so-called because it 'complements' the immune response. It consists of approximately 20 plasma proteins. Its activity can be destroyed by heating it to 55°C for 30 minutes, and restored by the addition of fresh serum. It enhances the effects of antibodies on bacteria; it lyses red cells; it 'recruits' the inflammatory system; it is involved in blood clotting; it breaks down fibrin; it forms kinins; it contains chemotaxins and anaphylaxotoxins. These plasma proteins are synthesised in the liver and in phagocytes.

The complement system nowadays is used to measure antigen or antibodies, and thus to diagnose many diseases, such as syphilis and glandular fever. Many of the diseases regarded as autoimmune (Table 116) are characterised by positive complements fixation tests. The complement system is regarded as being involved in inflammation. It is a very complex series of activators and cascades. Each protein has been characterised, and different ones have different effects as follows: binding to antigens, Clq; activating enzymes, C1r, C1s, C2b, Bb, D; membrane binding proteins and opsonins, C4b, C3b; peptide mediators of inflammation, C5a, C3a, C4a; membrane attack proteins, C5b, C6, C7, C8, C9; complement receptors, CR1, CR2, CR3, CR4, ClqR; complement regulatory proteins, C1INH, C4bp, CR1, MCP, DAF, H, I, P, CD59 (Janeway et al, 2001, pages 8:35) (Table 124).

The proteins of the human complement system

<i>Component-</i>	<i>Molec-</i>	<i>Subunit structure</i>	<i>Plasma</i>		<i>Rule</i>
	<i>ular weight</i>		<i>concentration</i>	(mg/L) (μ M)	
<i>Chemical pathway</i>					
C1q	460	6A chains (26 kDa), 6B chains (26 kDa), 6C chains (24 kDa)	80	(0.15)	Binds Ig; initiates classical pathway activation
Clr	83	Single, complex with Clq	50	(0.3)	Cleaves, activates Cl5
Cl _s	83	In plasma (Clq, Cl _r ₂ , Cl _s ₂)	50	(0.30)	Cleaves/activates C4 and C2
C4	205	1 α chain (97 kDa), 1 β -chain (75 kDa), 1 γ chain (33 kDa)	600	(3.0)	Binds C2 during activation
Cl _s	83	In plasma (Clq, Cl _r ₂ , Cl _s ₂)	50	(0.30)	Cleaves / activates C4 and C2
C4	205	1 α chain (97 kDa), 1 β -chain (75 kDa), 1 γ chain (33 kDa)	600	(3.0)	Binds C2 during activation
C2	102	Single chain	20	(0.20)	Cleaves/activates C3 and C5
<i>Alternative Pathway</i>					
Factor B	93	Single chain	210	(2.0)	Cleaves/activates C3 and C5
Factor D	24	Single chain	2	(0.05)	Cleaves/activates Factor B
Properdin	220	4 Identical subunits (55 kDa)	26	(0.10)	Stabilizes activation pathway convertases
Common C3	185	I α -chain (110kDa); 1 β -chain (75 KDa)	1300	(7.0)	Binds C5 in convertase; opsonisation/chemotaxis, etc.
<i>Membrane attack pathway</i>					
C5	190	1 α -chain (115 kDa); 1 β -chain (75 kDa)	70	(0.4)	Initiates membrane attack; C5a major chemotactic/anaphylactic peptide
C6	120	Single chain	65	(0.5)	
C7	110	Single chain	55	(0.5)	Self associate with cleaved

Component-	Molecular weight	Subunit structure	Plasma concentration (mg/L) (μ M)		Rule
C8	150	1 α -chain (64 kDa), 1 β -chain (64 kD), 1 γ chain (22 kDa)	55	(0.4)	C5 to form membrane sites to which C9 can bind
C9	69	Single chain	60	(0.8)	Major component to MAC
Soluble Control					
C1-INH	110	Single chain	200	(1.8)	Binds Clr + Cls, dissociates Cl
C4bp	500	8 identical subunits (70 kDa)	250	(0.5)	Accelerates decay of C4b2a cofactor for C4b cleavage by 1
Factor II	150	Single chain	450	(3.0)	Accelerates decay of C3Bb cofactor for C3b cleavage cleaves/inactivates C4b+C3b
Factor 1	80	1 α -chain (50 kDa), 1 β -chain (38 kDa)	35	(0.4)	
S-protein	83	Single chain	500	(6.0)	Binds fluid-phase C5b-7
Sp-40,40	70	2 subunits (35kDa)	50		Binds fluid-phase C5b-7
Membrane Control					
-CR1	160-250	Single chain			Accelerate decay of C3/C5 convertases; co-factor in C3b cleavage
DAF 70	70	Single chiain			
MCP	45-70	Single chain			
HRF/MIP	65	Single chain			
CD59	20	Single chain			Control of MAC formation / antigen activity

Table 124. Each of these proteins has been isolated and characterised chemically. This Table is from Morgan B P, (1990). *Complement-Clinical Aspects and Relevance to Diseases*, Academic Press, London, pages 10-11.

The complement system has a ‘classical’ pathway and an ‘alternate’ pathway. There is also believed to be a lectin pathway. Lectins are proteins which are believed to bind to mannin (glycoprotein) on the surfaces of bacteria. In addition to the

antigens and antibodies, there are believed to be 35 to 40 separate proteins involved in these pathways, in addition to the enzymes to synthesise, break down, and regulate them. The main elements of the complement system are illustrated in the diagrams of Clark WR (1986), reproduced here as Figure 70). These show the proteins involved, the order in which the reactions are believed to occur, and the effects of their final products. An anaphylatoxin produces an acute inflammatory response; each has its own receptor, can induce smooth muscle contraction, and increases vascular permeability. An opsonin coats the surfaces of bacteria and other particles, so that they can be phagocytosed or destroyed by macrophages. Chemotaxins from activated complement attract white blood cells. Membrane-attack complexes are believed to make the membranes of bacteria and other cells, including red cells, so porous that they lyse. Full treatments of the complement system can be found in Clark, (1986, pages 160-175), Roitt, (1994, pages 11-32), Janeway et al, (2001, pages 8:32 - 8:54); Roitt, Brostoff and Male, (2001, pages 54 - 61).

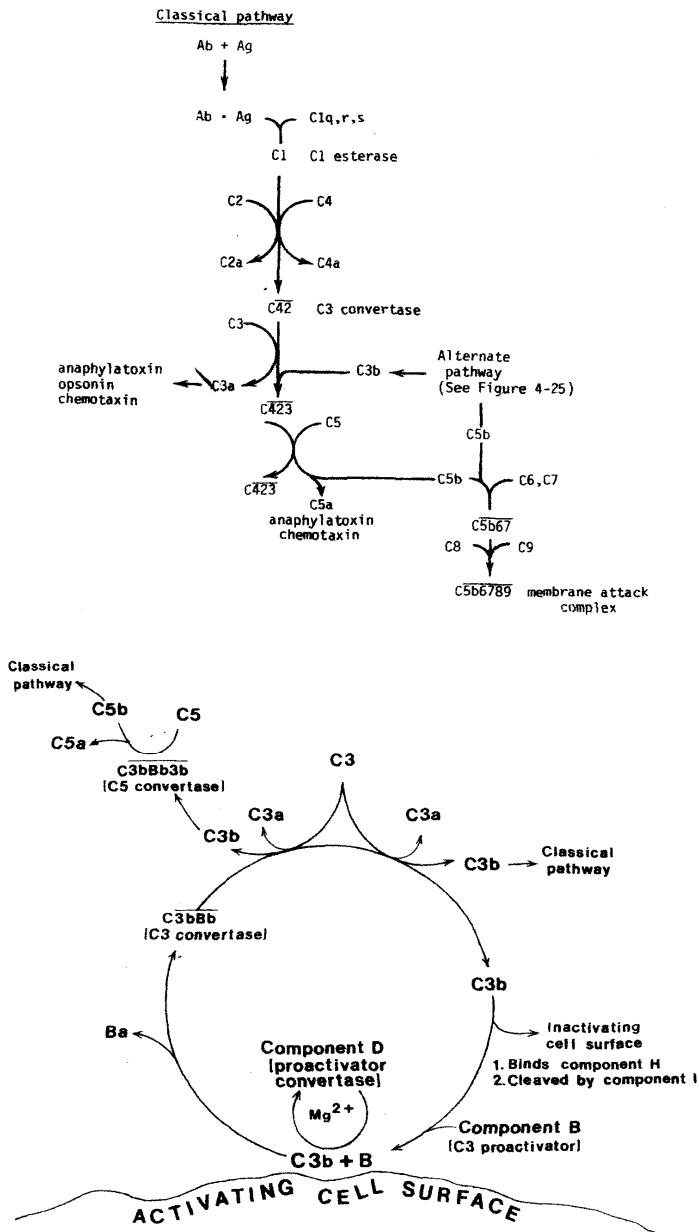


Figure 70. The complement system. *Upper*, classical pathway; *lower*, alternate pathway, from Clark WR, (1986), *The Experimental Foundations of Modern Immunology*, 3rd edition, Wiley, New York, pages 164 and 171, reproduced by kind permission of the Author and Publishers.

The complement fixation test is used to measure the presence and concentration of an antigen or antibody, and for deciding if a disease is autoimmune. It is based on the idea that if an antigen has reacted stoichiometrically with serum complement, the resultant bonded chemicals will absorb all haemolytic activity towards sensitised red blood cells, so that they will *not* lyse. If the antigen and antibody do not bind completely, some haemolytic activity will be left over and the red cells will lyse. In practice, a known quantity of antigen is incubated with serum containing suspected antibody, plus guinea pig or sheep complement. The three components bind together, so that there is no residual haemolytic activity. The mixture is then added to sheep red cells, which have been sensitised by exposure to rabbit anti-sheep red cell antibody. If the mixture does *not* cause lysis, it is concluded that the antibody has bound to the antigen and fixed the complement. Therefore, the antibody and antigen are mutually specific, that is the specific antibody is present at various titres of the serum; the test is called positive. On the other hand, if the sensitised red cells *are* haemolysed, it is concluded that all the antigen - antibody complex had not bound completely and fixed the complement, so that it was able to haemolyse the red cells. This is regarded as a negative result. The assay can detect 0.04 mg of antibody, and 0.1 µm of antigen.

When a column of blood from a healthy subject is allowed to sediment, the lymph appears clear and yellow. There is no measurable degree of haemolysis, so one must ask what the complement is doing in health. Obviously, it is not haemolysing. So it is possible that complement haemolyses blood only in the presence of inflammation (Table 125). Certainly, most of the diseases in which complement has been found to change are inflammatory (Table 126).

At this stage, one must ask whether it is reasonable to assume that all the reactions attributed to the antigen-antibody system (Table 119), and to the complement system (Figure 70) would have had time to occur, and, do, in fact, occur, during all rapid immune reactions. These would include anaphylaxis, hay fever, food allergy, asthma etc.

Some causes of haemolysis

<i>1. Iatrogenic</i>	
renal dialysis	pre-eclampsia
perfusion	blackwater fever
incompatible blood transfusion	disseminated carcinomatosis
mechanical prostheses	polyarteritis nodosa
drug induced e.g. penicillin, methyldopa, sulphonamides	systemic lupus erythematosus
ultra violet	Wegener's granuloma
x-rays	meningococcaemia
mercury salts	Wilson's disease
<i>3. Human diseases</i>	
haemolytic anaemia	<i>2. Experimental in vitro</i> (Ponder, 1924)
thrombocytopenic purpura	hypotonic solutions
septicaemia	heat
haemolytic uraemia	complement
burns	saponin
renal failure	strong acids and alkalis
glomerulo-nephritis	taurocholate
haemolytic disease of the new-born	glycocholate

Table 125. *In addition to the above causes, the end of the life span of red blood cells is the commonest, and the only naturally occurring, cause.*

Diseases in which complement changes significantly (continues over)

Acne rosacea	Behcet's syndrome
Acne vulgaris	Bronchiectasis
Acute haemolytic anaemia	Bullous pemphigoid
Acute oedema	Burns
Acute post streptococcal nephritis	Cancer
Adult respiratory distress syndrome	Chronic relapsing fever
Alzheimer's disease	Dermatitis herpetiformis
Anaphylactoid purpura	Dermatomyositis
Anaphylaxis	Diabetes, early onset
Angioedema	Echinococcus infection
Atheroma	Entamoeba infection
Autoimmune thyroid disease	Epidermolysis bullosa acquisita

Erythema multiforme	Pancreatitis
Glomerulo-nephritis	Para-influenza
Gonorrhoea	Parasitic diseases
Goodpasture's syndrome	Paroxysmal nocturnal haemoglobinuria
Gram negative infections	Partial lipodystrophy
Guillain Barré syndrome	Pneumonia
Haemolytic anaemia	Psoriasis
Haemolytic uraemia	Pyogenic infections
Haemophilus infections	Rectal carcinoma
Helicobacter infection	Regional ileitis
Hepatitis	Reperfusion injury
Hereditary angioedema	Rheumatoid arthritis
Herpes gestationis	Scleroderma
Herpes simplex	Shock
IgA nephropathy	Sjögren's syndrome
Immune complex disease	Staphylococcal infections
Influenza	Subacute sclerosing panencephalitis
Ischaemic reperfusion injury	Systemic lupus erythematosus
Leishmaniasis	Taenia infections
Leukaemia	Thrombocytopaenia
Lichen planus	Transplantations
Lipodystrophy	Trypanosomiasis
Lymphoma	Ulcerative colitis
Lymphosarcoma	Urinary tract infections
Many bacterial infections *(Würzner, 1999)	Urticaria
Measles	Vasculitis
Multiple sclerosis	Viral haemorrhagic fevers
Membranous neuropathy	Vitiligo
Meningitis	Xenotransplantation
Myasthenia gravis	Xeroderma pigmentosum
Myeloma	Waldenström's macroglobinaemia
Otitis media	

Table 126. The data for this Table comes from Whaley, (1987), Paul Morgan, (1990), Meri and Jokiranta, (1999), and Würzner, (1999).*

Forty years ago, in discussing the use of complement for the diagnosis of disease, Gray (1964) noted that "In practice, since they are all empirical tests employing a non-specific antigen the final interpretation must rest with the physician, who correlates the history, clinical findings and laboratory report to reach a diagnosis." This is true because many patients who have diseases believed to be autoimmune, do not show high titres of specific antibody, while others who do, do not suffer from the particular disease. Haemolysis occurs in a number of iatrogenic conditions, and several rare diseases (Table 125). Also, complement changes significantly in some not necessarily immune conditions (Table 126). However the complement fixation test is useful diagnostically for specific antibodies in very many suspect diseases. As with other ubiquitous clinical biochemical findings, it is extremely difficult to establish whether changes in complement are the causes or the consequences of the lesions seen.

An autoimmune reaction occurs when the animal body reacts to its own constituents - organs, tissues, cells and subcellular organelles (Ehrlich and Morgenroth, 1900). They suffer from 'horror autotoxicus'. The idea was conceived that the body produced natural toxins, that would destroy it, if it were not continuously protected by innate active immune mechanisms. Uhlenhuth, (1903) had shown that the eyes of rabbits could react to their own lenses and he proposed that different tissues of the same body contained different antigens. The concept of 'horror autotoxicus' may have come from 'Original Sin', but the belief that chronically ill people had toxic foci in their teeth, tonsils, appendices, salpinges, etc., led to the widespread excision of these organs, especially from innocent women and children, as recently as the 1960's (Boucher, 1894; Paczkowski, 1909; Smith JL, 1982). Of course, the body continuously produces a variety of agents, which if they reached high concentrations, and were not excreted in the bile and the urine, would be poisonous ('autotoxic', perhaps?). These include: K^+ , NH_4^+ , CO_2 , glucose, H^+ , CO_2 and a host of drugs, pollutants and food additives. The modern development of the concept probably started with the demonstration that the plasma of patients with Hashimoto's thyroiditis precipitated thyroglobulin from the thyroid extracts of healthy subjects (Roitt et al, 1956).

Criteria for autoimmune diseases

1. Hypergammaglobulinaemia.
2. High levels of IgG autoantibodies or self-reactive T cells in the blood or lymph.
3. Lymphocytic accumulation in infected tissues.
4. Existence of animal models of the diseases.
5. Transferability by antibodies or lymphocytes to animals.
6. Transferability by maternal antibodies in human diseases.
7. Induction of disease in animals by autoantigens.
8. Positive complement fixation test.
9. Immunofluorescence.
10. Transferability of disease to severe combined immunodeficient mice.
11. Association with other diseases believed to be autoimmune in the same individual family.
12. Treatable by corticosteroids or immunosuppressants.
13. Autoantibodies or self reactive T cells from organs believed to be targets of 'autoimmune attack'.
14. Presence of high affinity or clonally limited cellular autoantigens.
15. Clonally limited cellular immune response determined by T-cell receptor variable region gene or major histocompatibility complex class II restriction.
16. Static association with a particular major histocompatibility complex haplotype or aberrant expression of major histocompatibility complex class II antigens on the affected organ.

Table 127. These criteria are harvested from Witebsky, Rose, Terplan, Paine and Egan, (1957), Rose and Bona, (1993), and Rose and Mackay, (1998).

Clinical signs of allegedly immunological drug reactions

Drug eruptions	Pericarditis
Morbilliform exanthema	Pleurisy
Generalised urticaria	Eosinophilia
Erythema multiforme	Leucopenia
Pyrexia	Agranulocytosis
Lymph node and salivary gland enlargement	Thrombocytopenia
Jaundice	Anaphylactic shock
Haemolytic anaemia	Pulmonary infiltration
Arthritis	

Table 128. These signs are listed in Dash and Jones, (1972). *Not all of them are seen in all conditions regarded as allergic reactions to drugs. It is assumed that drug reactions are immunological.*

Several criteria have been drawn up for autoimmune diseases (Table 127), and one must ask whether particular criteria are believed to apply to all autoimmune diseases (Table 116). They are believed to have a repertoire of autoantigens (Table 116) and to produce a range of clinical signs in skin, blood, joints, etc. (Table 128). They are treated with an extensive pharmacopoeia, mostly empirically, of which the steroids are the most well known (Table 129). A battery of different methods are used to detect diseases, characterised as autoimmune (Table 130). Many different drugs are used as immunosuppressives for autoimmune diseases and transplantation (Table 131). A list of recognised autoantigens has been drawn up by Mackay (1992, page 180), and is reproduced here as Table 132.

Treatments of diseases believed to be autoimmune

<i>Conditions</i>	<i>Treatments</i>
Hashimoto's thyroiditis	thyroxin
Primary myxoedema	thyroxin
Thyrotoxicosis	antithyroid drugs
Parathyroid disease	parathormone
Myocardial infarction	streptokinase; oxygen; diamorphine; heparin; aspirin; β -blockers; ace inhibitors
Diabetes mellitus	insulin
Coeliac disease	glutelin-free diet
Ulcerative colitis	corticosteroids
Rheumatoid arthritis	salicylates; corticosteroids
Juvenile chronic arthritis	gold; penicillamine
Multiple sclerosis	beta-interferon; glatiramer acetate
Myasthenia gravis	anticholinesterases
Polymyositis	cyclosporine
Pernicious anaemia	B_{12}
Acute lymphoblastic leukaemia	methotrexate
Autoimmune neutropenia	steroids; antibiotics; splenectomy
Evans syndrome	corticosteroids; splenectomy
Autoimmune haemolytic disease	corticosteroids; vincristine; blood-transfusion
Idiopathic thrombocytic purpura	corticosteroids; intravenous immunoglobulins
Hughes syndrome	warfarin
Idiopathic haemochromatosis	phlebotomy
Adrenalitis	corticosteroids

<i>Conditions</i>	<i>Treatments</i>
Addison's disease	corticosteroids
Graves' disease	anti-thyroid drugs
Vitiligo	psolarens
Pemphigus	corticosteroids; cyclosporine
Pemphigoid	azathioprine
Psoriasis vulgaris	antihistamines; azathioprine; colchicine
Dermatomyositis	antimalarials; methotrexate
Scleroderma	ACE inhibitors
Sympathetic ophthalmia	ecothiopate iodide
Pancreatitis	pancreatin
Immunoproliferative small intestine disease	antibiotics
Autoimmune hepatitis	corticosteroids; liver transplantation
Glomerulo-nephritis	corticosteroids
Nephrotic syndrome	corticosteroids
Goodpasture's syndrome	plasma exchange; corticosteroids; cyclosporine
Wegener's disease	renal dialysis; renal transplant; methotrexate; cyclophosphamide
Vasculitis	gene therapy; corticosteroids
Systemic lupus erythematosus	chloroquine; corticosteroids
Discoid lupus erythematosus	chloroquine
Guillain - Barre syndrome	immunoglobulin
Polyarteritis nodosa	alkylating agents; corticosteroids
Sjögren's syndrome	hydroxychloroquine
Behcet's syndrome	azathioprine; colchicine; cyclophosphamide
Phagogenic uveitis	corticosteroids; cyclosporine
Schizophrenia	neuroleptics
Narcolepsy	modafinil
Syphilis	penicillin; tetracycline
Premature ovum failure	hormone replacement therapy
Premature onset menopause	oestrogens
Male infertility	gonadotrophins
Bulimia	psychological

Table 129. These treatments are described in Rose and Mackay, (1992), Hardman, Limbird, Molinoff and Rudden, (1996), Ledingham and Warrell, (2000) and British National Formulary, (2002). *Note the very wide variety of treatments of which the*

corticosteroids are generally used empirically.

Methods used to detect antibodies in autoimmune diseases

- Enzymes linked immunoabsorbant assay
- Immunofluorescence on fixed and unfixed, tissues, cultures and subcellular fractions
- Complement fixation tests
- Precipitation tests
- Passive haemagglutination
- C mediated cytotoxicity
- Stimulation of mouse thyroid in vivo
- Blocking TSH combination with receptors
- Stimulation of adenyl cyclase
- Induction of cell division in thyroid fragments
- Blocking combination with vitamin B₁₂
- Co-precipitation
- Blocking action of gastrin
- Blocking hormone binding to receptors
- Agglutination
- Radioimmunoassay
- Blocking radioassay with hydroxybenzylpindolol
- Blocking or binding radioassay with a -bungarotoxin
- IgG production of neuromuscular defects
- Histopathology
- Cytotoxic effects in culture
- Coombs antiglobulin test
- Waaler-Rose agglutination test
- Shortened platelet survival
- Immunoblotting
- Immunoelectrophoresis
- Antibody dependant cell mediated cytotoxicity
- Gel diffusion
- Antiglobulin tests
- Latex agglutination
- Erythrocyte lysis
- Counter immunoelectrophoresis
- Radio immunoelectrophoresis
- Crossed electrophoresis
- Immunoabsorbence
- Lupus erythematosus test
- Sheep red cell agglutination
- Counter current electrophoresis
- Western blotting
- Non-organ specific C¹ fixation test
- Probing of gene expression libraries
- Passive cutaneous anaphylaxis

Table 130. These methods are listed in Gill, (1970), Roitt, (1991, page 307) and

Mackay, (1994). Sensitivities vary from one molecule of antibody per cell, and from 0.2 µg/ml to 125 µg/ml.

Immune suppressant drugs

Prednisolone	FK506
Immunoglobulins	Basiliximab
Adrenocorticotrophic hormone	Daclizumab
Cyclosporine	Rapamycin
Monovalent vaccines	Desoxypregualin
Polyvalent vaccines	Viral IL-10
Cyclophosphamide	Murine IL-10
Actinomycin D	Human IL-10
Target cells used in gene therapy and in transplantation of heart, lungs, kidney	IL-12p 40
Azathioprine	Fos L
Antilymphocyte A globulin	CTLA41g
Tacrolimus	BcL-2
Mycophenolate mofetil	HSP TO
Anti T cell mitochondrial antibodies	Macrocyclic lactones
Antibodies to IL-2 receptors	3-hydroxyl-3-methyl- glutamyl coenzyme A
Calcineurin inhibitors	Reductase inhibitors
TGFβ1	

Table 131. Most of these drugs, cytokines and immune agents are listed by Salaman (1981) Thomson and Starzl, (1994), Wagner, (1999) and Schurmann, Feutren and Bach, (2001).

Autoantigens (continues over)

<i>Autoantigen</i>	<i>Related diseases</i>
Cell membranes	
Blood cells	Haemolytic diseases; haemolytic anaemia, thrombocytopenic purpura, neutropenia
Receptor structures for	
acetyl choline	Myasthenia gravis
thyrotropin	Thyrotoxicosis goitre
insulin	Diabetes, insulin resistant type
Nerve cells: myelin of oligodendroglia	Exptl autoimmune encephalomyelitis
Schwann cells	Neuritis
Liver cells: postulated membrane antigen	Chronic autoimmune hepatitis (CAH)

Cytoplasmic Organelles

Tissue specific

gastric H ⁺ , K ⁺ , ATPase	Autoimmune gastritis, pernicious anaemia
thyroid peroxidase	Hashimoto's thyroiditis
adrenal cortical cell	Addison's disease
pancreatic β islet cell	Type 1 diabetes mellitus
salivary duct cell	Sjögren's syndrome

Non tissue specific

microsomes, liver, kidney (cytochrome p450db)	Variant type of autoimmune hepatitis
mitochondria (2 oxo-acid dehydrogenase enzymes)	Primary biliary cirrhosis
ribosomes (phosphoproteins, PO, P1, P2)	SLE
cytoskeletal actin (smooth muscle antibody)	Autoimmune hepatitis
stress (heat shock) proteins	Rheumatic diseases
transfer RNA synthetases, including Jo1	Polymyositis and variants

Nuclei

DNA	SLE
Histones	SLE, particularly drug related; various rheumatic diseases
U (uridine-rich) RNA-protein complexes	Mixed connective tissue disease
U1-RNP, U1, U2, U4-6 RNI's (Sm antigens, various epitopes have been mapped)	SLE
Ro-La RNP complexes	
Ro (SS-A)	Cutaneous LE; fetal heartblock; primary Sjögren's syndrome; SLE(?)
La (SS-B)	Primary Sjögren's syndrome; SLE
Centromere	Scleroderma, crest variant
Sel-70 (topoisomerase 1)	Scleroderma, systemic sclerosis
Nucleolus	Systemic sclerosis; undifferentiated
fibrillarin	rheumatic diseases
nucleolar RNP	
Proliferating cell nuclear antigen-cyclin	SLE (rarely)
Ku	SLE and related diseases

<i>Specialised Tissue Products</i>	
Squamous epithelium	Pemphigus vulgaris
intercellular substance	Bullous pemphigoid
basement membrane	Myasthenia gravis
Striated muscle, 1 band	Myocarditis, rheumatic carditis
Cardiac muscle, sarcolemma	Rheumatoid arthritis
Chondrocytes, collagen Type II	Glomerulonephritis (Goodpasture type)
Glomerular basement membrane, collagen Type IV	Uveitis
Retina, S antigen	Male infertility; orchitis(?)
Sperm antigens	Ulcerative colitis(?)
Colon mucosal cell	Vasculitis
Myelomonocytic cells, cytoplasmic peroxidase	
<i>Secreted Products</i>	
Gastric intrinsic factor	Atrophic gastritis, pernicious anaemia
Immunogloblin G, Fc piece	Rheumatoid arthritis
Thyroid colloid	Hashimoto's thyroiditis
Insulin	Type I diabetes mellitus

Table 132. This list comes from Mackay I and Rose N.R., (1992), with kind permission from Elsevier Science. See also Ascherson and Cervera, (2001).

The concept of ‘tolerance’ implies an element of threat. A more powerful entity could destroy a weaker, but it does not. This is either because it does not ‘recognise’ it, or because the machinery for doing so is deficient. Nevertheless, the term ‘tolerance’ implies that it could. An antigen may not induce antibodies, either because: its *chemical* nature is such that it does not at present activate the immune system; the cells in the immune system do not react with ‘self’ (Table 121); some of the chemicals involved in the immune system are not present; the body contains mechanisms preventing the immune system from reacting to the antigen; the immunity has been suppressed therapeutically; the lymphocytes which would be ‘required’ for the immune reaction are not present or have been previously destroyed (clonal section); the animal or the patient has been ‘desensitised’ to the antigen or allergen; the change in the protein, which makes it into an autoantigen, has not yet occurred; since an antigen is defined as a substance, which induces an immune response, if there is no such response, the substance may simply not be an antigen -

that is, it may be characterised as self. Do all these represent tissue 'tolerance' to antigens?

Experimentally, several animal models have been used to study autoimmune disease. The most well known is experimental allergic encephalomyelitis. Rats or mice are injected with myelin basic protein, plus Freund's adjuvant; this is composed of killed mycobacteria (usually M tuberculosis), emulsified in mineral oil with mannoside monooleate. The adjuvant prolongs the action of the antigen, and induces the formation of granulomas. In about 3 weeks, animals of the same species develop a demyelinating disease, which looks like multiple sclerosis histopathologically. Of course, adjuvants have their own effects (Pearson, 1956; Petty and Steward, 1977). Using the same adjuvant, injection of acetylcholine receptor into rabbits produces a condition like myasthenia gravis. Injection of *Mycobacterium tuberculosis* causes a disease like rheumatoid arthritis, and thyroglobulin mimicks Hashimoto's thyroiditis in animals. The following other animal models are also used: NZB mice for haemolytic anaemia; NZB x NZW F₁ for lupus erythematosus with glomerulonephritis and anti-DNA antibodies; MRL/Mp-1pr/1pr for lupus erythematosus and arthritis; anti-DNA antibodies; MRL/MP-+/+0 for chronic glomerulonephritis; C57BL/KS-db/db for diabetes; NOD mice for diabetes; BXSB for lupus erythematosus - like syndrome with haemolytic anaemia and glomerulonephritis; BB rats for diabetes which may also develop autoimmune thyroiditis; Os chickens mimic Hashimoto's thyroiditis (Hay and Westbrook, 2002, pages 334-335).

Autoimmune diseases are believed to be organ specific, such as Hashimoto's thyroiditis and multiple sclerosis, or systemic, such as lupus erythematosus or rheumatoid arthritis. The suspected antigens to these diseases have been identified (Table 116, 117). There is a very important implication here, namely that an immune response is not just to a single protein - as occurs in immunisation (Table 118), but to the many different proteins in any organ. Also, the expression 'specific to an organ or a system' clearly implies that different organs in a single body have different immune properties.

Proposed mechanisms of induction of autoimmunity

1. A potential autoantigen is ‘sequestered’ in the embryo, for example, by the blood brain barrier or the cell membrane, so that it cannot induce an immune response. When the tissue is infected or inflamed, the autoantigen is released, and can then induce the immune response.
2. Lymphocytes clone and mutate. Some of the mutated cells have surface antigens, which act as ‘foreign’ proteins.
3. The proteins of the human host-tissues have some chemical groups; similar to those of bacteria or viruses ('mimicry'), so the immune system reacts to the infection as if there were autoantigens.
4. ‘Superantigens’ with molecular weights of 25-80,000 are products of bacteria and viruses. They react strongly with major histocompatibility complex (MHC) and T cell receptor molecules, and thus initiate an immune response.
5. Some autoantigens can act independently of T cells.
6. Endotoxin, Epstein-Barr viruses and Gram negative bacteria can induce B cells to produce IgM immunoglobulins in the absence of T helper cells. This is regarded as ‘non-specific’ activation.
7. Genetic control of MHC complexes has been shown in 22 diseases, linked to immune regulatory genes (Bellanti, 1978), and changes in these genes could alter the immune response.
8. A foreign determinant, such as a drug or virus may link covalently with helper T cells, and then bind to self - reactive B cells to cause them to proliferate and produce antibodies.
9. In experimental NZB mice, loss of T cell mediated suppression parallels the development of autoimmunity, and there is a hyperactive B and T lymphocyte response.
10. Antigens may act as antibodies by rearranging idiotypes, which are changes specific to a V_H or V_L gene.
11. ^HT helper cells may produce excessive IL2 cytokine, which stimulates B cells to proliferate and produce more antibody.

Table 133. The possible mechanisms for the induction of autoimmunity may be found in Bellanti, (1978, page 570), Rose and Mackay, (1992, pages 1-26), Roitt, (1994, pages 406-429), Kuby, (1994, pages 455-462), Klein and Horejsi, (1997, pages 651-658), Shoenfield and Isenberg, (1999), Lydyard, Whelan and Fanger, (2000), Janeway et al, (2001, pages 12.2-12.19), Abbas, Lichtman and Pober, (2000, pages 404-423).

It is generally believed that autoimmunity may be induced, either by suppression of the mechanisms, which normally inhibit it, or by the action of one of the several hypothetical mechanism, listed in Table 133. Most of them have been derived from experiments on animal models of human illnesses.

In addition to the criteria for autoimmune diseases (Table 127), they have other characteristics (Bowry, 1986). They have a genetic disposition. They are commoner in females than males. Autoantibodies such as antinuclear factor,

rheumatoid factor and thyroid antibodies, increase with age. Drugs and infections may cause transient increases of antibodies. Autoantibodies against adrenal, parathyroid, islet cells and mitochondria are rare in health at any age. Autoimmune diseases are not always clinically progressive.

One may now proceed to examine elements of the architecture of the immune and autoimmune responses.

The first point may only be semantic. Immunity in its original Latin sense meant that one did not have to pay tax. Immunity and autoimmunity in the present usage, means that the body *does* have a reaction against the antigen, to which it might previously have been tolerant.

Second, immunologists often compare the properties of B-lymphocytes from blood and lymphatic system, with those of T-lymphocytes from the thymus. Since they both originate from such different organs it is not at all surprising that they have different properties in the living intact animals, including solubilities, affinities, fragility, protein content, dimensions, etc. However, one is not looking at these lymphocytes in the intact animal, or having been isolated by non-energetic procedures. They are each isolated by quite different procedures, involving many reagents and agents. As in subcellular fractionation, no attempts have been made so far to measure how each of the steps in complicated and painstaking procedures (Hunt, 1987; Hay and Westbrook, 2002) affect the final answer. Thus one simply does not know either what the properties of the naive B and T lymphocytes were, nor how they were changed by the preparation. Each step in the preparation is empirical; the use of a particular reagent is not always justified chemically, and possible effects on the final measurements are not even considered.

It would seem to be highly desirable for one or several centres of immunology to examine the following: the effects of each agent and reagent used in the procedure on the properties of the B and T lymphocytes being studied; the recovery of the activities of their cells at each stage of the separation; the isolation by hand dissection without fixative, dehydration, disruptive or centrifugation of the two kinds of cells from the fresh blood, bone marrow and thymuses, and examining their immune properties; it would also be useful to examine the properties of red cells, hepatocytes and muscle cells, subjected to the same procedure as B and T lymphocytes.

Third, one of the basic assumptions of immunology is that different tissues of the body have different immune properties. For example, anti-glial fibrillary acid

protein is believed to react only with neuroglial cells in the brain. If different cells in the same body contained different antigens, one would expect that each antigenically different kind of cell would have reactions to other different antigens, which it did not possess. This happens in a Rh⁺ baby which induces antibodies in its mother, which kill it. Why do not the neurons produce antibodies to the glial cells? This assumption is so widely held, probably because different tissues stain differently by histochemical procedures. *Prima facie*, it seems to me reasonable to entertain the assumption, that if the same DNA is present in all the cells of the same animal, they each do produce, or are capable of producing, the same proteins. It is usually assumed that some proteins are not 'expressed' or are 'switched' off by suppressor genes, but this represents an explanation. Certainly, in a review of enzyme and protein markers in different regions of the brain, Pevzner, (1979) rarely found any region completely devoid of the marker, although some had very small concentrations or activities indeed. He did not have results for all markers in all preparations and regions. Of course, if a gene for a particular enzyme or protein were absent, one would not expect any of the enzyme or protein to be present at all, unless it had moved from another tissue during preparation.

Fourth, it is an important assumption, very likely to be wrong, that soluble substances do not diffuse during cell lysis, homogenisation, centrifugation, washing, dilution, etc.

Fifth, the concept that an autoimmune response results from a withdrawal of tolerance to a particular antigen, implies that mechanisms monitor the activities of both the antigen and the mechanism withholding or suppressing, the immune response, in a homeostatic 'feedback'. It would be far simpler to suppose that an inflammatory agent or external antigen initiated the response.

Sixth, although haemolysis does occur in a few infections (Table 125), it is not a feature of such common conditions as tonsillitis, pneumonia, gastro-enteritis or cystitis, that is, it is not a general concomitant of infections.

Seventh, if one transfuses incompatible blood, or transplants an unmatched organ, the recipient reacts very rapidly, and the donor organ such as blood, kidney, liver or heart, is attacked by the recipient's immune system. It shows necrosis rapidly and is rejected. This may result in the death of the recipient. Lupus erythematosus affects the skin, the pericardium, the pleura, the membrane covering the liver, the kidneys and the lymph glands. Rheumatoid arthritis affects several particularly

peripheral joints and skin. Myocardial infarction is also believed to be an autoimmune disease, affecting the myocardium. One must ask, if the latter 3 examples show that the body is rejecting its own tissues, why are not all the organs found to be involved clinically, simply rejected completely? Granted that a person with rheumatoid arthritis has inflamed joints, and a sufferer from myocardial infarction may have a poorly functioning heart, all the relevant organs are still present; they are inflamed, but do not slough away, and are not rejected.

Eighth, many patients who have the autoimmune diseases clinically, do not show antibodies. Also many healthy people have significant concentrations of autoantibodies.

Ninth, in respect of autoimmune diseases there is such a wide range of clinical syndromes (Table 116), autoantigens (Table 132), treatments (Table 129), and allergens (Tables 122, 123) that it seems extraordinarily unlikely that they share the same chemical steps (Table 119).

Tenth, the concept of 'self' is somewhat unclear. Obviously, bovine blood is unlikely to be 'self' and would be rejected, if it were transfused in to a human being. However, the same blood could be drunk with impunity. The Peyer's patches in the intestine acting as part of the 'defence' system could have rejected the incompatible blood, just as the mouth of a sensitive person may react to a single peanut.

Eleventh, one must repeat the question. 'Can one believe that all the steps of the immune reaction (Table 119), including complement activation (Figure 70) and production of a battery of cytokines (Table 120) have sufficient time to occur within the trice of the cross match of blood, an anaphylactic shock, or an attack of asthma?

Twelfth. For over 90 years, cancer has been regarded as an immune disease (Schöne, 1912; Tyzzer, 1916; Woglom, 1929; see Silverstein, 1989, pages 275-304), and it fulfils some of the criteria listed. It seems extremely unlikely that cancer can induce an immune reaction. Unlimited growth is exactly the opposite of an immune reaction, which would have the effect of destroying the tumour. Probably, the non-specificity of the criteria for autoimmunity (Table 127) allows cancer to be included in this category, bearing in mind that complement fixation and fluorescence are two of the popular techniques used for detecting it. In a beautifully written review on the evidence for tumour antigenicity, Hewitt, Blake and Walder (1976) concluded that "Isotransplants of 27 different leukemias, sarcomas and carcinoma all *of strictly spontaneous origin* in laboratory bred mice of lower cancer strains CBA/Ht and

WHT/ht, has revealed no evidence of tumour antigenicity. Of 20,000 maintenance transplants, *none* have failed and none regressed.” Please see also Hewitt (1979). Earlier findings of antigenicity had come from artificially induced cancers. It was previously believed that tumour growth *in vivo* might be controlled by the immune system (Burnet, 1970), and that T cells ‘surveilled’ antigens in the blood and reacted against them, so that clinical tumours which appeared were those that had ‘escaped surveillance’ (Doherty, Knowles and Wettstein, 1984; Kripke, 1988; Vile, Chang and Dowdi, 1997; Finn, 2006). Surveillance requires a detector on the T cells, a signal from the tumour cells, and also an effector mechanism.

Thirteenth. It is widely believed that lymphocytes possess antibodies to all possible antigens, including foods, drugs, toxins, and metals (Tables 122, 123) to which they have never been subjected before (Burnet, 1969a,b; Jerne, 1974). This seems extraordinarily unlikely, even although their membranes have adequate molecular space for them. Many of the drugs are not even proteins, so, presumably, they have to conjugate with proteins before they can induce an immune response. The antibodies will then be produced as a consequence of the binding with the proteins. Thus, one must add this assumption to the hypothesis the idea that non-protein antigens can induce antigen behaviour by binding to a natural protein in the body. A much earlier and simpler hypothesis was that each antigen itself acted as a template itself for antibodies (Bail and Tsuda, 1909; Silverstein, 1989, page 67).

Fourteenth. Ehrlich and Morgenroth, (1900) originally assumed that antibodies were on the outer surface of cell membranes, a generalisation, which has accumulated belief with age. However most experiments show fluorescence throughout the cytoplasm.

Fifteenth. Binding of antibodies, MHC molecules, ligands and receptors, only means reaction with them chemically. The event could last only a fraction of a second, but have triggered a metabolic cycle or pathway, while it reacted. The binding could be so rapid that it could not be detected, so that failure to find such a reaction is not sufficient evidence that it has or has not occurred.

Sixteenth. Much of the understanding of immune processes depends upon the *specificity* of the antigens, antibodies, receptors, ligands and inhibitors. The distinction often made between a ‘specific’ and a ‘non-specific’ bond is somewhat difficult and usually subjective, since the naive tissue probably can not tell the difference. ‘Specificity’ *should* mean that the particular substance reacts with only

one site on one kind of molecule or ion, and not with any of the other molecules or ions naturally occurring at that site, or under the changing chemical conditions of the experiment.

Seventeenth. The proteins of the complement system (Table 124) the antigens (Tables 116, 117 and 132), as well as the immunoglobulins, are all macromolecules believed to be present on the outside of the lymphocytes, and within the resolution of the electron microscope (Pressman and Grossberg, 1967). Nevertheless, it is difficult to find in the literature sections showing these molecules by transmission electron microscopy; they are occasionally seen in fractions. When they are seen by scanning electron microscopy, the white spheres are identified, as the molecules which the microscopist wishes to label, with no certainty whatsoever. One must insist on the unpopular thought that an electron microscopist is looking at the *heavy metal* with which she or he has adorned the cells.

Eighteenth, the clearest evidence for the antigen - antibody reaction is the hugely successful use of vaccines and immunoglobulins, *which are specific to one single protein* (Table 118). Classically, each single protein protects against a single disease. With the exception of Jenner's cow pox protecting against small pox, most of the other vaccines and immunoglobulins are believed to be single identified antigens inducing the production of single specific antibodies. However, the antigens of autoimmune diseases are believed to be several organs, an individual organ or a subcellular organelle (Tables 116, 117 and 132). Each of these would contain their own rich repertoires of hundreds of proteins. Thus, any mechanism suppressing an autoimmune response would have to act on many proteins, unless one were to believe and have demonstrated that the body reacts to particular proteins common to several organs, tissues and organelles.

Nineteenth. Obviously the characterisation of a disease as being autoimmune, or cancer as being an immune condition, implies that the whole immune response is involved in these conditions. It also implies that this understanding will facilitate the design of rational measures for treatment. So one must ask if immune suppressant drugs (Table 131) are effective against diseases believed to be autoimmune, or against cancer. It is true that steroids have been found empirically to be effective against many autoimmune diseases, but they are not by any means the only treatments (Table 129). Steroids also improve the symptoms of cerebral oedema, dying and osteoarthritis, but these are not considered autoimmune diseases. The demonstration

that any diseases, including cancer, could be treated effectively and routinely by 'specific' antibodies and antigens would justify their classification as immune diseases.

Various areas of immunology may now be considered (Table 114) in light of the discussion in this chapter.

Infectious diseases such as human immunodeficiency virus, glandular fever and meningitis are diagnosed by specific serum tests. Vaccination and immunisation are successful applications of the understanding of immunology to the prevention and treatment of a large number of human (Table 118) and animal diseases. The same is true of the success of characterisation of blood groups for transfusion, and of the Rhesus factor in neonatal morbidity. It must be stressed, that all these applications of the 'classical' - one may say 19th century and early twentieth century - immunological findings, are all based on single proteins acting as antigens and antibodies. They did not require a battery of proteins in theory or in practice.

Inflammation is a characteristic of nearly all diseases. Clinically, one sees heat, pain, swelling, redness and loss of function. One also sees a chart of local and general biochemical changes (Chapter 49). Here one must ask three unanswerable questions. Do the biochemical changes *cause* the inflammation, or are they consequences of it? What is the difference between inflammation and an immune response? By what experimental criteria can they be distinguished?

In considering allergy or sensitivity to particular animals, drugs and foods, one can watch the consequences to a sensitive patient. The person is in the presence of a cat, is given penicillin for pneumonia, or eats one biscuit containing nuts. He or she immediately has difficulty in breathing, may show urticaria, anaphylaxis and their hearts may stop. They may require intravenous anti-histamine, or intramuscular adrenaline. They may even require urgent tracheotomy. These represent true allergies. Such patients are to be distinguished from those who say that they are allergic to milk, but that they like cheese, biscuits, bread, etc. Some foods may make them retch or feel sick, but they are not true allergies. A person who is sensitive to a particular animal, food or drug, becomes very ill immediately after being exposed to the most minute quantity of the particular protein, even if the latter has been processed subsequently.

Tissue transplantation is another successful use of immunology. Rejection depends upon the age and health of the donor, and the time of transplant, as well as

the tissue match.

One must ask two questions in respect of the immune response, and they are also difficult to answer. Do immunologists maintain that the whole immune response (Table 119) occurs with every vaccination, immunisation, autoimmune disease, incompatible blood transfusion, rejection of transplanted tissue, drug sensitivity, food allergy, anaphylactic reaction? Also, how can one know whether the immune responses cause the biochemical changes or vice versa - or, perhaps, it does not matter.

After transplantation, recipients take a variety of medicines to suppress the immune response (Table 131). These represent a wide range of chemical structures, which may reflect the different parts of the immune system, which they are suppressing.

The concept of autoimmune diseases has been questioned considerably in this chapter, and it is probably untenable. The main grounds for rejecting it may be summarised: it is based on the assumption that normally the immune system *suppresses* the proclivity of some of its own tissues to act as autoantigens; that it is very unlikely that the body would produce antibodies, not just to a protein, but to a nucleus, membrane, organ, tissue and several tissues, when each cell in a particular animal or person has been shown to contain the same DNA. The large variety of diseases, aetiologies and treatments makes it extremely unlikely that all the phenomena studied by immunologists share a common immune mechanism. Autoimmune reactions seem to overlap inflammation.

Immunofluorescence and immunocytochemistry depend upon tissues fluorescing, either themselves (for example, tyrosine, tryptophane, phenyl alanine or lipofuscin), or being made to fluoresce by the addition of fluorochromes. Regrettably, the fluorescence is not very specific and many of the steps of preparation could change its intensity (Chapter 10).

Monoclonal antibodies are useful for diagnosing diseases, and they are being developed optimistically for the use of anticancer drugs to focus the destruction of tumour cells.

Anaphylaxis can occur in seconds, and an animal or human being can die rapidly. It is widely believed to involve the release of histamine and the immune system. Whereas there is time for the former, it is unlikely that the whole immune reaction could occur in such a short time.

Chapter 52

Dying and Death

This is a drama in six acts. First, a number of events determine that death shall occur. Second, the animal gradually dies. Third, the process becomes irreversible, and this is called the point of death. Fourth, a number of events follow death. Fifth, one has putrefaction. Sixth, the soft tissue disappears.

Death and dying can be seen as a gradual loss of organisation (Feldman and Hillman, (1969). The animal or human being dies, but its organs, can be excised and either be transplanted, or function, after isolation. Skin, kidneys, hearts, livers and lungs, can be transplanted successfully into recipients. Isolated kidneys produce urine. Isolated hearts beat and can do work. Isolated udders can produce milk. Although the donors may be dead and buried, their organs may go marching on.

When the latter fail, one can cut tissue slices of, brain, liver or kidney, and they concentrate K^+ , respire linearly, and metabolise, for 2-3 hours. They are clearly not dead. One may pluck out nerve cells, hepatocytes, ganglion cells, rods and cones, and they also respire linearly and exhibit many enzyme activities, but fail within a few hours. The organ may even be homogenised, and the fragments respire and show enzyme activities for a few hours. Here one sees the descent of organisation to the oblivion before creation.

There are several well known causes of death of animals, organs, tissues and cells (Table 134), some of them unnatural. However, it is generally believed that cells die because of hypoxia, often caused by local ischaemia. At a cellular level, hypoxia stimulates anaerobic glycolysis, the accumulation of lactate, the exit of K^+ from cells, and the entry of Na^+ .

These triggers of death may not necessarily be irreversible, because death can be prevented or delayed by cooling the tissue, providing more oxygen, vigorous perfusion or resuscitation.

Known causes of death

<i>Agents</i>	<i>Examples</i>
Tissue destruction	explosion; decapitation; gunshot wounds
Ischaemia	cardiac infarction; pulmonary embolus; thrombosis
Hypoxia	asphyxia; lung disease; gassing
Dehydration	lack of water; massive burns; vomiting; diarrhoea

Tissue denaturation	excessive heat; immersion in strong acid or alkali
Freezing	drowning; hypothermia; frost bite
Poisons	cyanide; poisonous gases
Radiation	x-rays; ultraviolet rays; ionising radiation
Loss of homeostasis	damage to hypothalamus; destruction of brain stem
<i>At a tissue level the following occur</i>	
Accumulation of lactate	in tissue cultures
Infections	bacteria and fungi producing toxins
Lytic enzymes	break down proteins and lipids
Organ or blood rejection	kidney transplantation; mismatched blood
Shedding of cells	from skin, mouth, alimentary tract, uterus, vagina

Table 134. In addition to these causes, it is widely held that cells are ‘programmed’ to die (apoptosis).

Dying and death can be observed in single cells and in tissue cultures. They generally swell initially and then shrink. Refractile particles can be seen in the cytoplasm. The cell and nuclear membranes become less clear. The refractive index of the cytoplasm increases. Intracellular movements stop. The mitochondria disappear. Blebs appear on the cell membrane. The transmembrane potential falls. Respiration decreases. The cell explodes. Its remains may be incorporated into vacuoles of other cells. However, if the cell has been isolated and kept sterile in a cavity slide, it may not show any changes over years, so that one can not know whether it is alive or dead. I remember celebrating the 4th anniversary of one such cell, whose unchanged appearance was clear evidence to the neurobiologist that it was the cell’s birthday. A list of cell changes is given (Table 135).

Of course, one can not examine the *progress* of dying and death in histological sections or electron micrographs, because the act of fixation kills the cells.

No one really knows which step is irreversible during dying. One may speculate that the cell proteins denature, possibly due to the low pH consequent upon the accumulation of lactate in the cells; this occurs if one does not change the medium sufficiently often in microbial or tissue cultures.

Events during the dying of cells

Cyanosis appears in the tissues
 Ischaemia occurs
 Homeostasis fails
 The temperature falls
 The tissue becomes hypoxic
 Glycolysis increases
 Lactic acid accumulate
 The pH of the tissue falls
 The cells depolarise
 K^+ leaves, and the Na^+ and Ca^{2+} enter the cells
 The viscosity of the tissue increases
 The tissue swells, or shrinks in a dry atmosphere
 Proteins denature
 The cells become stainable with methylene blue
 The nuclei shrink and break up
 The organelles break up
 Stained tissues appear blurred
 Parts of cells appear more darkly stained
 Autolysis occurs
 The permeability of membranes decreases
 Small molecules move down their concentration gradients
 Co-factors, which move, may stimulate or inhibit enzymes
 Microbes viruses and fungi invade, producing toxins and proteolytic enzymes
 Vacuoles are produced by anaerobic bacteria
 Phagocytosis occurs in the intact animal
 Immune responses disappear
 Gases, including ammonia, carbon dioxide and sulphides, are liberated
 The cells disappear

Table 135. These events have been observed. *Note that their exact order is not known. The terms 'suicide, pyknosis, karyorrhexis, karyolysis, necrosis and apoptosis' have not been used.*

One of the biggest mysteries for cell biologists is the real nature of what is called necrosis. It appears in histological sections as shrunken dark particles in ‘disorganised’ tissue, but the reagents used in histology and electron microscopy take such a toll of their chemical constituents (please see Chapter 8), that one does not know the chemistry of the tissue before staining. Von Virchow, (1859) distinguished between ‘dry’ and ‘wet’ narcosis. Observations on stained sections of gangrenous tissue showed shrinkage (‘pyknosis’) then cells broke up (‘karyorrhexis’), then, they

finally disappeared ('karyolysis'). Particles were observed 'engulfed' and white blood cells and bacteria were noted in the region. It is tempting to believe that naming the observations in neo-Greek would add to understanding their biological nature, but it should be regarded as a classification prior to further analysis.

Councilman, (1890) ligated the portal veins of rats to study necrosis, and he observed dark bodies, which were named after him. Kerr, (1971) showed that these bodies contained mitochondria and endoplasmic reticulum. Councilman bodies have also been seen in viral hepatitis, and in Civette bodies in the skin in lichen planus (Staunton and Gaffney, 1998).

In 1972, Kerr, Wyllie and Currie described what they regarded as a previously undiscovered type of cell death, which they called, 'apoptosis' or 'programmed cell death'. It was described as an 'active' process. It was a physiological, rather than a pathological phenomenon, in which some cells participated in their own death. It was sometimes reversible. It could be caused by a large number of agents, including hypoxia, cancer, toxins, Alzheimer's disease, etc. (Table 136), and was involved in other processes (Table 137).

Causes of apoptosis reported in the literature

<i>Biological</i>	
Programming by genes	Cell stress
Development of organs and limbs	Loss of matrix attachment
Congenital malformations	Addition of tumour necrosis factor
Organs during metamorphosis	Growth factor withdrawal
Graft versus host diseases	Regression of tumours following treatment
<i>Pathophysiological</i>	
Mild heat (44°C)	Liver after carcinogenesis
Hypoxia	Ultraviolet or ionising radiation
Wound healing	Cytotoxic drugs for malignancy
Endocrine deficiency	T cell killing
Atrophy of sex organs after castration	Autoimmunity
Organ atrophy and failure	

<i>Unnatural reagents</i>	Cytosine arabinoside
Ethanol	Actinomycin D on Hela culture cells
Fixatives	Oncogene and tumour suppressive gene products
<i>Diseases</i>	
Cancer	Parkinson's disease
Degeneration of the liver	Amyotrophic lateral sclerosis
Myocardial infarction	Alopecia areata
Heart failure	Basal cell carcinoma
Myocarditis	Acute peritonitis
Arteriosclerosis	Skin sunburn
Stroke	Bowen's disease
Leprosy	Lichen planus
Sarcoidosis	Regressing keratoacanthoma
Tuberculosis	Melanoma
Crohn's disease	Bacterial and virus toxæmia
Lupus erythematosus	Burkitt's tumour
Foreign body granulomatosis	Fixed drug eruption
Alzheimer's disease	Viral diseases

Table 136. This list of causes is not comprehensive. It is mainly drawn up from Kerr, Wyllie and Currie, (1972), Majno and Joris, (1995), Thompson, (1995), Harmon and Allan, (1996), Sluyser, (1996), Wyllie, (1997), Haunstetter and Izumo, (1998) Apoptosis Issue, (1998) and Malmusi and Ackerman, (1999).

Categories of biological processes in which apoptosis is believed to be involved

Adjusting cell numbers	Inflammation
Atrophy of endocrine glands	Involution of hyperplasia
Caspase activation	Metamorphosis
Cell death	Phagocytosis
Embryonic development	Signalling
Genetic expression	Teratogenesis
Immune cell ontogeny	Toxin exposure
Immune killing	Tumour cell deletion

Table 137. Each category consists of many different processes. They are listed more fully in Arends and Wyllie, (1991), and sources quoted there. See also Lockshin, Zakeri and Tilly, (1998). *These all involve some cells dying.*

Apoptosis or programmed cell death was alleged to be different from the previously recognised necrosis. By histology and electron microscopy, it was seen to occur in single cells, rather than in groups. Chemically, apoptosis required energy and protein synthesis, and a ‘cascade’ of proteases (caspases) occurred as a result of it. Soon, a cascade of differences between necrosis and apoptosis was observed, many of them dependant upon whether they were seen to occur early or late in the process (Table 138). Differences included shrinkage of cell membranes, blebbing, chromatin condensation and nuclear fragmentation. In contrast, necrosis is characterised by mitochondrial swelling, nuclear flocculation and the loss of plasma membrane integrity, which results in uncontrolled cell lysis. Time lapse studies show that apoptosis takes about 1 - 3 hr, and apoptotic structures persist for about 1 hr (Gorman et al, 1994).

Differences between necrosis and apoptosis

<i>Necrosis</i>	<i>Apoptosis</i>
A. Structural	
Death of groups of cells	Death of single cells
Cell membrane disrupted	Cell membranes intact
Cells swell	Cells shrink
DNA fragmentation late	DNA cell fragmentation early
Few spaces between cells	Prominent spaces between cells
Cell lysis and disintegration	Fragmentation of nucleus and cytoplasm
Phagocytosis by macrophages	Phagocytosis of apoptotic bodies
Acute inflammation	No inflammatory response
No loss of microvilli and desmosomes	Loss of latter
Dyes enter cells	Dyes do not enter cells initially
Swollen mitochondria and dense matrix	Compact intact organelles and swelling of cytoplasm
Dilated organelle profiles	Condensed endoplasmic reticulum
Lysosome breakdown	Lysosomes and mitochondria intact
Nuclear swelling	Condensed chromatin
Nuclear pores adjacent to membranes disappear	The nuclear pores do not disappear
Nucleoli compact until advanced degradation	Nucleoli break up into granules
	Blebs are reported around cells

<i>Necrosis</i>	<i>Apoptosis</i>
B. Biological and biochemical	
Non genetic	Genetic
Accidental	Physiological or pathological
Irreversible	Sometimes reversible
Non-energy dependant	Energy dependant
Cessation of protein synthesis	Requires protein synthesis
Random DNA degradation	Non-random oligosomal length
Release of acid hydrolases	Release of caspases
	Cell mediated immune killing

Table 138. These differences are reported by Kerr, Wyllie and Currie, (1972), Wyllie, Kerr and Currie, (1980), Cohen et al, (1992), Bowen, (1993), Wyllie, (1997), Staunton and Gaffney, (1998), Kroemer, Dallaporta and Resche-Rigon, (1998) Wyllie and Duvall, (1999). See also Cotter, (2003).

Among the techniques used to detect apoptosis were trypan blue exclusion, staining with acridine orange and uptake of ethidium bromide. It can be detected also by scanning and transmission electron microscopy, flow cytometry (size and granularity of cells) and Percoll density gradient centrifugation. Apoptosis has been reported to be seen in lesions in many diseases, which may be listed in alphabetical order (Table 139).

Diseases in which apoptosis is believed to be involved

Adrenovirus	Colonic cancer
Adrenal atrophy	Cytotoxic T cell killing
AIDS	Follicular lymphoma
Alcoholism	Graves disease
Alzheimer's disease	Haemorrhagic liver necrosis
Amyotrophic lateral dystrophy	Heart failure
Aplastic anaemia	Herpes
Atherosclerosis	Huntington's disease
Autoimmune thyroiditis	Immunomediated glomerulonephritis
Basal cell carcinoma	Irradiation
Breast cancer	Liver necrosis
Carcinoma p53 mutations	Lung cancer
Cerebellar degeneration	Myocardial infarction

Neuro-degenerative disease	Retinitis pigmentosa
Non-Hodgkin lymphoma	Rheumatoid arthritis
Ovarian cancer	Thyroid cancer
Pancreatic duct obstruction	Sarcoma
Parkinson's disease	Stroke
Pox virus	Systemic lupus erythematosus
Prostate cancer	Viral diseases
Reperfusion injury	

Table 139. ‘Apoptotic’ activity is increased or decreased in these conditions. The data comes from Thompson, (1995), Winkler, (1999) and Lin, (2001). *These involve growth of some cells and dying of others.*

Between 1972 and 1998, there have been about 20,000 publications on apoptosis, (Goldstein, 1998). Very few have questioned the fundamental concept of the phenomenon critically (Farber, 1994; Malmusi and Ackerman, 1999). A recent ‘Essay in Biochemistry’ (Cotter, 2003) was entitled ‘Programmed Cell Death’. I am indebted to Dr Malmusi and Dr Ackerman for their very useful insights into the concept of apoptosis. It has many difficulties.

1. One can not use any procedure which kills, fixes, homogenises cells, or subjects tissue to histology or electron microscopy, to study the process of dying or death (please see Chapters 8,9). Another reason for which histology and electron microscopy can not be used, is that *sections* are made of *shrunken* tissues, and one can not measure the dimensions of any cells in such situations.

2. There is no doubt that longevity of an animal species has some genetic element, since the natural life history of most species and varieties falls within a limited range - many of them being known to zoologists. Nevertheless, the mechanisms controlling ageing, longevity and life span are not known. Red cells, white cells, myometrial cells and squamous epithelial cells, all have different life spans in the same animal. If, say, the average life span of an Englishman is 75 years, it is not known, if this represents the maximum regenerative power of continuously working organs like the heart, the lungs, the intestines, the kidneys and the retinas or if the body contains a master programme organ, which switches on at birth. Of course, there are other possibilities. The older an animal is, the more traumas, stresses, pollutants, illnesses, accidents and mutations, to which it has been subject. Nevertheless, the relationship between the life expectancy of any particular animal,

and each of its variety of cells is simply not known.

When one looks at the list of activities in which apoptosis is believed to be involved (Table 137), it seems that the development of organs and metamorphosis in some species are the only elements, which might be programmed. The others may be consequences of changes in the environments of the cells. It is not known how changes in protein quantity or conformation, enzyme activities or cellular metabolism, affect the life span of the cell.

The idea of apoptotic cell death implies that the cells have already inherited a programme, which causes them to die at a particular time. However, one can draw up a long list of *pathophysiological* causes of apoptosis (Table 136). Many of the experimental models of apoptosis involve powerful agents such as radiation, ethanol, hypoxia etc.

3. When one examines the differences between necrosis and apoptosis (Table 138), each criterion has problems: from a single section, one can not tell whether a single cell or a group of cells is affected; for the same reason, one cannot know whether the membrane of a particular cell is disrupted or not, when it is said that some cells swell, while others shrink. The fragmentation of DNA can not be assessed in a single section; the amount of space between cells can not be assessed, because it depends upon the plane of the section, as well as the staining system used; cell lysis and ‘disintegration’ are not necessarily different from ‘fragmentation’ of the nucleus and cytoplasm; the presence or absence of phagocytes would be an important difference, if it had been shown to occur by rigorous and statistically controlled observations; the same is true for the presence or absence of the inflammatory response; the loss of microvilli and desmosomes should also be compared statistically, if they did not suffer from the disability of being artifacts (Chapter 33); dyes like trypan blue enter necrotic cells, apparently rapidly, but not, *initially*, apoptotic cells. Presumably this depends upon whether the preparation was made soon after the experimental or clinical lesion, and on several sections of the same lesion; the swelling of the mitochondria, or of the cytoplasm can not be assessed in a single section, and merits serial sections and statistical demonstration; the same holds for organelle ‘profiles’; ‘lysosomal breakdown’ means the presence of many granules, where one might have been previously; nuclear swelling is a significantly different finding from chromatin condensation; nuclear pores would be of interest in comparing the two phenomena, if one believed that they were not artifacts (please see Chapter

31); the ‘compactness’ of the nucleoli can not be assessed in a single section, nor can the number of granules into which it breaks up; ‘blebbing’ is a sad characteristic of apoptosis, shared unfortunately with many cells dying in tissue culture.

Apoptosis is thought to be genetically controlled because defects in certain genes, believed to be involved in apoptotic pathways, cause developmental defects (Yoshida et al, 1998; Ranger, Malynn and Korsmeyer, 2001); the characterisation of necrosis as ‘accidental’ means that is is not believed to be pathological, a view hardly likely to be embraced by pathologists; whereas necrosis is regarded as irreversible, apoptosis is believed to be reversible. It is difficult to know how one could arrive at the latter conclusion. When one examines a lesion under a microscope, there is absolutely no way of knowing whether particular cells are dying, dead, recovering, necrotic or, for that matter ‘apoptotic’, to coin a term; the energy dependence of apoptosis presents a serious problem. Cells die, *inter alia*, when they run out of energy. Dying is regarded as a catabolic process. Thus the idea that a dying piece of tissue would be able to ‘recruit’ energy, say, from mitochondria in the presence of a diminishing supply of oxygen, substrate and blood, seems somewhat unlikely; the same would be true for the energy and metabolic machinery for protein synthesis, when metabolic pathways would be in disarray. Differences in oligosomal lengths between necrosis and apoptosis would be of significance if they had been shown statistically. The release of acid hydrolases is regarded as a sign of necrosis, and caspases are markers of apoptosis. The 14 caspases in mammals are cysteinyl aspartate proteases, believed to be involved in inflammation as well as apoptosis (Zhivotovsky, 2003). They can be arranged in pathways, called ‘cascades’. Their activation is seen at the same time as the histological changes in apoptosis, and so it has been supposed that the latter cause them. Although the many lysosomal enzymes have been detected in catabolising tissue, while the caspases are regarded as specific for apoptosis, the monopoly of both enzyme groups to either of these processes has not yet been demonstrated; apoptosis is also believed to be involved with the T-lymphocyte killer cells, thus forming a connection with the immune system.

A few general remarks are appropriate in respect of the differences between necrosis and apoptosis. Firstly, in neither case does the histology give any information about the chemistry of the subcellular organelles, granules or ‘apoptotic bodies’ seen. Secondly, most of the morphological statements about appearances using these techniques would require serial sections to justify, and, of course, the

assumption or belief that none of the steps in their preparative procedures would change such parameters as, the size of the organelles, the intactness of the membranes, the shapes of the cells, etc. Thirdly, most of the characteristics distinguishing the two different kinds of death should be identifiable in populations of those cells, not just somewhere in the tissue, which is then labelled as undergoing necrosis or apoptosis. Fourthly, expressions like ‘usually’, ‘mostly’, ‘early’, ‘late’ are sometimes subjective. Fifthly, histology and electron microscopy still remain two of the few areas of biological sciences, which are not regulated by statistics. All criteria alleging differences should be shown preferably double blind and significant.

4. It is conceivable that the chemical reactions, pathways and cascades, described as occurring in apoptosis, occur, for example, during the relatively slow process of development, but it seems *extremely* unlikely to have time or specificity to occur, for example, when a tissue is fixed with buffered osmic acid, when an animal is killed by rapid freezing to - 196°C, or blown to pieces by a bomb. This sort of reasoning leads one to suggest that these ‘non-specific’ rapid deaths do not need all the fanfare of caspase cascades. The same also applies to the concept of death receptors (Wajant, 2003). One does not know the chemical triggers of anabolism, catabolism, dying, life or death.

5. If apoptosis were an important element of dying, one would expect that inhibition of caspases (Zhivotovsky, 2003) would delay death, and also that they would be effective against some of the diseases, in which it is believed to be involved (Table 139).

6. It is not surprising that, during dying, proteases and caspases, and possibly several other enzyme systems and metabolic pathways, are activated. Perhaps other systems would be inhibited because death causes a redistribution of all ions, and any other substances to which the living membranes might have been impermeable, but dying membranes are not. Thus if Na^+ activated enzymes in life were in the cytoplasm, - and not in the membrane, as is commonly believed, - when tissue was dying, the higher concentration of Na^+ would enter the cells, and it would appear to biochemists that dying activated Na^+ ATPases. So, one must be extremely wary of extrapolating localisations of activities found in dead and disrupted tissues back to the state *in vivo*. In general, one may say that any activating ion or co-factor which can cross the cell membrane in life or which might be redistributed during dying or disruption of a tissue, may appear to be activating or inhibiting an enzyme or a

metabolic pathway, during dying or death.

7. The apparent dark staining of necrotic particles, Councilman bodies, apoptotic bodies, nuclei or nucleoli could result from: shrinkage due to dehydration; precipitation by histological reagents; greater affinity for a particular stain; breaking up of the particle by proteolytic enzymes; cell lysis; change in cytoplasmic pH. Unfortunately, there is no way of distinguishing between these possibilities, but some of them may be artifacts of preparation.

8. The disappearance of cells during embryonic development and the opening of webs, which are attributed nowadays to apoptosis, could alternatively be due to: insufficient blood supply to keep all the cells in the tissue alive. This is reasonable in view of the fact that the genes control the development and the adult architecture of the body. However, these anatomical changes are also controlled by the stem cells, by the endocrine system, and, perhaps, the brain, rather than by the target organ, engaging in apoptosis.

9. DNA measurements can not distinguish between apoptosis, necrosis and autolytic cell death (Grassl Kraupp et al, 1995), because the DNA is extracted from dead tissues.

10. One must be very careful about extrapolating the biology and chemistry of dying in unicellular animals or plants, or in tissue cultures, to dying in mammalian cells in intact animals. The chemistry and the environments of the different systems are so very different (please see Chapter 12).

11. The tissues breaks down due to the activities of proteolytic enzymes, and the invasion by aerobic and anaerobic bacteria, fungi and viruses. These digest the soft parts of the tissue and give off gases, such as carbon dioxide, ammonia and hydrogen sulphide. The tissue swells and may explode. If the bodies are in the open, they may be eaten by other animals, invaded by insects, and eggs may be laid in them. If the vapour pressure of water outside the body is low, its subsequent loss of water may allow it to be mummified, when the fats turn into wax. If the temperature is below the eutectic point of the body fluids, the body may freeze solid. The act of freezing will keep the water in the body, but will separate it from the solutes in the body fluids, so that the cells become effectively dehydrated, although the ice crystals are very close by. The rest is archaeology.

12. There are a number of ways which have not yet been explored sufficiently, to study the dying process. These include: studying clinical biochemistry of blood

and, perhaps, urine during and after dying; studying post mortem tissues, immediately after death, and for several days, to observe changes in animals, of enzymes, proteins, lipids, carbohydrates etc, (Hardy and Dodd, 1983); phase contrast, anopteral, confocal and video-enhanced light microscopical observations on fresh, unfixed single cells such as red cells, lymphocytes, sperm, ova, ganglion cells, bronchial cilia, bronchial washings, cervical biopsies and buccal epithelial cells, while they are dying or being killed, or while these processes are being delayed or prolonged; cinematographic observations, combined with clinical examination of dying animals (Feldman and Hillman, 1969); similar microscopic examinations of injected single cells to watch intracellular particles and their movements; observations on brain windows and living web capillaries during dying of animals; retinoscopy of dying animals; reflectance and split-beam spectrophotometry of the corneas, brains, livers, intestines, peripheral nerves, etc., of dying animals.

Section D

Consequences to Cell Biology

Chapter 53

Relevance of Cell Research to the Biology of Normal and Ill Animals

This chapter can be divided into several topics. First, the relative validity of the different procedures used in biological experiments. Second, the relevance of the animal experiments to human physiology and pharmacology. Third, the relationship between healthy and diseased tissues.

The dimensions, physiology and chemistry of any part of an organism should not vary with different procedures used to discover them. This means that the thickness of a membrane, the activity of an enzyme or the concentration of a nucleotide, should each be described within a small range, by all the procedures used to measure them. If two do not agree, one or both of the procedures must be erroneous. It is one's duty to attempt to explain and correct any significant difference. It is useful to arrange the different categories of procedures into two hierarchies, based on their chemical, thermodynamic (Hillman, 1976) and biological complexity. They give information, which is limited by the position of the preparation within the hierarchy. The categories of experiments are arranged upside down, so that the simplest preparations head the lists, while the most complex lie at their feet.

Chemical hierarchy of preparations

<i>Organisation</i>	<i>Comments</i>
Chemical constituents in natural concentrations and with natural activities	may be unmeasurable
Natural chemical mixtures inside and outside cells	the properties of chemical constituents should be studied under these conditions
Metabolising mixture	may respire and show enzyme activities, but not their rates
Isolated cells	should be separated with little energy
Tissue slices	partially metabolising, partially disrupted
Tissue culture	chemistry depends on growth media
Isolated organs	may function as in intact animal
Anaesthetised animal	anaesthetic may affect chemistry
Conscious, unstressed, unrestrained animal	may be affected by experimental conditions

Table 140. *The nearer the organisation is to the bottom of this list the more its chemical conditions reflect those in the healthy living animal.*

Biological hierarchy of preparations

<i>Tissue</i>	<i>Comments</i>
Cytoplasm	withdrawn from cell under direct vision
Insoluble fractions	contains all cell membranes
Extracellular fluid	clinical biochemistry, or small sample withdrawn from extracellular phase
Homogenates	broken membranes; mixed compartments
Homogenates, plus added reagents	may not show the same chemical activities as in the intact animal
Tissue cultures	chemistry completely dependant on growth medium
Tissue slices	surrounded by unknown proportion of disrupted tissue and metabolites
Tissue slabs	should be in media imitating those in intact animal
Isolated organs	may not be perfused as in vivo
Whole animal	most accurate

Table 141.

Even if the tissue components could be extracted and purified completely, when they were added together in the same proportions as they were believed to be in the living organelles, they would react with each other, and establish new bonds and equilibria with each other. Since the membranes between the cells and the organelles are believed to be semi-permeable, the equilibria, may be rather brief, - indeed, they may change so rapidly as to be unmeasurable. Whole animals are open systems.

There are many tissues, such as liver, muscle, heart, cornea, lens, which have relatively small extracellular phases, and they contain very uniform cell populations. Therefore the whole organs may be used to study the chemistry of their particular cells. Of course, when they can be examined in perfused organs, the properties found will approximate to those of the whole organ in life. In the far distant future, it may be possible to decide if, the properties of an organ add up to more than the properties of its components.

Some tissues such as skin, intestine, retina, have several histologically identifiable layers. One may study the physiology of the whole tissue, regarding it as dangerous to separate different layers, but it may be more informative to know what the individual layers do. One may separate the layers gently, and attempt to elucidate the biology of each layer.

Tissue slices are of limited value, even when they respire linearly and show enzyme activities, since one does not know the effects of pressure during cutting, the contact with the media, and the metabolism of dying tissue, on the chemistry of the subcellular organelles. However, they do have some real uses in studying anaerobic glycolysis, protein synthesis and K⁺ accumulation (please see Chapter 13).

Cells in tissue cultures change as they grow and their biochemistry is completely dependant upon their growth media. It is also highly likely – although it has not yet been tested adequately – that a parent tissue changes its shape, volume, staining and immunological properties, during recovery, growth and division (please see Chapter 12).

Isolated limbs or organs, such as legs, hearts, kidney, brains, etc, can be extremely good preparations for studying tissues either perfused artificially, or by other animals' circulations, or by their own circulations. One can study arteriovenous differences, reflexes, excretions, electroencephalograms, in systems in which cells have not been disrupted.

The chemical hierarchy is not quite the same as the biological hierarchy. Very important information can be derived by withdrawing minute quantities of extracellular fluid, cytoplasm and axoplasm, and studying them by microchemical procedures (Osborne, 1974). Nuclei and membranes can be dissected out and their enzymes measured manometrically using the microdiper (Chapter 14). It is simply not known how these manipulations change the properties of the cellular parts examined. However, they do involve remarkably little energy, and, so little movements of chemicals or changes of entropy may be expected.

Homogenates may take up oxygen linearly and demonstrate enzyme activities. Their relevance to the study of living tissues is that they may be used to demonstrate single enzyme activities, and what substances in physiological or pharmacological concentrations enhance or inhibit the particular enzyme activities. However, until the effects of homogenisation have been tested on tissues systematically, any measurements must be regarded as qualitative, rather than quantitative.

Tissue slabs were developed in brain (Burns, 1958). These were small areas of cerebral cortex, cut off from surrounding brain, but still perfused by superficial blood vessels. They were neurologically silent, but the effects of drugs and transmitters could be studied on the cerebral cortex. The separated pieces of brain reacted to electrical stimulation with a 'burst' response. This original preparation has

not been exploited by other neuroscientists. Unfortunately, it could only be used in organs such as the brain and spinal cord, whose blood supply comes from the surface.

The second relationship which requires examination is the relevance of the findings of experiments on animals to human beings. It is probably a fair generalisation that the physiology and biochemistry of mammals, such as rats, mice, cats, dogs, sheep, cows and monkeys, etc, can be extrapolated to human beings. However, animal and human physicians do not necessarily practice the same pathology and pharmacology as each other, since different mammals are susceptible to different diseases, and they sometimes vary in their responses to different drugs by several orders.

Anti-vivisectionists assert that many experiments on animals are cruel, and irrelevant to human disease. It is true that confining animals in small spaces, not feeding them properly, killing them painfully, restraining them and carrying out experiments on unanaesthetised animals, can be very traumatic. However, most of these procedures can be avoided by planning experiments appropriately. Furthermore, the whole chemistry of an animal may be distorted by pain or stress, and these may mask the physiological phenomena, which the research workers are intending to study.

One of the uses of animals is to study the toxicity of drugs, about which antivivisectionists complain. Whereas it is true that some drugs, which are not toxic to animals, would harm human beings, it would generally be agreed that a drug which was found to be dangerous to mammals at low doses, would never be tested on human beings; this alone justifies some experiments on animals.

The relationship between a healthy and the diseased tissue has to be examined. Disease does seem to pick out organs, for example, *Salmonella* and *Campylobacter* attack the intestine; multiple sclerosis affects the nervous system; alcohol causes gastritis and cirrhosis of the liver; muscular dystrophy causes muscle to degenerate. Traditionally research into a disease takes the following route: the natural history of the disease is recorded; activities which allay or intensify it are noted; the social and family history of the patient is recorded; the doctor examines the patient; the chemistry of the body fluids may be analysed; x-rays, respiratory tests, electrocardiographs, computer assisted tomography, electroencephalographs and nuclear magnetic resonance scans, may be carried out; a tissue biopsy may be necessary; a diagnosis is made; specific or symptomatic treatment is prescribed; the

patient may come to post mortem; epidemiological studies may be pursued. It should be noted that the physician and the pathologist are generally looking at the *consequences* of diseases, and do not know their geneses, except in the case of infectious diseases.

Attention to this drama of disease suggests an outline for a rational approach to research into it, in the following steps.

1. The susceptible organs are identified by symptoms and signs, pathological findings and detection of sites of action of effective drugs.
2. The chemistry of the affected organs is compared with that of unaffected, organs from the same patient or animal, by (a) if possible, perfusing both organs and comparing the chemical differences between the arterial and venous blood of the two; (b) cutting tissue slices of the two organs, incubating them and comparing the chemistry of the slices and fluids in which they have been incubated; (c) if possible, comparing the latter two (b) with the organs of an allied species subjected to the same condition, which is not afflicted by the disease; (d) comparing the enzyme activities of the tissues in (b) and (c), including the effects of successful therapeutic agents on them. In these experiments, the tissues should be incubated in plasma, lymph, cerebrospinal fluid, or incubationg solutions, such as Krebs-Ringer, imitating them. All drugs used should be administered to the intact animals or human beings in pharmacologically effective doses.

Fresh, dissected and teased small pieces of unfixed tissues in life like media (Table 148) can be examined by phase contrast, anopteral, dark ground, interference, Rheinberg, video-enhanced and confocal microscopy (Chapter 11). It is quite surprising that despite the distortions of histological procedures being known for the major part of the 20th century, so little attempt has been made to examine unfixed lesions in so many different diseases (Tables 142, 143). In addition to seeing cells in conditions approximating to those in the intact patients or animals, one may also be able to observe the evolution of cells from healthy, through precancerous to cancerous or sarcomatous states.

Types of lesions which have not been examined, unfixed and unstained, by light microscopy

Precancerous, cancerous and normal cells of cervix uteri
 Early cancerous cells from all tissues
 Sarcoma cells
 Normal and malignant blood and bone marrow in leukaemia
 The development of plaques in the nervous system during the evolution of multiple sclerosis
 Neurofibrillary tangles and plaques in Alzheimer's disease
 Degenerating and degenerated fibres in the extra-pyramidal system in Parkinson's disease
 Cells in the nervous system in amyotrophic lateral sclerosis
 All cells in the brain regions, which have been reported to be changed in schizophrenia
 The evolution of cells during cirrhosis
 Neurons and neuroglia in dementia
 The evolution of islets of Langerhans in diabetes
 The evolution of muscle fibres in muscular dystrophy
 The degeneration of retinal cells during macular degeneration
 Necrotic tissues

Table 142.

Diseases where cells have not yet been examined fresh by light microscopy

Acquired immune deficiency disease	Colitis
Addison's disease	Creutzfeldt-Jacob disease
Alcoholism	Cushing's disease
Allergy	Dandruff
Alopecia	Dupuytren's contracture
Alzheimer's disease	Eczema
Amyloid disease	Epidermolysis bullosa
Amyotrophic lateral sclerosis	Epilepsy
Aneurysms	Exfoliative dermatitis
Ankylosing spondylitis	Friedreich's ataxia
Atherosclerosis	Glial scars
Autoimmune diseases	Gliomata
Blood dyscrasias	Gliosis
Burns	Haemolytic anaemia
Cachexia	Hashimoto's thyroiditis
Cancers	Hepatolenticular degeneration
Cardiac infarction	Hodgkins disease
Cataracts	Huntington's chorea

Ileitis	Osteoarthritis
Inflammation	Parkinson's disease
Jaundice	Psoriasis
Macular degeneration	Senescence
Meniere's disease	Metastases
Multiple sclerosis	Spongiform encephalopathy
Muscular dystrophy	Subacute combined degeneration of the cord
Nephrosis	Tourette's syndrome
Neurofibromatosis	Vitiligo
Non-Hodgkins lymphoma	Virus disease
Pemphigus	Ulceration
Precancerous cells	

Table 143. *This list is not comprehensive.*

4. Examination of whether effective drugs reverse the microscopic and biochemical characteristics of the lesions.

5. Identification as in 2, of any biochemical differences in metabolic pathways or cycles between normal and ill tissue, with a view to finding drugs for reversing the abnormalities found.

6. Injection of small lesions into litter mates and watching their development, using boiled pieces of the same specimens as controls.

One of the aims of all biological research workers is to distinguish between the biochemical changes *preceding* such phenomena as fertilisation, resting potentials, action potentials, conduction, meiosis, mitosis, transmission, muscular contraction, inflammation, stress, sleep, ageing, dying, death and necrosis, and those *consequent* upon these phenomena. The same is also true of virtually all biochemical lesions found in association with diseases. The Holy Grail is the programme of the mysterious events which initiate them. Thus, it is useful to try to list some criteria, which may be able to help to distinguish between the two.

First, a cause of a physiological phenomenon or a disease will have to be evident *before* the phenomenon or lesion is manifest. It may or may not continue to be seen as the phenomenon or lesion progresses.

Second, animal models of human diseases should only be considered adequate, if they show nearly all the clinical signs and the biochemical lesions of the human diseases, and the human treatments are effective on the animals.

Third, a lesion is likely to be a consequence, of the disease, rather than part of the mechanism generating it, if the lesion is present in many clinically similar diseases, not if it is found in rather than a few. The latter is the case for diseases in which the following are believed to be involved: mitochondria, (Table 62); lysosomes (Table 71); G-proteins (Table 102); peroxisomes (Table 72); free radicals (Table 107). The diseases are so widely different in their aetiologies, natures, histories, pathologies, clinical biochemistry and treatments, that it is extremely unlikely that they share common initiating mechanisms.

Fourth, as a corollary to the latter, and by analogy with endocrine diseases, any conditions regarded as deficiency diseases should be treated successfully by active addition of the inadequate substances. Further any disease, in which an excess of the substance found, should be curable by inhibiting its synthesis or activity chemically.

As a footnote to discussion of biochemical lesions in disease, it is worth remarking that it is extremely unlikely that any major disease which does not cause the patient to die, involves such widely distributed properties and substances, as membrane permeability, DNA conformation, the adrenergic system, the cholinergic system, ATP, K^+ , Ca^{2+} , or Na^+ , as they are all parts of ubiquitous systems accurately controlled in life by homeostasis. The exception to this would appear to be the anti-cholinergics being very successful in the treatment of myasthenia gravis. It seems rather strange that although acetylcholine is believed to be a transmitter for the heart, bronchi, intestine, etc., its deficiency affects mainly the ocular and other skeletal muscles, but not the other cholinergic systems.

Chapter 54
Summary and Conclusions

1. Whereas thermodynamically living systems are open, most biochemical experiments are carried out in largely, but not completely, closed systems.
2. Changes in entropy of the systems being studied must affect their chemical activities relative to those in living intact animals.
3. Measurements of concentrations in a whole homogenate give no information about the concentrations or activities in subcellular organelles in life.
4. Disruptive procedures, including subcellular fractionation, can not be assumed to give reliable chemical information about the original subcellular organelles in the intact animals, until comprehensive control experiments on the effects of the procedures on the reactions studied, have been completed. Such experiments have now been awaited for 60 years.
5. Low energy procedures yield information about living tissues more accurately than high energy procedures.
6. Similarly, the effects of all reagents used in biochemistry, histology, histochemistry, electron microscopy and immunocytochemistry on reactions, will have to be examined, before conclusions can be drawn relevant to the chemistry of living cells.
7. Soluble components will move to different compartments during any histological procedures, including fixation, dehydration, heating, freezing, etc.
8. The ambient temperature, and the source of illumination used to observe Brownian movement, streaming and diffusion, provide sufficient energy for these movements, which also occur in colpoids, so that it is unnecessary to assume the existence of molecular motors.
9. In living cells, intracellular movements of light microscopically visible particles preclude the existence in life of a cytoskeleton, as does its two dimensional nature.
10. Geometrical considerations show that the ‘unit’ membrane consists of one layer in life. The metal stains deposited on both faces make each layer appear ‘trilaminar’.
11. The thickness and chemistry of membranes in life are not known, and are possibly unknowable.

12. Ion channels are probably artifacts.
13. Nuclear pores are artifacts.
14. The myelin *lamellae*, the endoplasmic reticulum and the Golgi body are artifacts.
15. Light microscopy of living or fresh unfixed unstained cells, both from healthy tissues and from diseased organs, needs to be carried out.
16. There are no anatomical macromolecular receptors. Usually, it is assumed that a reaction with a transmitter, hormone, drug, toxin or ligand, means that each of these has one or a family of preformed dedicated receptors. Since the evidence for the receptor is usually only that a reaction has taken place, this represents a circular argument. Macromolecular and transmembrane receptors are not seen by electron microscopy, although the sequencing of the molecules indicates that they are large enough to be seen using this instrument.
17. The chemistry of receptors is created by the ligands.
18. The cristae of the mitochondria are artefacts, as is the apparent trilaminar appearance of the inner and outer mitochondrial membranes. Mitochondrioplasm is fluid in life. The mitochondrial membrane is one layer thick.
19. The mechanisms by which particles and macromolecules enter and leave cellular compartments are not known.
20. There is no pump that keeps Na^+ higher outside the cell membrane than inside it. No such mechanism is housed in the cell membrane. The gradients are caused by the higher affinity of substances in the extracellular phase for Na^+ , and the higher affinity of substances in the cytoplasm for K^+ .
21. The passage of action potentials along axons is due to excitation passing down the axoplasm, rather than across the cell membrane or myelin sheath, which are believed to insulate the axon. The excitability appears across the membrane, because the current in the axon induces a current in the extracellular phase.
22. Apoptosis is not a meaningful concept, because swelling or shrinkage can not be assessed in a single section, or in a dehydrated tissue. Furthermore, the microtome may cut sections in any orientation.
23. Transmission is not a chemical phenomenon causing release of transmitters but excitation is communicated by neuroglia to the nearest neurons. Release of transmitters may be a cause or a result of passage of current between excitable cells.

24. The chemiosmotic hypothesis is based experimentally on subcellular fractionation, and contains too many untestable elements to be warranted.

25. The sliding filament hypothesis of muscle contraction depends upon the existence in life of thin and thick filaments, which do not seem to be governed by the laws of solid geometry, and of cross bridges, which probably consist of heavy metal deposited during preparation for electron microscopy. The hypothesis as a whole is unwarranted.

26. The body probably does not contain a mechanism which suppresses a response to its own proteins, which, when it fails, permits it to react against its own tissues. Autoimmunity is a very unlikely concept.

27. The immune response can not be distinguished from inflammation.

28. The many chemical changes, which have been found during inflammation, may be *consequences* of the phenomenon, rather than initiators of it.

29. Antigens act as templates for the antibodies they induce. The lymphocytes are not covered by a huge number of antibodies to antigens, known, unknown, and to be discovered.

30. The central nervous system consists of a relatively small number of neurons in a ubiquitous neuroglial syncytium, in which naked nuclei can move. All cells with processes are neurons. The neuroglial nuclei are the only other cellular elements, in addition to the capillaries and the blood.

31. The anatomical synapses are artifacts, consisting of two dimensional deposits of heavy metals. The dendrites rarely, if ever, make contact with each other or with other cell bodies.

32. Free radicals are released both in normal metabolism, and in a number of pathological conditions, including hypoxia and cancer. Therefore, free radicals as such, can not be considered to be harmful, necessarily contributing to the illness of an animal.

33. The chemical event which makes death irreversible is not known.

34. All biological procedures, especially disruptive ones, must be accompanied by parallel accurate and sufficient control observations, *before* the validity and interpretation of the experiments are accepted.

35. All research workers should list systematically all the hypotheses *inherent* in their procedures, units, calibrations, experiments, interpretations, and the theories derived from the experiments. The warrantability of the crucial hypotheses and

assumptions should always be tested by further appropriate experiments. The necessary control experiments must be done before such experiments are accepted as complete.

36. Many frequently used procedures have not been examined physicochemically or thermodynamically. They are regarded as empirical steps, whose effects need not be examined (Table 144).

37. Radioactive isotopes, which have no significant effects on the chemistry or biology of tissues are to be recommended.

38. Heating above 60°C, freezing below the eutectic point of tissue fluids, and addition of unnatural reagents or reagents in unphysiological concentrations, are undesirable.

39. Inhibitors and activators of enzymes, transmitters, receptors and drugs, should only be used in experiments, in which they have been shown to have the same effects and to be 'specific' in vivo, as well as in vitro in the same concentrations.

40. Stressful and painful experiments should be avoided, not only because an animal in stress or pain many have different biochemistry than one at ease, but also because it is unkind.

41. Many operational terms used in biochemistry have not been defined (Table 145).

Not always recognised effects of experimental procedures

<i>Manoeuvre</i>	<i>Other effects</i>
Stressing the animal	blood creatine, adrenalin, and noradrenalin, rise; hypoxaemia; hypercarbia; lactate acidæmia
Killing the animal	tissue ischaemia, hypoxia; lactic acidæmia; hyperkalaæmia; hyponatraæmia
Stunning the animal	severe stress; spastic paralysis; unconsciousness
Pithing or sticking the animal	pain; convulsive movements; flaccid paralysis
Immersion in freezing agents	as in killing; crystallization in cells; dehydration; shrinkage
Cutting out the organ	ischaemia; cooling; physical damage to organ
Cutting tissue slices	as in cutting out organs; pressure; ischaemia; cutting connections
Perfusion fixation of the whole animal	anesthesia; ischaemia; operation; denaturation of tissues; inhibition of enzymes
Maceration or homogenisation	compression; heat; breaking natural compartments; diffusion

<i>Manoeuvre</i>	<i>Other effects</i>
Incubation	alteration of cellular environment; exchange with media
Dissection	breaking connections; mechanical damage to cells; killing cells
Addition of unnatural substances	denaturing proteins; changing the chemistry; diluting the tissue
Addition of unphysiological concentrations of natural substances	altering the osmotic conditions; diluting the tissue; changing the chemistry
Fixation	killing the cells; diluting the tissue; denaturation; aqueous extraction; enzyme inhibition
Drying	removal of water, fats and other labile substances; denaturation; shrinkage; heating; subjection to low pressure
Centrifugation	pressure; heating
Permeabilisation	addition of powerful detergents

Table 144. *Many of these effects are recognised, but not necessarily taken into account, when drawing conclusions from experiments.*

Undefined steps in procedures

Activation	Precipitation
Binding	Flocculation
Centrifugation	Sublimation
Denaturation	Freezing
Temperature changes	Drying
Homogenisation	Solubilisation
Electrophoresis	Sonication
Chromatography	Vaporisation
Extraction with solvents	Concentration
Rinsing	Dialysis
Dilution	Elution
Concentration	Filtration

Table 145. *These steps have not been defined sufficiently chemically or thermodynamically.*

The overall conclusion of this monograph have been summarised under the headings of inappropriate procedures, anatomical artifacts and problematic concepts, with reference to the pages in which these have been discussed in the text (Table 146).

Overall conclusions

<i>Inappropriate procedures</i>
Subcellular fractionation
Histology
Histochemistry
Electron microscopy, including freezing techniques
Immunocytochemistry
Binding in homogenates and tissue slices
Tissue slices
Use of 'specific' inhibitors
Disruptive techniques
Use of ligands
Dehydration
Deep freezing
Freeze drying
Heating and boiling
Use of extracellular markers
Intracellular pipette measurements
Patch clamp measurements
<i>Anatomical structures which do not exist in living intact organisms</i>
Macromolecular membrane receptors
Transmembrane molecules
Ion channels
Membrane carriers and transporters
Nuclear pores
Gap junctions
Intermediate junctions
Desmosomes
Tight junctions
Myelin lamellae
Microsomes
Synapses
Synaptic thickenings
Synaptic vesicles
Golgi bodies
Cytoskeleton

Endoplasmic reticulum
Lysosomes
Microtrabeculae
Polysomes
Ribosomes
Molecular motors
Thick, thin and intermediate filaments in living muscle
Cross bridges in muscle
Phagolysosomes
Mitochondrial cristae
Inner and outer mitochondrial membranes
Vesicles on inner mitochondrial membrane
Astrocytes
Oligodendrocytes
<i>Concepts which have been questioned</i>
Davson-Danielli hypothesis of membrane structure
Singer-Nicolson hypothesis of the fluid mosaic of membranes
Pre-existence of antibodies to all possible antigens
Preformed receptors
Active transport
Signalling
Chemiosmotic hypothesis
Apoptosis
Death receptors
Chemical transmission
Sliding filament hypothesis of muscle contraction
Tolerance
Autoimmunity
Proton pumps
Cell stress*
Excitotoxicity*

Table 146. *These concepts have not been considered here. *The doubts about these structures and the assertion of the inadequacy of the concepts, apply to living cells in intact animals.*

Chapter 55
Practical Consequences to Cell Biology

A. Microscopy

The case for looking at living or unfixed and undisrupted cells by a variety of light microscopic techniques has been made (Chapter 11). One may draw up a list of the optimal conditions for these studies: the cells should be either naturally single, or could be separated by non-energetic procedures; their cytoplasm should be translucent; intracellular movements should be visible, if necessary by using time lapse photography; any reagents, or agents such as light, heat, cold or centrifugation should not kill the cells, stop intracellular movements, denature them, increase the refractive index of the cytoplasm, or interfere with the uptake of oxygen by the cells; the same agents should not cause the cells to swell or shrink; few or no bacteria or fungi should be visible; the specimens should not be malodorous; the concentrations of reagents used during the observations should be limited to the physiological range; all drugs used should be tested within the ranges found pharmacologically effective in the intact animals, unless, of course, toxicity is being examined; non-invasive techniques are to be preferred, including microscopy, time lapse photography, split beam spectrophotometry; the cells should respire linearly during the course of the experiments. In similar conditions; resting potentials should be recordable, and, preferably, could be lowered by hypoxia or deprivation of substrate; enzyme activities should be measurable in the cells, using microdivers, but the presence of enzyme activities alone is not, itself, sufficient evidence of the life of cells; if it can be measured, the cytoplasmic viscosity should be low; microscopic preparations should be exposed to the lowest light intensity and heat possible; low intensity images may be enhanced by using image intensification, monochromatic light, video-enhancement, sensitive film and Rheinberg illumination. The cells should be in equilibrium with the incubation media.

A few cells, such as blood cells, sperm, ova, buccal epithelial cells, cervical and bronchial epithelia, etc. have been examined in these conditions. The appearances of most cells in the body in the unfixed or living state are simply not known. For example, it is difficult to identify the cytoplasm of lung epithelial cells; the membranes of gut epithelia and salivary glands are not clear, when the cells are

secreting; the entry and exit of particles may be seen in tissue cultures, or in single cells.

Electron microscopy gives a clear view of the exoskeletons of diatoms, because they are composed of silica, which obstinately resists dehydration and electron bombardment. The instrument can also be used to identify viruses, when they have been isolated in homogeneous groups, and when identification has been facilitated by clinical examination, immunology and epidemiology. However, one must be careful examining necrotic tissues, pus, faeces, etc., which contain a pot-pourri of particles, granules, bacteria and viruses, many of them not identifiable.

In electron micrographs biologists should be more careful about describing vesicles, particles, granules, bodies, fibrils, pores, somes, etc., when such appearances may be due to drying, precipitation, boiling, burning, bubbles, evaporation, etc. The preparations examined consist of tissue, *minus* soluble materials, *plus* reagents, electron bombardment, heat and radiation.

One of the most obvious indications of artifacts in electron microscopy are the two-dimensional images often seen. Many others are only depicted in diagrams, and rarely or never in micrographs. These lists (Tables 84, 85) represent an invitation to any one in the field to publish micrographs showing the structures in three dimensions.

It has been concluded here that all the structures only seen by electron microscopy, plus the Golgi apparatus, are artifacts of preparation. These include the trilaminar appearance of the cell membrane (the unit membrane), the nuclear pores, the myelin lamella and the synaptic vesicles. One may soon be in a fortunate position, when one may demonstrate that these conclusions are wrong. The maximum resolution of the light microscope in optimum conditions is 200-250 nm. Recently, use of sub-micron optics points the way to imaging molecules and very small structures, down to nanometre dimensions (Carr and Atkinson, 1999). Quantum dot fluorescence may track single molecules (Michalet et al, 2003; 2005). In particular, such techniques may bring evidence in living or unfixed cells about the following: whether all cell membranes are one layer thick, or trilaminar; their real dimensions; the existence of Golgi apparatuses, cytoskeleton and lysosomes; the existence of the myelin lamellae; the presence of molecular motors on the surface of mitochondria and other moving cytoplasmic particles; the presence of a cell membrane around the cytoplasm enclosing neuroglial nuclei; the presence of cross-bridges between thick

and thin filaments of muscle; the adherence of macromolecules to the external surfaces of membranes; the demonstration of transmembrane molecules spanning the membranes; the location of antigen-antibody reactions; the passage of transmitters across the synaptic clefts; the demonstration of ionic channels and nuclear pores.

If it should transpire that the objections in this monograph to the structure of cells, as deduced by electron microscopy - are not sustainable, research workers could continue studies based on the current consensus. On the other hand, if contemporary cytologists were persuaded by the case presented here, or if nanomicroscopy in the future were to show that this case is correct, then study of the properties of the structures listed in Table 146 should be abandoned.

The development of histology as a branch of pathology during the late 19th and early 20th centuries, in parallel with the introduction of such dyes as cresyl violet, methylene blue, malachite green and rose bengal, ensured that tissue was embalmed in an aesthetically appealing fashion, and their stained appearances became immortal. Consequently, the appearances of fresh tissues, as closely as possible to their states in vivo, has been rather neglected. One simply does not know what most cells would look like under those conditions (Table 147). It would be highly desirable to observe the tissues in isotonic biological fluids made up to mimic them chemically (Table 148). At the risk of being regarded as cynical, a further list of reasons which has been given for particles or structures, expected to be present in the tissue, *not* to be seen, is also given (Table 149).

Useful examinations of unfixed and unstained cells

Tissue	Reason
Lung	it is not clear whether parenchymal cells have cytoplasm
Liver	to establish whether sinusoids are shrinkage artifacts
Muscle	to see whether sarcoplasmic reticulum and T-tubules are visible
Chemoreceptors	to examine sensory mechanisms
Megakaryocytes	they have rarely been seen unstained
Platelets	they have rarely been seen unstained
Bowman's capsule	to find out whether the subcapsular space is an artifact
Cells in urine	to establish their morphology and origin (Birch et al, 1994; Fogazzi, Ponticelli and Ritz, 1999)
T cells	to find out their morphology
B cells	to find out their morphology

Tissue	Reason
Killer cells	to find out their morphology
Buccal cells	to study the effect of agents and staining on structure
Bronchial epithelium	to study ciliary movement
Paccinian corpuscles	to examine sensory transduction
Free nerve endings	to examine sensory transduction

Table 147.

Recommended incubation media

Serum	Transfusion fluids
Plasma (plus anticoagulants)	Ocular fluids
Cerebrospinal fluid	Isotonic glycerol
Lymph	Isotonic buffered aminoacids
Tears	Isotonic propylene glycol
Krebs Ringer solution	Insoluble oils
Culture media	Synovial fluid
Normal saline	Silicone fluids
Buffer solutions	Eye bathing solutions

Table 148. *These solutions should not cause immediate gross changes in tissues, and should be sterile, if possible. Tissues should be examined rapidly, as there will be some exchanges between them and the media, even if they are oxygenated, buffered and isotonic.*

Reasons for which structures and substances believed to be present in living animals, may not be seen under the microscope

Reason	Examples
they are broken down by lytic enzymes during dying	labile phosphates; glucose; lipids
they diffuse away during preparation	water; K ⁺ ; Ca ²⁺ ; Na ⁺ ; O ₂ ; water soluble substances dissolving in organic solvents
they are broken down by agents used in histology	proteins; nucleolar membranes in neurons
their concentrations depend upon the availability of oxygen and substrate	glucose; labile phosphates; metabolic intermediates
they are broken down by microorganisms	substrates of enzymes
they may be too small to be resolved by the microscope	receptors on membranes; nuclear pores; ribosomes; vesicles

<i>Reason</i>	<i>Examples</i>
their existence arises from a hypothesis	natural killer cells; transmembrane molecules; thick filaments in smooth muscle;
they may be inaccurate diagrams, or represent molecular arrangements impossible to see	A few examples are given in Figure 11
they may not be present at all in the cells in the intact living animal	see Tables 84, 85, 146

Table 149.

B. Histology, histochemistry and pathology

These disciplines are useful for diagnosing diseases, and for afflicting students who have to learn them. However, they are not appropriate for measuring cell characteristics (Chapter 8; Table 27). Furthermore, the chemistry of the tissue in a histological or electron microscopical section is simply not known, because the reagents extract substances soluble in water, fixatives and organic solvent, and add dyes, embedding agents and metal deposits. A list of cell characteristics which can not be studied by disruptive or histological procedures is given (Table 150).

Cell characteristics which can not be studied by disruptive or histological procedures

<i>Class of property</i>	<i>Properties</i>	<i>Reasons</i>
Dimensions of subcellular elements	length, width, thickness	they are homogenised or sectioned
Structure	shape	they have been compressed or are in sections
Physicochemical	diffusion, endocytosis, exocytosis streaming, Brownian movement, convection, secretion, phagocytosis, pinocytosis, viscosity, solubility, flagellar movement, ciliary movement, direction of movement, permeability of membrane, tonicity, osmotic pressure	movements stop; chemistry is altered
Chemical potential	concentrations of any chemical constituent, enzyme activity, subcellular localisation of soluble materials, respiration, activity in fractions, co-locations of substances, chemical potential, degree of naturalation	tissue is homogenised; dehydrated; cut; subjected to many chemicals; substances move during preparation
Staining and binding	intensity, fluorescence	affected by other chemicals in tissue

<i>Class of property</i>	<i>Properties</i>	<i>Reasons</i>
Optical properties	absorption, contrast, transmission, colour, texture, flocculence	change when tissue is homogenised or fixed
Incidence of process	whether there is swelling, oedema, shrinkage, dying, death, muscle contraction, ageing	cells shrink and are sectioned

Table 150. *Histological procedures are not usually regarded as disruptive.*

If one wishes to discover the status quo ante of the tissues before histology one must first establish not merely what substances the whole section has gained or lost, but also what the individual phases and organelles of the tissue have gained or lost. The following may be used to approach this problem; (a) measurement of the proteins, lipids, carbohydrates and ions in the tissue, at every stage of the staining; (b) chemical measurements in separated subcellular organelles, before and after preparation; (c) examining the dimensions of simply shaped red cells, white cells and ova, by phase contrast microscopy, and then subjecting them to whole histological staining procedures. The dimensions are then measured again in the stained sections. The histologists may then use them to calculate from stained what the dimensions might have been originally; (d) pieces of tissue, which are necrotic, inflamed and oedematous, can be dissected out and analysed by microchemical procedures. Instead of extrapolating back to the original cells, perhaps, it would be better to use fresh untreated cells, and thus keep the door of the stable closed.

C. Biology, biochemistry and cytology

Biochemists have traditionally ignored the Second Law of Thermodynamics (Chapter 3). The failure to recognise such derogations puts modern biochemists and biophysicists in a very difficult position indeed. The sincere newcomer to a research career may *either*: continue to ignore fundamental laws, in the knowledge that he or she is in the good company of well-established authorities; *or* carry out the suggested control experiments in the hope of extrapolating the final results back to the status quo ante in the living intact animal. In those steps in which the changes in entropy or free energy were found to be significant, it might be necessary to modify procedures to minimise such changes, for example, avoid powerful reagents, homogenisation, centrifugation and electrophoresis, etc.; check up that the same biochemical reactions are occurring *in vitro*, as *in vivo*, while being aware that their rates and equilibria are not necessarily the same.

There is also a widespread failure to appreciate the difference between the breakdown of substrates due to their instability, and that due to enzyme activity. This difficulty arises from the fact that enzyme activities are normally measured by their effects on substrate disappearance or product accumulation, rather than by measuring the *quantity* of enzyme present. In a sense, of course, the distinction between the two causes of substrate breakdown may be meaningless chemically, as one assumes that both occur simultaneously in the intact animal. However, it does underline the importance in experiments, of copying as closely as possible the precise chemical conditions found *in vivo*.

The difficulties of subcellular localisation of chemical activities have been summarised in considering the problems with cell membranes (Table 151).

Summary of the difficulties of location of substances in cell, mitochondrial or nuclear membrane

Problems associated with subcellular fractionation
Knowledge of thickness and extent of the membranes by weight or volume
The inability to see on electron microscopy the vast majority of macromolecules believed to be present in or near the membranes
The impossibility of knowing the quantity or proportion of fluids in individual compartments of cells in intact animals.
The imprecision of the knowledge of the values of the membrane potentials, and their precise location
The lack of knowledge about changes in entropy during biochemical experiments
The unknown physiological status of tissues <i>in vitro</i>
The imprecision of knowledge of distribution of markers believed not to enter cells.
The uncertainty about whether findings on such tissues as red cells, ova, cultured cells, giant axons and protozoa can be generalised to all membranes in all mammals, vertebrates and animal cells.

Table 151. *Each of these difficulties represents a cornucopia of valuable experiments.*

There may be some comfort to research workers in poor countries, in all countries who have difficulty in obtaining funds, and to those who are persuaded by the case put forward here. The devices exposing tissues to large quantities of energy are expensive. These include centrifuges, electron microscopes, freeze dryers, gas-liquid chromatograms, electrophoresis apparatuses, etc. These may be abandoned in favour of cheaper humbler devices, such as phase contrast microscopes, chromatograms, microdissection wires, and simple chemistry.

If one abandons the use at present of subcellular fractionation, histochemistry, histology, electron microscopy and immunocytochemistry, to find out how living cells work, it is useful to try to design a protocol to examine how a transmitter, hormone, drug or toxin might act. Let us call the substance X.

The first step is to administer radioactively labelled X in physiological or pharmacological dosage, until it has an effect; for example, sodium pentothal is injected intraperitoneally into a rat, until it becomes anaesthetised; adrenalin is injected intramuscularly until the heart rate increases; glucose is fed to a rat in hypoglycaemic coma, until it begins to regain consciousness. The animal is scanned, *as soon as possible after the effect is detected*, to see where the radioactive isotope is located. The animal is then guillotined, and the organs in which the isotope is found are excised, and cut into thin slices. They are placed on thin cover slips on sensitive film for a few seconds, and examined autoradiographically; the films are then developed. The slides can be compared with the films under the microscope, to see if the radioactivity can be localised microscopically.

When the target organ is localised, a similar organ from another animal is excised, and perfused. The arteriovenous differences of the concentration of X are measured. If it is too low to be measured, the possible metabolites may be identified by chromatography. If neither X nor its breakdown products can be detected in a particular organ, then one must look at other, not necessarily 'target' organs, where X is found in higher concentrations, to see if its effects occur first on a primary organ, and subsequently on another target organ.

In an organ, X may act on: the vasomotor tone and thus the blood flow; a particular anatomical site; a biochemical reaction, pathway, cycle or enzyme; more than one of these.

The following problems attend this scheme. Firstly, X may move rapidly during dying. Secondly, this procedure shares the untestable assumption of most current pharmacological procedures, namely that drugs and other substances act at the sites at which they are found at high enough concentrations to be measured.

D. Pharmacology and toxicology

Pharmacology is the study of the effects of drugs on the body in health and disease, and elucidation of the mechanisms by which they cause these effects. It has been much more successful in the former than in the latter endeavour. With the exception of infectious diseases, the treatments of most major symptoms and diseases

have been found by accident or empirically (Table 152). The mechanisms of action of relatively few drugs has been worked out and demonstrated on chemical grounds (Table 153). This is at a time when almost certainly more research is devoted to drug discovery and pharmacology than ever before in history.

Treatments discovered accidentally or empirically

<i>Disease or condition</i>	<i>Treatment</i>
Smallpox	vaccination
Anaesthesia	nitrous oxide
Pain and inflammation	salicylates, opium
Heart failure	digitalis
Skin inflammation	poultices
Infected wounds	maggots
Scurvy	fresh fruit
For paralysis	curare
Gout	colchicine
Malaria	quinine
Goitre	iodine
Anxiety	lobotomy
Depression	lithium salts
Appearance of eyes	belladonna
Thrombosis	warfarin
Amoebic infections	emetine
Pernicious anaemia	raw liver
Staphylococcal infections	penicillin
Epilepsy and anxiety	barbiturates
Angina	amyl nitrate
Asthma	adrenalin
Diabetes	pancreas extract; sulphonyl urea
Anxiety	benzodiazepines
Schizophrenia	neuroleptics
Dermatitis, asthma, arthritis and other diseases	corticosteroids
Allergy and anaphylaxis	antihistamines
Cancer	nitrogen mustards
Gram positive and negative infections	cephalosporins

Table 152. *Many later drugs of these classes have been chemical variants of those first found to be effective.*

Drugs based on known mechanisms, or designed

Disease	Drug
Syphilis	salvarsan (Ehrlich and Hata, 1910)
Myasthenia gravis	anti-cholinesterases (Walker, 1934)
Pernicious anaemia	B ₁₂ (Minot and Murphy, 1926)
Addison's disease	corticosteroids (Britton and Silvette, 1931)
Myxoedema	thyroxin (Murray, 1891)
Hypopituitary diseases	pituitary extract (Raben, 1962)
Stomach ulceration	histamine receptor antagonists (Black, 1993)
Hypoxia	oxygen (Beddoes, 1794)
Parkinson's disease	dopa (Cotzias, Van Woert and Schiffer, 1967)

Table 153. This list is not comprehensive. *Several of these diseases are deficiency diseases.*

The reasons for which the mechanisms of the actions of drugs have been so difficult to elucidate, probably include: (a) the assumption that homogenisation, centrifugation and preparation for histology and electron microscopy, etc, have no significant effect on the affinity of drugs and the distribution of chemical constituents in tissue; (b) the belief that the site at which a drug or ligand is found after gross chemical interventions is the same as that at which it acts in the living intact animal; (c) the possibility that the reaction between a drug and its target is so rapid and so different in life, that it may not be possible to detect the drug tissue reaction by current rather slow procedures; (d) the naïve view that the tissue can distinguish between 'specific' and 'non-specific' receptors; (e) the use of ligands, instead of the drugs themselves; (f) the failure to appreciate that effects of agents in vitro are not necessarily the same as those in vivo, for several reasons, (Table 154); (g) the assumption that receptors are nearly always involved in drug actions; (h) there are several other differences between cells in vitro and in vivo (Table 39).

Actions of drugs in vitro and in vivo

<i>Drug</i>	<i>In vitro</i>	<i>In vivo</i>
Dinitrophenol	uncouples oxidative phosphorylation	slimming agent
Aspirin	uncouples oxidative phosphorylation	analgesic and anti-inflammatory
Fluoride	‘blocks’ respiratory enzymes	reduces dental caries
Actinomycin	inhibits RNA synthesis	radiomimetic
Ouabain	partially inhibits ATPase	treatment for heart failure
B-blockers	constrict small blood vessels	lower blood pressure
Tetracycline	impairs protein synthesis	used as antibiotic
Proton pump inhibitors	inhibit proton pump	treat peptic ulcers (Leontiadis, Sharma and Howden, 2005)

Table 154. *The differences indicate that the mechanisms found in vitro may be modified in vivo.*

A further possible method of examining where drugs work, and the organs they affect, is to examine susceptible animals, not only to watch the clinical chemical and pathological effects of the drugs – as is already done – but also to administer overdoses to the animals. Then one would find the concentrations in each organ affected, and study the effects of the drug at a range of concentrations on preparations of the isolated organs.

Toxicology is properly a study of the substances and mechanisms which poison whole animals. The term is often also used to describe the effects of substances on tissues and fractions in vitro. The difficulty here is that when drugs in their concentrations in vivo have little effect in vitro, toxicologists sometimes raise the concentrations tested experimentally until they show effects. Unfortunately, the addition of low concentrations of toxins may inhibit complex processes, such as oxygen uptake or glycolysis, or relatively simple enzyme reactions, such as the hexokinase or ATPase activities, (Hochster, 1963-1973).

The effect of toxins may be examined in three stages. As in all biochemical and disease processes, one would like to know the initial reactions which cause the toxic effects. Then there are the tissue reactions, and, thirdly, there are those which cause a particular organ to fail and the animal to die subsequently. It is relatively easy

to identify in a whole animal liver failure or renal failure, and irreversible coma may be regarded as failure of the brain.

Several aminoacids, such as glutamate and kainic acid are regarded as excitotoxic, because when immature mice ingested them, some retinal cells degenerated (Olney, 1988; Lipton and Rosenberg, 1994). Whatever the explanation for the latter phenomenon, it seems highly unlikely that aminoacids normally present in the blood would be toxic, unless they have not been detoxicated by the liver or excreted by the kidney. K^+ , NH_4 , and H^+ in high concentrations may produce deleterious effects. Are they cytotoxic?

E. Immunology

The first question to examine is the assumption that different tissues in the same body are antigenically different. If this were true, the injection of say, rat neurons and neuroglia cells from the same rat into a rabbit should produce *two* different antibodies. This relatively simple experiment would test the hypothesis – a hypothesis that does not seem to have been stated explicitly, despite the availability of fat catalogues of antisera believed to be specific for many tissues in the body. This is such an important question that testing it carefully and comprehensively would seem to be an early and overdue duty.

Similarly, questions have to be addressed and answered experimentally about immunology. How does the body produce antibodies to a whole tissue, organ or group of organs (Chapter 51)? Why do autoantigens not affect all similar organs in the bodies, for example, all joints in rheumatoid arthritis, all muscles in polymyositis, and all arteries in polyarteritis? Does not each similar tissue in the same body house the same antigens? Why has the identification of these diseases as autoimmune not yet been translated into the design of effective clinical treatments, whereas the understanding of individual specific antigens and antibodies, has been so successful in immunisation, vaccination and blood grouping? The demonstration of effective immunological treatment of autoimmune diseases would be a powerful vindication of the whole belief in the concept of autoimmunology.

The hypotheses that the body was continuously suppressing its proclivity to react against its own tissue, and that when this suppression ceases, the body reacts against its own proteins, need critical examination, both theoretically and experimentally. Even if certain diseases seem to be a reaction to the body's own tissues, it seems a much simpler hypothesis to suggest that the tissues in the body

acquire immune responses a few weeks or months after birth, rather than that they are born with suppressive machinery. The evidence for autotoxicity is rather poor.

The possibility that tissues change immunologically in incubation or culture suggests a practical idea. A potential donor tissue, which would be antigenically different from the recipient, could be incubated or grown in tissue culture. In the latter conditions, the potential donor tissue could be incubated or grown in a medium derived from say, the recipient's serum, to 'wash out', precipitate, or neutralise, antibodies from the donor, so that it would no longer be rejected by the recipient.

It is a proper question to ask whether there is a case for regarding an immune reaction as a different phenomenon from inflammation. Both of these phenomena need to be examined by rapid non disruptive procedures, especially ones in which it is not assumed that powerful chemical agents used in the experiments do not affect the affinity of the antigen for the antibody.

A comprehensive critical analysis of immunological procedures is long overdue. Immunoassay is used very widely. Please see Wild, (2001).

The concept of malignant diseases as immune diseases should be much more firmly supported or abandoned. The properties of spontaneously occurring malignant tumours require examination. One would expect that immune suppressive agents would be much more successful in treating malignant disease, if malignancy were an immune phenomenon.

F. Neurobiology and neurology

The structure of the nervous system described here is very different from that found in neuroanatomy textbooks. The first problem is that originally neurons, astrocytes, oligodendrocytes and microglia were distinguished by their shapes, and the stains which showed them up (Hillman, 1986a). Hýden and Pigon, (1961) and Hamberger (1963) claimed that neuroglial clumps contained oligodendroglia, astrocytes or microglia, but they did not state the criteria by which they distinguished them. It would seem to be highly desirable for those who maintain that there are four different kinds of cells, besides the blood vessels, to isolate a few examples of each, preferably unfixed and unstained, and demonstrate their characteristic differences.

It has been concluded here that most of the brain is a syncytium with mobile nuclei moving around in it. This is contrary to the general consensus that each of the four different kinds of cells, including the three kinds of neuroglial cells are invested by cell membranes of their own (Chapter 34). In my studies of the literature which

shows such alleged cells, I have been quite unable to find such a membrane. A focussed study should be done with the express intention of demonstrating these neuroglial cell membranes. It is as well to recollect that neuroglia in the sense of Von Virchow, (1846; 1854) was an amorphous glue, which he did not claim to be cellular. Hodgkin and Lister's, (1827) observation of granules in brain, which we have called fine granular material (Figure 40), is really only an aggregation of the glial clumps of Hýden and Pigon, (1960). It has been completely ignored, in favour of the general view that if each of the four elements could be stained separately virtually the whole brain would be coloured (Nauta and Feirtag, 1979). This was shown not to be the case (Hillman, 1986a; pages 98-115). If the greater part of the brain and the spinal cord consists of fine granular material, which has not been recognised, one wonders of what the different fractions believed to be neuron-rich and astrocyte-rich, are composed.

The unfixed neuroglial clumps deserve appropriate attention from chemists. With no supporting evidence whatsoever, one may speculate that the very large number of fine granules in the syncytium could be the digital sites for memory, analogous to the bytes of computers.

The absence of membranes around the syncytia of the brain and spinal cords and the mobility of the neuroglial nuclei in life, is analogous to the apparent lack of membranes around glial tumours and their rapidity of spread. This could suggest the possibility that direct introduction of anticancer agents into the syncytium might slow down the progress of the glial tumours. This possibility could be tested experimentally in animals.

The neuroglial or glial cells have generally been regarded as 'satellite cells', 'supporting cells', connective tissue, scar cells or a sort of filler. The assumption has been widely held that the glial cells were somehow second class to the neurons, which were excitable. Of course, it is generally believed that in life, most tissues are parts of the normal economy of the body, and its properties would be grossly modified, if it were deprived of them, or if they grew disproportionately. However, objectively, there is no sense in which any cells which co-exist with or are adjacent to others, can be considered to be more important than any other.

Whereas the four kinds of cells were originally distinguished by the different staining procedures which showed them up, different markers gradually came into use. These were believed to characterise the kinds of cells, often in tissue cultures.

However, as pointed out (please see Chapter 12), there is a peculiar shortage of experiments, (a) showing that the staining properties of the parent cells in the intact animals are the same as the cells in culture; (b) that the neuroglial markers are specific to the particular kinds of stained neuroglial cells (c) that the neuroglial stains do not stain neurons – it was shown that they do (Hillman and Deutsch, 1979). (d) many of the studies on astrocytes, have looked at those believed to be present in the optic nerve (Raff et al, 1979; 1980). The cells in the optic nerve have been assumed not to be neurons. It is difficult to know why the latter authors are so sure that the cells they examine in the optic nerves are astrocytes, and why they have not experimented on a wider range of cells believed to be astrocytes elsewhere in brains and spinal cords.

It has not been sufficiently appreciated that cultures of the four allegedly different cells grow in such different culture media, that it would be surprising if they did not show different shapes, rates of growth, changes in structure with time and biochemical properties, so that evidence from tissue cultures must be regarded as rather weak. A very crucial series of control experiments would be to incubate each of the supposed different cell types in all four of the media considered to be characteristic of all the cell types, and then compare the cells in respect of the above properties.

Hydén and his collaborators compared the properties of neuron cell bodies with those of adjacent neuroglial material. However, when Hertz, (1966) used the micro diver, he found the *qualitative* difference, that raised K⁺ in the incubating medium increased the oxygen uptake of the neuroglia, but not of the neurons. In general, it should not be considered surprising that two adjacent types of tissue, which stain differently, should be chemically different.

The understanding that the main elements of the central nervous system are neurons with processes, and neuroglia composed of fine granular material rich in mitochondria and mobile nuclei, focuses on the necessity to analyse each of these populations chemically. They could be examined in embryonic, young, adult, aged, senile and diseased brains. One of the questions which could be addressed is why neuroglia seems to be prone to tumours, and why the neuron cell bodies are not. Other questions are, ‘What is the chemistry of gliosis?’ ‘What is the cytology and chemistry of unfixed neurofibrillary plaques and whorls?’

It is often said that no serious experiments are possible if one does not use disruptive techniques, histology or electron microscopy. One may compare tissues

from young animals and human beings, with tissues, from adult animals themselves, from apparently normal tissues adjacent to lesions and from the lesion themselves. (Tables 3, 155). The tissues should neither be sullied by other reagents, nor insulted by other manoeuvres.

Methods of study of nervous tissues

<i>A. Tissue</i>	<i>B. Procedures</i>
Neuron cell bodies	Light microscopy (Chapter 11)
Neuronal nuclei	Refractive indices
Neuronal cytoplasm	Oxygen uptake
Neuronal membranes	Enzymes by manometry
Fine granular material*	Membrane potentials
Neuroglial nuclei	Excitability
Ependymal cells	Chemistry
White matter fibres*	Effects of K ⁺ , Na ⁺ and Ca ²⁺
White matter nuclei	Effects of hypoxia and lack of substrate
Capillaries*	Specific gravity
Choroid plexuses*	Mobility
Brain sand	Viscosity
Ganglion cells	Spectrometry
Glia in ganglia	Microspectrophotometry
Myelinated fibres	Microspectrophotofluorimetry
Unmyelinated fibres	Conduction properties and chemistry
Axoplasm	Microelectrophoresis
Myelin sheath	Conductivity
Schwann cell nuclei	Capacitance
Remak fibres	Heat conductivity
Cerebrospinal fluid*	Heat capacities
Aqueous humor*	Staining properties
Vitreous humor*	Chromatography
Serum*	Incubation
Slices*	

Table 155. Asterisks* indicate tissues in which large enough samples, may be obtained without using micromethods. Most of the procedures can be used on all tissues. Many of these methods of study are found in Bailey and Smith, (1968).

It has been concluded that anatomical synapses are artifacts (Chapter 34) and that chemical transmission is very unlikely (Chapter 44). One of the central tenets of the chemical hypothesis is the view that the tips of the dendrites of one neuron have swellings ending on the cell bodies of other neurons. In preliminary unpublished experiments, I have observed that very few dendrites indeed actually contact knobs on other cell bodies. Of course, one can not tell from a two dimensional photograph whether any two fibres seen in a field are actually touching each other or not. The way of establishing this is to take Golgi or Palmgren stained sections, which show fibres, and focus on particular fibres and observe, if they do, indeed, end on a cell body, or, if not, what is the vertical or horizontal distance between the two? It would be well worthwhile to repeat this experiment, bearing in mind that, if my findings were confirmed, the chemical hypothesis of transmission, would be thrown into serious doubt.

The assertion that the anatomical synapse is an artefact of staining, does not mean that the electrical signals in a junctional region detected by a micropipette, which are called synaptic excitatory or inhibitory potentials, are artefacts. The physiological properties can be detected in living tissue. I am casting doubt on the assumption that the electrical transients occur at the same *sites* as those called anatomical synapses.

Conduction of action potentials along axons is believed to occur at nodes along the myelin sheath, which is also regarded as insulation. At the same time, the node of Ranvier is considered to be a region of low impedance and resistance between the axoplasm, and the extracellular phase. Excitability is believed to ‘saltate’ from one node to the next (Tasaki, 1939; Huxley and Stämpfli, 1949; Stämpfli, 1952; 1956). The excitability is believed to arise from the sudden increase in permeability to Na^+ and K^+ at the nodes of Ranvier. This permits the latter ions to move across, carrying current in and out of the nodes.

Since action potentials pass along unmyelinated fibres, which have neither myelin sheaths, nor nodes of Ranvier, it is clear that neither the insulating function, nor the jumping at the nodes, is necessary for conduction. Furthermore, if the impedance and the resistance are lower at the nodes of Ranvier, why does the action potential not short circuit there, rather than jump to the next node? Since the impedance and resistance of the axoplasm, are so much less than those of the myelin sheath, Kirchhoff’s Law dictates that nearly all the current will travel along the axon,

and much less will cross the myelin sheath, although a large proportion of it will shunt across the nodes. The current escaping through the nodes of Ranvier should make the action potentials decrement. These problems suggest several experiments: (a) to confirm that particles in Brownian movement can be seen in myelin sheaths by high power light microscopy of peripheral nerves; (b) if one collated the values for the impedances and resistances, of the extracellular phase, the myelin sheath, the membrane of the non-myelinated nerve fibres, the axoplasm and the nodes of Ranvier, one could determine what proportion of the current would cross at each of these sites; (c) to study the electrical properties of the axoplasm from myelinated nerves, and from axoplasm of non-myelinated fibres of mammals, as has been done in cephalopods, if that can be obtained in sufficient quantity. *Prima facie*, it seems likely that excitability originates from the cytoplasm of the neuron cell body, and spreads down the axoplasm of the axons and dendrites. Also, experimentally, it should be possible to show radioactive Na^+ entering the nodes of Ranvier under direct vision during the progress of action potentials.

It would be highly desirable to show light micrographs of the Schwann cell wrapping round the axons, not just in tissue culture, but in developing myelinated nerves. It is generally assumed that each Schwann cell is invested with its own cell membrane in whole animals. In peripheral nerves, the double lamellae of each Schwann cell membrane in this model appears as two lines, but, of course, the Swiss roll model should appear as *four* lines. The failure to do so is covered by the unproved and unprovable assertion that two axonal membranes 'fuse' together.

The new promise of nanotechnology should help to demonstrate chemical transmission from synapses directly in living cells, that is, one should be able to record (i) the presence of synapses containing synaptic vesicles; (ii) the existence of pre and post synaptic thickenings; (iii) the passage of the transmitter through the presynaptic thickening and its attachment to the postsynaptic thickening; (iv) the speeding up of movement of vesicles, when the synapses are excited (v) the presence of channel through which the transmitters are thought to pass; (vi) the effect on synaptic 'blockers' of all these changes; (vii) the failure of the transmitter to leave the synapses except at the presynaptic thickenings. Indeed, the main assumptions of the chemical hypothesis of transmission could be tested directly.

White, Albin and Verdura (1964) and White (1968) have kept the isolated heads of monkeys alive for up to 8 days, and the bodies for up to 11 days.

Unfortunately, during this time, the brains have not yet regenerated to grow their axons down the spinal cords to control them. It is possible that the fine granular material, which is so ubiquitous in the central nervous system (Figure 40), could be modified so as to lose its antigenic properties, and thus act as a brain 'glue', encouraging the latter relationship. It may be necessary to add nerve growth factor, protamine, steroids, fresh serum or peptides *locally* to encourage the ends to join up.

Finally, it is worth listing several psychological and philosophical phenomena, which probably can not be studied in biological experiments, firstly, because they are unlikely to occur in homogenates and, secondly, one does not know the relevant physiological or biochemical parameters, which can measure them (Table 156).

Whole body activities which can not be studied by disruptive biochemical procedures

<i>Psychological phenomena</i>	<i>Philosophical phenomena</i>
Thinking	Soul
Learning	Mind
Memory	Good
Personality	Evil
Love	Religion
Hatred	Morality
Jealousy	Appreciation of arts
Dreams	Aesthetics
Sleep	Criminality
Depression	Will power
Anxiety	Consciousness of self
Personality	Poetry
Homosexuality	Music
Meditation	Taste
Fantasy	Inspiration
Paranoia	Pleasure

Table 156. *The assignment to the two categories is arbitrary. The location of the psychological activities in whole animals may be sought by magnetic resonance imaging and positron emission tomography (Foster and Hutchison, 1987; Jezzard, Matthews and Smith, 2002).*

G. Undergraduate and popular biology

There are common problems for the following: writers of undergraduate textbooks; undergraduate students; journalists; broadcasters. They arise from the fact that they take the consensus view as the true one, and the most appropriate to represent to the public. They are in a very difficult position if this view is challenged, especially with evidence from geometry and microscopy, which they can understand themselves. Usually, those holding the consensus view assert that the science is too complicated for the students and the public to understand controversies. Unfortunately, this is not the case with the present situation. However, a chief examiner of biology for the Ordinary Level of the London Examination Board, informed me that a student, who answered an examination question doubting what

was in the textbooks, would fail the examination. Furthermore, journalists and broadcasters have to give brief accounts of beliefs, and so do not have the possibility of representing more than one view. Thus, established views acquire their own inertia. The material taught at examination level, before students have time or experience to question it, acquires a biblical authority.

One way to challenge dogma is for professional research workers and all other ‘stakeholders’ to ask crucial questions, and to insist on obtaining answers to them. A list of these have been previously published (Hillman, 2003) reproduced her as Table 157). Regrettably, refusal to recognise them goes no way to answering them, nor to resolving apparent anomalies in viewpoints.

Chapter 56

Crucial Questions

Cell Biology

Methods

1. Can one calibrate substances extracted from tissues, using pure solutions of those substances?
2. To which parameters of the tissue should concentrations be referred?
3. Can one use a single pure protein to calibrate different subcellular fractions, each containing different mixtures of proteins?
4. Does the ‘enrichment’ of a subcellular fraction or cell type, change its chemical properties?
5. Does one assume that the preparation of tissue for histology, histochemistry, immunocytochemistry or electron microscopy does not change its chemical or biological properties?
6. What control experiments have ever been carried out to warrant the assumptions of 4 and 5, or is it not necessary to do them?
7. Does the finding of a chemical activity or a structure in a subcellular fraction, or in a subcellular location, prove that it has not moved during the preparation?
8. What is the evidence that solutes, liquids and particles do not relocate during fixation and freezing?
9. Does the shrinkage of tissues and sectioning in any histological technique permit one to measure the dimensions of cells and subcellular organelles?
10. Can an electron microscopist looking at a heavy metal deposit on a dehydrated tissue derive any information about its biochemistry in life?
11. How can one assess the dimensions and structure of cell membranes by electron microscopy, when the preparation involves the use of ethanol, acetone and propylene oxide, which extract lipids, which are believed to be major components of the membranes?
12. Can one know the thickness in life of cell membranes?
13. How valid is the use of agonists, antagonists, inhibitors and ligands, to detect receptors, in preference to the transmitters, hormones, drugs and toxins, themselves?
14. Why is it assumed that homogenisation, washes and centrifugation, do not affect the affinity of receptors for transmitters, hormones, drugs, toxins and ligands?
15. Why should it be necessary to tilt the stages of electron microscopes to see randomly orientated membranes and other structures in all orientations, when this is not necessary with the light microscope?
16. Why have so few experiments been done to compare the chemistry and staining properties of the *same* cells in parent tissue and in tissue cultures subsequently?
17. Is it reasonable to believe that incubation of tissues does not result in exchange of substances between them and incubating media?
18. Does growth of tissues in culture change their structures, biochemistry, staining or immuno-reactivity?
19. Can intracellular pipettes measure activities of a single intracellular ion, or membrane potential accurately?

Structure

20. Why do the cells, the nuclear and the mitochondrial membranes, as well as the mitochondrial cristae, appear in electron micrographs nearly always to be cut in near perfect transverse sections?
21. If the cell membrane is fluid mechanically, how can cells maintain their shapes?

22. How is intracellular movement of light microscopically visible particles in the cytoplasm, possible in life, when it is believed to contain the cytoskeleton, lysosomes and mitochondria, and it has a low viscosity?

23. Why has only *one* of the many *thousands* of receptors, cell surface antigens and channels, which have been isolated, sequenced, and characterised, their structures elucidated, and their dimensions calculated, been seen by electron microscopy on the cell membranes, when, they are believed to be located there, and it is claimed that the thickness of the membrane is within the resolution of the electron microscope?

24. Why do the lamellae of the myelin sheath always appear to be uniformly distant apart on longitudinal section?

25. What is the evidence that the microsomal fraction consists of cell membranes and endoplasmic reticulum?

26. Where are the liposomes in life?

27. Can a lysosome be a particle or a vacuole?

28. Why is it assumed that each cell of a particular organism contains the same quantity of DNA despite the huge variation in dimensions of their nuclei?

29. Where do protein synthesis and acid hydrolysis occur in cells, in which ribosomes and lysosomes cannot be seen?

Physiology, biochemistry and pathology.

30. How can carriers assist the passage of ions and amino acids across membranes, when the complex of the carrier and the substance carried *must* be larger than the substance carried itself?

31. How can the nuclear pores prevent ions and small molecules crossing the nuclear membrane, when the messenger RNA moves from the nucleus to the cytoplasm?

32. How do the ions cross the membranes of the non-excitable cells, if a signal does not cause the ionic channels to open and close?

33. Do different cells in the same body contain different antigens?

34. What evidence is there that the body produces proteins, which are toxic to itself?

35. In diseases believed to be auto-immune, either *organ*-specific or *tissue*-specific, why does the body not reject the whole organ or tissue, as it rejects incompatible transplanted organs or blood of the wrong group, often making patients ill, or even killing them?

36. Is it possible to study the chemistry of necrosis by disruptive biochemical procedures, or procedures in which tissue is killed?

Theoretical

37. Should one entertain a hypothesis, some of whose elements are unproved or unprovable?

38. Are *living systems* open, partially open, or closed, thermodynamically?

39. Are tissues studied by *biochemical and biophysical procedures* open, partially open, or closed, thermodynamically?

40. How can one distinguish between the causes and effects of a disease process, that is, identify its genesis?

41. Should one ever accept the results, interpretations or hypotheses arising from experiments in which no, or inadequate, controls have been carried out?

42. Is it a reasonable assertion that scientists have a duty to enter into dialogue with all interested parties?

43. What is the difference between autoimmunity and inflammation?

Neurobiology

Methods

1. Why are what are believed to be different kinds of neuroglial cells rarely seen by light microscopy of healthy central nervous systems?
2. Why is there no common agreement about the staining procedures, which are believed to identify neurons, astrocytes, oligodendrocytes and microglia?
3. Does the use of the term neuroglia or glia implies that neurobiologist and pathologists cannot distinguish between the alleged sub-types, or that there are no differences?
4. Since the three alleged types of neuroglial cells were *originally* classified using histological techniques, does not this imply that anyone using antibodies to identify each type, should correlate these two criteria for identification of what they believe to be the different cell types?
5. Is it surprising that allegedly different neuroglial cells grown in completely different cell culture conditions show different morphological, biochemical and immunological properties?
6. Is it reasonable to believe the neuronal and astrocytic cell bodies contain different antigens then their processes?
7. How specific are antibodies and other markers to neurons and different neuroglial cells?
8. Why are synapses not seen in unfixed and unstained preparations of the nervous system by light microscopy?

Structure

9. Why are the dimensions and numbers of synapses so different by light and electron microscopy?
10. Why is it so difficult to find the non-myelinated segment of the neuron continuous with the cell body and the myelinated sheath of an axon?
11. Why does one not see the bifurcation of myelinated fibres, except in diagrams?
12. Why do light micrographs in the literature never show the connection between the dendrite of one neuron to the presynaptic fibre and synapse on the next neuron?
13. Why are synaptic vesicles so uniform in diameter in sections of the nervous system?
14. Why do there not appear to be cell membranes around the cytoplasm of what are believed to be neuroglial cells?
15. Why do axons from ganglion cells of the autonomic nervous system appear to have only one axon, rather than an afferent and efferent fibre?
16. Why do synaptic clefts, tight junctions and desmosomes nearly always appear in electron micrographs to be equally spaced apart, and why does one rarely, if ever, see them obliquely or face on?
17. Can one measure dimensions of cell bodies and axons in sections?

Physiology, biochemistry and pathology.

18. How is axonal transport of particles possible when the axon contains networks of endoplasmic reticulum, and microtrabeculae?
19. Why is it assumed that evidence derived from experiments on neuromuscular junctions is relevant to understanding of transmission in the central nervous system?
20. How can reactive astrocytes move within hours to the sites of injury of the brain and spinal cord, if these structures are packed with neurons and neuroglial cells?
21. What evidence is there that high concentrations of 'excitotoxic' transmitters damage adjacent cells?

22. If high concentrations of K^+ , low concentrations of Ca^{2+} and mechanical damage can induce neurons to fire, are they not transmitters?

Theoretical

23. How can synapses store information, when they are also believed to transmit it?

24. What evidence – as opposed to hypothesis – is there that learning, memory, self-consciousness or mind affect synapses?

25. Can one devise experiments on living animals that test *only* learning or memory, without inducing other behavioural and physiological changes?

26. Does the chemical theory of transmission contain unproved and unprovable sub-hypothesis?

27. Why are synapses regarded as necessary for the transmission of information, when electric conduction conveys information without crossing synapses?

Table 157. This table is reproduced from Hillman H, (2003) by kind permission of Elsevier Science Ltd.

It seems necessary to identify assumptions inherent in experiments, since they have significant impacts on results (Table 158).

Common assumptions and hypotheses

<i>Assumption</i>	<i>Comments</i>
1. Assumptions inherent in calibrations	untested
2. Assumptions in referring measurements of constituents to tissue parameters	empirical measurements
3. Experiments do not need comprehensive controls for the effects of procedures	widely assumed
4. The fixation, disruption, or death of cells does not affect their biology	widely ignored
5. Enzyme activities are not altered by dilution	sometimes ignored
6. Changes in entropy in closed systems do not affect the equilibria of the reactions	ignored
7. Thermodynamics are difficult to calculate in open biological systems	ignored
8. Soluble constituents do not diffuse during preparation	ignored
9. Compartmentation can be demonstrated by subcellular fractionation	the compartments are mixed by homogenisation
10. There are 'specific' enzymes for cellular organelles	assumed, but difficult to prove
11. The similarity of an organelle in an intact tissue and in a fraction is evidence that it has not changed chemically	unlikely
12. The Davson-Danielli hypothesis	some compatible evidence but impossible to prove
13. The Singer-Nicolson hypothesis	some compatible evidence but impossible to prove
14. Most membranes have similar structure and chemistry	assumed

<i>Assumption</i>	<i>Comments</i>
15. Most transmitters, hormones, drugs, ligands, signals and toxins, bind to receptors	assumed in most cases, but difficult, to demonstrate and hardly ever demonstrated conclusively
16. These receptors are on cell membranes, nuclei and, occasionally, cytoplasm	very unlikely, not demonstrated beyond doubt
17. A ligand occupies the same receptor as a transmitter, hormone, drug or toxin	probably some of the same chemical groups
18. Results from experiments <i>in vitro</i> can be applied to whole animals	their properties are different (Chapters 12 and 13)
19. The chemical theory of transmission	many elements unprovable
20. The Dale hypothesis that each synapse has its own transmitter	unlikely and unprovable
21. Transmission at a synapse is a similar process to that at a neuromuscular junction	unlikely, although widely assumed
22. The synapse is the site of learning	assumed but no proof – possibly unprovable
23. The myelin sheath is a scroll of cell membranes	assumed, but lamellae are artifacts
24. The Geren model shows how myelination occurs	assumed, but very unlikely; not seen in whole developing peripheral nerves
25. The properties of cells in culture reflect those of their parent tissues, in respect of morphology, movements, biochemistry, staining, immunology	there are many differences between them
26. Dimensions in sections of stained tissues are approximately equal to those in living tissues	they have been demonstrated many times not to be
27. Each subcellular organelles shrinks to the same extent during a histological procedure	they each contain different quantities of water, and other compounds soluble in the reagents used
28. Intracellular ionic concentrations or transmembrane potentials can be measured quantitatively by intracellular pipettes	results must be qualitative
29. Different cells from the same animal contain the same quantity of DNA	unproved, but unlikely, due to the very large range of sizes and shapes of nuclei
30. RNA passes through nuclear pores	unlikely, although it may cross the nuclear membrane
31. Different tissues from the same animal contain different antigens	very unlikely indeed, as circulating antibodies would destroy own cells
32. Microsomes consist of cell membranes and endoplasmic reticulum	unproved and probably unprovable

<i>Assumption</i>	<i>Comments</i>
33. Cells can be immune to proteins nearby or within them – autoimmunity	they would destroy the whole tissue
34. Macromolecules can aid the passage of ions across membranes	carriers or transporters would make them larger
35. The chemiosmotic hypothesis explains proton transport	it contains too many unprovable assumptions
36. Second messengers can explain the action of ‘signals’ on cells	unnecessary hypothesis
37. The effects of hypoxia, dying, swelling, shrinkage, organelle movement, or necrosis, can be studied in stained tissue sections	the volumes of cells and their organelles change during dying of the animals and histological preparation
38. The sliding filament hypothesis of muscle contraction	has many anomalies
39. ‘Apoptosis’ is a mechanism of cell death	very unlikely
40. A substance which can influence a reaction in vitro, controls it in the same way in the intact animal	it is more complicated in vivo
41. The animal’s rejection of its own tissues is normally suppressed	there is a much simpler hypothesis
42. There are autoreceptors	unlikely to have opposite effects
43. Purification of substances does not affect their chemical activity	extremely unlikely
44. A crystal of a pure substance behaves the same way as in a dilute solution in vivo	unprovable
45. Detergents have no effects on tissue chemistry other than extracting proteins	they are very active chemically

Table 158. *Many of the assumptions and hypotheses have never been identified.*

Chapter 57

What Is Known, Not Known, and Perhaps Unknowable in Cell Biology

A useful approach to a difficult problem is to list the areas of ignorance about it. Some of the parameters are unknowable (Table 159). Many of the uncertainties originate from the fact that life is a labile property, so that one must beware of altering the biochemistry of tissues, to find out their properties before they were examined. At present, one may be fairly certain about the structures present in living cells (Figure 66), and probably about overall metabolic pathways. However, cellular dimensions and the rates and equilibria of reactions in cells and their organelles in living intact animals, are not known. The big mistake of the 20th century was to believe that these parameters were, indeed known. Perhaps, the relevant experiments to try to complete these experiments will be carried out in the 21st century. Further lists of important relationships which are not known, are given (Tables 160).

Finally we have a list of incomplete investigations (Table 161).

In the final analysis, biological research, - like academic work, politics and religion – will only advance mankind, when their practitioners exercise intellectual honesty. This includes examining evidence dispassionately, not evading awkward questions, not indulging in casuistry, and according a higher priority to the pursuit of knowledge than to personal ambition.

At present unknown and some unknowable parameters.

<i>Parameter</i>	<i>Reason</i>
Thickness of cell membranes	probably beyond the resolution of the light microscope: electron microscopy requires dehydration: perhaps nanomicroscopy in the future will be able to measure it
Subcellular distribution of chemical histology and activity	subcellular fractionation changes distribution and activity of chemicals
Activities of enzymes or unstable materials <i>in vivo</i>	most procedures examining them are disruptive, and change the free energy or entropy of reactions in which they are involved
Dimensions from any histological procedure involving staining or cutting	the procedure involve dehydration, which shrinks tissue
Movements or their rates by any histological procedure	these involve killing the animal and fixing tissues

<i>Parameter</i>	<i>Reason</i>
Dimensions and shapes of subcellular organelles below the resolution of the light microscope	preparation for electron microscopy changes them; perhaps nanomicroscopy in the future
Permeability of membranes in fixed tissue	fixation alters membrane permeability
Morphology of cells from their appearance in culture	morphology changes during culture
Chemistry of tissues in histological or electron microscopic slides	this does not seem to have been investigated
How macromolecules and particles cross cell membranes	the fact that they do in phagocytosis and pinocytosis does not describe the mechanism
The location of chromatin during interphase	it is assumed to break up in the nucleus but does not absorb ultraviolet light
What effects ionophoretic currents have on the cell membrane	these currents may effect the temperature, the activity of the substance injected, and the rate of diffusion between the micropipettes and cytoplasm
Movements of synaptic vesicles	they can only be seen by electron microscopy, in which movements can not occur
Dry weight of tissue and tissue water	heating to constant weight does not only extract water
Movements across nuclear pores	pores cannot be seen in living cells
Tissue swelling or shrinkage, dying, death or vacuolation, by histological procedures	tissue is dehydrated during histology
Chemistry of membranes	the membranes are normally extracted during preparation
Concentrations of unstable materials or materials broken down during dying	it is impossible to know the concentrations before the materials break down, unless one can show – rather than assume – that one can inhibit the breakdown before it occurs
‘Non-specific’ effects	‘specificity’ is a questionable concept
Structures which can not be seen in living cells	their existence has to be deduced
The chemical changes during dying which become irreversible	they have not been examined

Table 159. *This list is not comprehensive, and it would be useful to explore any possible experiments to clarify in what ways the measurements of the parameters might be made with greater certainty.*

Important relationships not known

Connection between intention and motor pathway
 Connection between stress, pain, psychological phenomena and the autonomic nervous system
 Mechanisms of sensory transduction at nerve endings*
 Relations of the properties of extracted DNA and RNA to those in living cells*
 How macromolecules and particles enter and leave cells
 Concentrations or activities of enzymes, unstable and short living molecules in different cellular compartments
 Whether ligands bind to the same sites as transmitters, hormones, drugs and toxins
 Causes of Brownian movement and streaming*
 The relationship of the chemistry of macromolecules in a living animal and purified crystals of the same molecules
 Location of chromosomal DNA in interphase cells
 Whether biochemical changes found in cancer, aging, dying, malnutrition, etc are causes or effects
 Mechanisms of conduction*
 Mechanisms of transmission*
 Forces which separate chromosomes at anaphase
 Effects of subcellular fractionation on the properties of organelles and their apparent localisations
 The existence of astrocytes and oligodendrocytes*
 Natural triggers of illness, malignancy and death
 Mechanisms of initiating cell division*
 Chemistry of necrotic particles
 Reversible and irreversible chemistry of dying
 Mechanism of contraction of voluntary, involuntary and cardiac muscle*
 Whether adult neurons can regenerate*
 Whether most phenomena claimed to be immune share the same mechanisms*
 Whether different cells in the same body can be antigenically different*
 Whether autoimmunity is a viable concept*
 Connection between the longevity of the individual and its individual types of cells
 Relationship between the dynamics of single simplified reactions and the same reactions in the whole intact animal
 Relationships between dying and death of a whole animal with those in its tissue and cells
 Relevance of findings in cell and tissue culture to the properties in the whole intact animal
 The mechanism of gradients of ions and soluble molecules*

Table 160. *Some of these* are believed to be known already, but they have been questioned in this monograph.*

Important investigations not yet carried out comprehensively

1. The effects of *energetic procedures* on the biochemistry of tissues (c).
2. The effects of all *histological procedures* including histochemistry, electron microscopy, freezing and immunocytochemistry on the dimensions, shape and chemistry of subcellular organelles.
3. The effects of *each manoeuvre* and each reagent used in *subcellular fractionation* on the dimensions, shapes and chemistry of organelles, and whether these effects are reversible (c).
4. The effects of all natural *chemically active reagents* used during biochemical procedures in concentrations outside the physiological range, and of all unnatural chemicals upon the biochemical and physiological properties of the systems studied (c).
5. Assessment of whether *solubulisation* of receptors, membranes etc., affects their chemical activity.
6. Assessment of the effects of *heat generated* within tissue during and after homogenisation and centrifugation (c).
7. The effects of *culture media* on the chemistry of the different cells, especially of those whose growth occurs in particular media.
8. Comparison of the staining, enzyme and immunocytochemical properties of cells in intact tissues and the same cells in *tissue culture* (c).
9. Examination of the *chemical exchange* between tissue slices and the media in which they are incubated, and an assessment of what these changes do to the properties of the slices (c).
10. The properties of *colpoids* to find out what further explanations are required in the properties of living cells (c).
11. Assessment in particular experiments of the degree to which they are *open or closed* (c).
12. Examination of *smears, unfixed tissue, isolated cells* and *tissue culture* by light microscopy of the properties of cells from lesions due to cancer, multiple sclerosis, Alzheimer's disease, schizophrenia, Parkinsonism, as well as conditions, such as tissue rejection, inflammation, necrosis, atrophy, etc. (c).
13. The *chemistry* of histological and electron microscopic sections in healthy and diseased tissues.
14. Comparison of the differences of properties of macromolecules in low concentrations in tissues and their properties after *crystallisation* (c).
15. Measurement of the quantitative and qualitative effects of *electrophoresis* and *chromatography* on the chemistry and recovery of macromolecules (c).
16. Measurements of the effects of the procedures for *extracting DNA and RNA* on their subsequent chemistry and recovery (c).
17. Measurement of the *movements* of transmitters, drugs and receptors during subcellular fractionation and histology (c).
18. Assessment of 'non-specific' binding in vivo (c).
19. Measurement of the *rates of diffusion* of solutes, solvents and particles in colpoids mimicking conditions in living cells (c).
20. Assessment of where the *energy* in the brain and spinal cord is dissipated in vivo (c).
21. Measurement of the *heat conductivity* and *heat capacity* of different biological tissues (c).
22. Effects of freezing, cooling and heating on the *stability of unstable constituents* in tissues (c).

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| 23. Effects of <i>freezing</i> for electron microscopy on the dimensions and structures of cells (c). |
| 24. Measurement of the <i>stability</i> of substrates and unstable substances in tissue, in the absence of enzyme activity (c). |
| 25. Assessment of the <i>specificity</i> of activators and inhibitors over a wide range of reactions in intact tissue (c). |
| 26. Investigation of tissues to find out whether there are structures which have <i>no affinity</i> for stains. |
| 27. Attempt to explain why <i>macromolecules</i> are not seen by electron microscopy on or near the membranes in which they are believed to be located. |

Table 161. Some of the kinds of investigations, which have not yet been carried out, but the results of experiments have been interpreted assuming that they have. (c) represents control experiments.

Glossary

Terms marked with asterisks are considered misleading.

<i>Term</i>	<i>Definition</i>	<i>Comment</i>
Acoustic microscopy	Microscopy observing differences in acoustic impedance of tissues	has poor resolution
Action potential	Brief, all or none, electrical transient in a nerve or muscle cell	believed to originate in the cell membrane
Activation	An increased biochemical activity or an initiation of a physiological reaction	
Active process	A biochemical reaction or gradient requiring energy	often tested by depriving the system of oxygen or substrate. Such tests are not conclusive
Active transport	Distribution of ions, aminoacids and sugars apparently against the electrochemical gradients	there are other possible explanations
Adapted*	Change believed to occur as a result of evolution	often means that the phenomenon is just present. Often post hoc explanation
Aggregate*	Particles found close to each other	the particles may have precipitated during the procedure for seeing them
Anopteral microscopy	Reverse phase contrast	very little used; advantages of phase contrast
Antagonist	A substance which inhibits the action of a ligand, by binding to the same site as the ligand	mostly demonstrated in vitro, and assumed to act similarly in vivo. Often assumed to be 'specific'
Antibody	A glycoprotein immunoglobulin produced in the body in response to the presence of a 'specific' antigen. The antibody reacts with an epitope on the antigen, and precipitates it	the reaction was originally believed to be due to one 'specific' foreign antigen or autoantigen
Antigen	A protein or polysaccharide from outside the body, which causes the body to react to it by producing antibodies	sometimes believed to originate from the tissues of the same animal

<i>Term</i>	<i>Definition</i>	<i>Comment</i>
Antioxidant	A compound which ‘protects’ a biological system from ‘excess’ oxygen or free radicals	it combines with free radicals
Apoptosis	Death of cell in which the programme to kill it resides in the cell itself	relationship between life duration and cell death, is not known. Evidence for the phenomenon is extremely weak (Chapter 52)
Array or* Assembly	A repeating pattern in electron microscopy	one has to be sure that it is a three-dimensional object and is not a crystalline form resulting from dehydration or electron bombardment
Artifact	A structure not seen in the living tissue, resulting from the procedure used to examine it	many of them are due to biochemical, histological or electron microscopic procedures
Assumption	This is a relationship or law governing a system, which is taken for granted	most assumptions in calibrations, observations, experiments, conclusions and interpretations, have not been recognised
Autoantibody	An antibody believed to be produced as a reaction to the antigens of its own tissues	this concept has been questioned (Chapter 51)
Autoantigen	A protein in a tissue, which is believed to induce antibodies in the same individual	this concept has been questioned (Chapter 51)
Autoimmune disease	Chronic disease, believed to result from a tissue of the body acting as autoantigen	this concept has been questioned (Chapter 51)
Autolysis	Breakdown of a dying tissue due to proteolytic enzymes, and sometimes infection	this is very difficult to measure, because it is changed by disruption

<i>Term</i>	<i>Definition</i>	<i>Comment</i>
Autophagic vacuole*	A vacuole containing a cytoplasmic particle, which fuses with a lysosome to digest it	vacuoles are seen in unicellular organs and in tissue cultures, but may not be present in the intact adult mammalian tissue
Autophagosome*	A membrane limited cytoplasmic body in which autolysis develops	it is only seen in diagrams, and can not be identified for certain in cells
Autoreceptor*	Presynaptic receptor acted on by transmitter it itself releases	an unprovable hypothesis
Axonal flow* or transport	The movement of ions, molecules and particles, between the cell body and axon in both directions	it is a portmanteau term which may include diffusion, streaming, Brownian movement, and movement due to temperature
Binding	A reaction between two compounds, which may or may not produce effects	may not be detectable; binding is assumed to have occurred if a substance produces effects
Biochemistry	Chemistry of living intact cells in intact animals	should not imply that the chemistry of living tissues obeys different laws than normal chemistry of its constituents
Biology	Study of living tissues	strictly speaking, should only apply to properties of living tissues
Biopsy	Piece of tissue taken from a live or recently dead animal	usually used for diagnosis but may be used for research
Brownian movement	Random movements of small particles in fluids	occurs in colpoids
Calcium ion antagonists	Substances believed to stop movements of calcium ions across voltage sensitive or receptor operated calcium ion channels in myocardium and smooth muscle cells	they should be shown to be directly preventing the entry of calcium ions into cells

<i>Term</i>	<i>Definition</i>	<i>Comment</i>
Carrier or transporter	A substance believed to be present in or near a membrane, which facilitates the passage across the membrane of ions, aminoacids and sugars	this must make the ions or molecules transported larger than the compound transported
Cascade*	A metabolic pathway	seems only to be used for caspase enzymes during the dying of cells
Cell-cell* inhibition	The prevention of the expansion of one cell in a culture by an adjacent cell	presumably the cell body is attached to the culture medium that it can not be moved easily by a process from an adjacent cell
Cell movement	Movement of the contour or body of a whole cell, or substances within it	this is seen in circulations and in cells in cultures
Cell stress*	Dying of cells believed to be due to proteins	this is a very unclear phenomenon, originally meaning the appearance of cold proteins
Chelator	A substance binding divalent ions	it is assumed that it has no effects other than diminishing Ca^{2+} or Mg^{2+}
Chemiosmotic hypothesis	A hypothesis to explain proton distribution believed to be controlled across mitochondrial membranes	many of its sub-hypotheses can not be proved or disproved (Chapter 47)
Chromatolysis	Visible break up of nuclei, chromosomes or chromatin (Boyd, 1932)	could be loss of stainability
'Classical' transmitter or reaction*	One found earlier	meaningless term
Cisterna	Gap between the endoplasmic reticulum seen on electron micrographs	artifact
Closed system	A thermodynamic system which can not exchange energy with its surrounding	most biochemical experiments are largely or completely closed

<i>Term</i>	<i>Definition</i>	<i>Comment</i>
Colloid	A mixture of salts, oils and water, mimicking some characteristics of living cells	their properties should be exhausted, to find out which of them may occur in living tissue
Compartment	This houses particular metabolic pathways	may be an organelle or a chemical reaction; usually discovered by subcellular fractionation
Complement	A protein in fresh serum, which restores the ability to lyse activated red blood cells	assumed to be lysing red cells in intact animals
Conduction	Passage of action potential along axons	
Contractile proteins	Actin, myosin, tropomyosins and meromyosins, believed to be involved in contraction of muscle and intracellular movements	they are apparently present in most cells, including those which do not contract
Control experiment	Observation to see the effects of procedure used on experimental results	these have been totally inadequate in many experiments
'Controversial' view*	A term used to indicate a view with which the author does not agree	it is not usually used for the consensus view. It is an unscientific term
Convection	Movement of small particles and fluid due to temperature gradients	it may be caused by the illumination used to examine the cells
Conventional*	Previously accepted	unscientific term
Critical point drying	The drying of tissue at a particular pressure and temperature	drying would not occur if solid, liquid and gas, phases were in equilibrium
Cross bridges	Myosin heads believed to cause muscle contraction by being attached to thick and thin filaments	the apparent structures are probably artefacts of electron microscopy
Cytoplasm	The fluid between the cell and the nuclear membrane	it is widely believed to be traversed by the cytoskeleton
Cytoskeleton	A system of tubules, fibres and filaments believed to be present in the cytoplasm	it is an artifact (Chapter 28)

<i>Term</i>	<i>Definition</i>	<i>Comment</i>
Cytosol	The fluid in the cytoplasm between the cytoskeleton	assumes that the cytoskeleton is not an artifact
'Damage'	This is believed to be chemical changes in cells which are sometimes reversible, but may lead to death of the cell	is not measurable
Death	Irreversible loss of properties of life of the whole animal, tissue or cell	it is difficult to study, because most procedures kill tissues
Degeneration or degradation	Irreversible changes of staining	occurs in atrophies, dystrophies and ischaemia
Denaturation	Largely irreversible changes of protein, can be induced by heat, cold, dehydration and strong acids or alkalis	should be avoided in biochemical experiments
Deoxyribonucleic acid (DNA)	The genetic material of animals, viruses and plants	believed to be present only in nuclei and mitochondria
Depletion	The diminution of number of granules seen in a histological section	the chemistry of the granules is not necessarily known, and they may be dehydration artifacts
Desensitization	The disappearance of an immune response	could have several causes
Desmosome	A kind of junction between cells	seen on electron micrographs; may be an artifact
Detergents	Powerful chemical reagents which solubilise proteins	have many other chemical effects
Dialysis	Separation of soluble materials by a semi permeable membrane	has membrane properties
Diapedesis	Cell crossing through capillary walls	very rarely seen happening
Diffusion	Movements of ions, molecules or small particles as a consequence of concentration gradients	the simplest kind of movement; only stops at absolute zero temperature
Domain	Many macromolecules are believed to have extracellular, membrane and intracellular domains	they are not seen on electron microscopy of whole tissues

<i>Term</i>	<i>Definition</i>	<i>Comment</i>
Dying	Series of reactions, resulting in death.	it is a descending process (Chapter 52)
Electron microscopy	Visualisation of heavy metal deposit on a structure	must be carried out at a very low pressure
Endocytosis and exocytosis	Entry of particles and their exit from cells	these can not be seen occurring in fixed or dead tissues
Endoplasmic reticulum	A system of lamellae or networks believed to be present in the cytoplasm of all cells	it is an artifact
Enriched fraction	A subcellular fraction believed to be enriched in respect of a particular cell type or organelle	it is difficult to study its chemistry, as the enrichment may have changed it
Entropy	Amount of energy unavailable for work in a thermodynamic system	changes in this property are often ignored (Chapter 3)
Enzyme activity	Ability of an enzyme to accelerate or slow down a chemical reaction	depends upon the enzyme and substrate concentrations, temperature, co-factors, pH, and other substances present
Epiphenomenon*	An experimental finding which the author wishes to ignore, or does not consider relevant to the interpretation	it is difficult to know in the following examples which came first: current flow and magnetism: heat and light: chicken and egg. It is an inappropriate subjective term.
Epitope	The site on an antigen to which an antibody binds	has to be separated by chemical procedures
Evidence	Observations and findings, considered in light of reason	Critical evidence should be sought in comparing the validity of two opposing or incompatible theories. The degree of certainty about the validity of evidence is subjective
Excitable tissues	Nerve and muscle, which respond to electrical stimulation, and which produce action potentials	

<i>Term</i>	<i>Definition</i>	<i>Comment</i>
Excitotoxic compounds	Aminoacids, which are believed to be toxic to the cells which secrete them	extremely unlikely; ammonia, K ⁺ and lactate could be considered cytotoxic
Expression of protein*	Its presence	
Extracellular fluid	Plasma, cerebrospinal fluid, lymph and serous fluid assumed to be perfusing extracellular, including intercellular, phases	it is extremely difficult to measure accurately in the whole or in tissue, because no ideal extracellular markers have been found
Facilitated or carrier diffusion	Movement of ions or molecules across cell membranes believed to be facilitated by a carrier in the membrane	carriers are not seen on electron microscopy
Fixation	Treating tissue with substances which kill bacteria, fungi and viruses, and are intended to preserve structure	they denature tissue and do not prevent diffusion, unless they precipitate cell components
Fluid mosaic model of cell membrane	Cell membranes are believed to be fluid between their two layers	the evidence proves only that diffusion occurs on a wet surface
Free energy	The energy available to do work	it drives all chemical reactions
Free radical	A short living species capable of independent existence that contains one or more unpaired electron	sometimes believed to be toxic although they occur in the electron transport system
Freezing techniques	Cooling tissue to -70°C to -200°C to preserve its chemistry, or to make it suitable for cutting sections	dehydrates; widely believed to have no morphological or chemical effects of its own
Freund's adjuvant	Mineral oil, antigen, emulsifying agent, plus killed Mycobacteria, to enhance the immunogenicity of the antigen	used experimentally in animals
Function*	What an organ, tissue, cell or organelle <i>does</i>	should not be used to mean why it is there, which is an unanswerable question

<i>Term</i>	<i>Definition</i>	<i>Comment</i>
Gap junction	A junctional tissue, seen on electron microscopy	it is not three dimensional and is an artifact (Chapter 33)
Geren model	The belief that during development Schwann cells wrap around axons	is not seen in intact axons; Schwann cell membranes are not seen
Glioma	Tumours of any neuroglial cell	the term implies that the pathologist can not distinguish between astrocytoma, oligodendrocytoma and microglioma
Ground substance	Originally cytoplasm, also used for substance between neurons in central nervous system	can be seen histologically, characterised here as fine granular material in central nervous system
G-proteins	A family of proteins, which bind guanosine triphosphate, and are believed to be located on cell membranes, where they transduce signals and hydrolyse proteins	they are seen on the membranes only in diagrams, or in subcellular fractions
Heat shock	Phenomenon includes the production of heat, shock or stress, proteins	it is difficult to know what 'shock' or 'stress' mean in relation to cells
Homeostasis	The regulation of the extracellular biochemistry and temperature within narrow limits	occurs in living intact animals
Hypothesis	A theory based on experimental findings	its warrantability is measured by the accuracy of its forecast of the results of future experiments. It should be provable and disprovable
Implicated or Involved*	A substance is implicated or involved in a reaction, if it can alter the rate or equilibrium	many substances can affect reactions
Immunity	Resistance to antigens	
Immunocompetent	The ability of an animal to respond to antigens by cellular or chemical reactions	

<i>Term</i>	<i>Definition</i>	<i>Comment</i>
Incubation	Keeping tissue sections or specimens in media in which they metabolize linearly	they exchange components with the incubating media
Inhibition	Diminution of an enzyme, hormone, immune or physiological, reaction	it is often assumed to be ‘specific’ to a particular system, in which it has been demonstrated in vitro
Interface	Boundary between two phases which are insoluble in each other	can not be distinguished from a membrane by microscopy
Intracellular movements	These occur in cytoplasm, in the nucleus, and in the nucleolus	they are visible by light microscopy in living unicellular animals and tissue cultures, and are assumed to occur in all living cells
Intravital or supravital staining	Staining of living cells, which is not believed to change their biology or chemistry	often this is not tested
Ion channels	Very fine channels believed to be present in cell membranes and regulated by particular ions, their diameters, their voltage, and by receptors	their opening is believed to generate minute currents of pA
Ion pumps	Chemical mechanisms, believed to be located in cell membranes and to distribute ions, such as the Na^+ against the electrochemical gradient	they are large molecules; there are other explanations for the uneven distribution of ions
Ionophoresis	The driving of current from a micropipette to the outside or inside of a cell body	it is assumed that the current driving the ions has no effect on the permeability of the membranes
Isolated organs	Organs perfused artificially or connected by blood supply to the rest of the body	can be studied to demonstrate living physiology

<i>Term</i>	<i>Definition</i>	<i>Comment</i>
Isometric contraction*	Contraction of muscle without shortening, because the muscle is held tightly at both ends	it would not be seen as contracted, if it remained at the same length
Isotonic contraction*	contraction of muscle without increase in tension	it would not contract, if the tension was not increased
Karyolysis	Break up of the nucleus	seen in cell division and necrosis
Karyorrhexis	Shrinkage of the nucleus	seen in cell division and necrosis
Killer T cells	Lymphocytes are believed to recognise and kill cells infected by a pathogen	these cells are difficult to characterise anatomically
Lectin	Plant proteins which have an affinity for 2 or more sites on sugars	animal tissues are not normally exposed to these
Life	Life exhibits several properties (Table 87)	
Ligand	A molecule that is believed to occupy the same site as a transmitter, hormone, drug or toxin	even if it competes with the latter at the same sites, it is chemically different
Liposome	Spherical structure believed to have phospholipid membranes and to contain lipids and salts	they are only seen in vitro, not in intact cells
Lysosomes	Bodies in the cytoplasm, seen by electron microscopy and believed to contain acid hydrolases	not seen unfixed by light microscopy; are artifacts
Meiosis	Cell division to produce two haploid daughter cells	seen in life
Membrane	Solid structure surrounding cells, nuclei and mitochondria. They are semi-permeable. Believed to be trilaminar, and be 7-10nm thick on electron microscopy	it is not trilaminar, and its thickness in life is not known (Chapter 20)
Message	It carries signals from one part of tissue to another	any environmental or bodily change can be considered a message or signal
Messenger RNA	RNA believed to leave nuclear pores and to initiate protein synthesis in ribosomes	nuclear pores and ribosomes are artifacts

<i>Term</i>	<i>Definition</i>	<i>Comment</i>
Microsomes	Subcellular fraction believed to be composed of cell membranes and endoplasmic reticulum	they do not exist in the intact animal
Mitochondrioplasm	The fluid content of mitochondria	not the same as matrix in mitochondria
Mitosis	Division of a cell to produce two diploid cells	seen in living cells
Molecular biology	Molecular changes in biological tissue	a synonym for the chemistry of the living cells
Molecular motors	These are believed to be composed of contractile proteins, and to be attached to mitochondria and to other particles, which they move	movement would occur from Brownian movement, streaming, convection and diffusion, without motors being necessary; they can not be seen by electron microscopy
Naked nuclei	Nuclei in syncytia without membranes between the cells	occur in syncytia (Table 25)
Necrosis	Changes seen histologically, following the death of cells	these changes are non-specific, and the chemistry of necrotic tissue is not known
Nucleolonema	Fibrillar structure in nucleolus	seen in unfixed tissue, not seen by histology or electron microscopy; neglected by biologists
Nucleolar membrane	Membrane around the nucleoli of neurons and, possibly, other cells	seen in unfixed neurons, not seen in histology or electron microscopy; neglected by biologists
Nucleolus	Body or bodies seen in nucleus	it appears as amorphous granule(s) by electron microscopy
Organisation*	Can mean structure or physiological response	can not be studied in dead tissues
Oxidative phosphorylation	The phosphorylation of ADP to ATP	generally believed to occur in mitochondria

<i>Term</i>	<i>Definition</i>	<i>Comment</i>
Pars amorpha	Part of the nucleolus showing no structure	very little is known about its chemistry
Partial antagonist	An antagonist, for example, such as ouabain, which partially antagonises an enzyme, such as ATPase	this concept is difficult to understand enzymologically
Partitioning	A solute partitions itself between different phases	it will partition between each phase differently (Table 7)
Permeabilisers	Substances which make cell membranes more permeable	in fractionation, it is assumed that permeabilisers have no chemical activity other than making cell membranes more permeable
Permeability of membranes	The ease of passage of substances across membranes	should be measured in living cells
Peroxisomes	Organelles seen by electron microscopy which are believed to break down fatty acids and to produce peroxides	they can not be seen in unfixed cells by light microscopy
Phase contrast microscopy	Form of microscopy detecting different refractive indices in tissue, and translating them into differences of density	can be used to examine unfixed, unstained tissue
Plasma membrane*	Cell membrane, believed to be continuous with endoplasmic reticulum	the membrane is not continuous with endoplasmic reticulum, which is an artifact; not a useful term
Proton pump	Mechanism, which is believed to transport protons across mitochondrial membranes	conditions of experiment are quite different from those <i>in vivo</i>
Purification	Separation of a chemical species from tissue	always involves changes in entropy of reactions in tissues
Putative transmitter*	A substance believed to be a transmitter, which does not yet fulfil the agreed criteria for a transmitter	many substances, which are not transmitters, excite nerve and muscle cells (Table 96)

<i>Term</i>	<i>Definition</i>	<i>Comment</i>
Pyknosis	Shrinkage of the nucleus	seen in living and dying cells
Receptor	Macromolecule, believed to be mostly on the cell membrane, which reacts with ligands, transmitters, hormones, drugs and toxins to produce effects; the reaction with the latter may be activated or inhibited	not seen on cell membranes; often studied using ligands, and in subcellular fractions; often not specific
Regulation	Feedback mechanism controlling metabolic pathway or physiological process	agents are believed to control reactions in reactions in intact animals
Resting membrane potential	Potential differences across all living cell membranes, is believed to be created by the K ⁺ gradient across the cell membrane	can not be measured accurately
Ribosome	A particle lining the endoplasmic reticulum, which is believed to be the site of protein synthesis	many cells do not have ribosomes, but all synthesise proteins; synthesis of proteins is demonstrated in subcellular fractions
Sarcomere	the smallest unit of muscle contraction	believed to consist mainly of thick and thin filaments
Sarcoplasmic reticulum	A system of networks believed to be located between muscle cells	it is an artifact
Second messenger	An intracellular signalling molecule stimulated by binding with a receptor on the outer surface of the cell membrane	most evidence comes from subcellular fractionation. It is impossible to demonstrate conclusively
Self and non-self	Proteins, tissues and organs, to which antibodies are not, or are produced	very difficult to define
Serial sections	Sequence of sections from embedded stained tissues, designed to enable three-dimensional reconstructions of shapes to be made	the tissues have already shrunk and been distorted by histological procedures
Side effects	Other symptoms, in addition to the therapeutic effects of the drugs; some of them are undesirable	the concept is anthropomorphic

<i>Term</i>	<i>Definition</i>	<i>Comment</i>
Signal*	Physiological, hormonal, electrical or light stimulus, which conveys information across the cell membrane, passing through transmembrane molecules	the concept is too wide to be useful, because each kind of stimulus acts by a different mechanism
Sol-gel hypothesis	A proposed early mechanism of muscle contraction	actin and myosin contract when mixed
'Specific' activator or inhibitor	Such a substance that affects one particular enzyme or pathway, with few other similar substances producing similar effects	specificity is in doubt
Stochastic	Arrived at by skilled and logical conjecture	" "
Stoichiometry	Quantitive relationship of the reactions and products in the proportions, in which they appear in equations	
Stress fibres	Contractile proteins found in cytoplasm believed to support the shapes of cells	these cytoplasmic components are artifacts
Structure-linked*	An enzyme activity found in a fraction, which was not revealed, until it was treated by detergents or freezing	the detergents and freezing probably activate the enzymes
Stunning an animal	Renders an animal immobile	does not make it insensible immediately
Synapse	A connection between one neuron and another, and the supposed site of chemical transmission	does not exist as an anatomical structure in life
Synaptic activity	Transient electrical activity found between neurons	pre and post synaptic potentials are assumed to arise at the anatomical sites
Syncytium	A tissue containing nuclei sharing the cytoplasm, not divided by membranes between the cells	these are much more widely distributed than has been recognised (Table 25)
Tight junction	Close apposition of adjacent cells, seen in electron microscopy	these are artifacts
Trafficking*	The intracellular or transmembrane movement of particles or chemicals in one or both directions	not a useful term; means only movement

<i>Term</i>	<i>Definition</i>	<i>Comment</i>
Transducer	A mechanism changing one form of energy into another form – a chemical reaction or electrical signal	
Transmitter	A substance which leaves a nerve cell, diffuses to another nerve cell or muscle, binds to a receptor, and excites or inhibits the second cell	each of the criteria for transmitters is very weak (Chapter 44)
Transport*	Movement of ions, molecules and granules within one phase or across to another	this is a portmanteau term
T-tubules in muscle	Fine tubules which are believed to conduct excitability from the neuromuscular junctions to individual sarcomeres	they are difficult to see, and are not three dimensional
Ultrastructure	The structure of tissues studied by electron microscopy	it is assumed that this information is more accurate than light microscopy; only heavy metal deposits are viewed
'Unit' membrane	The cell membranes are trilaminar	this contradicts the laws of solid geometry
Vacuole	Spherical or oval clear space in cytoplasm, containing fluid, which is immiscible with the cytoplasm	seen in unicellular animals and tissue cultures; difficult to find in higher animals; can not be assessed in tissues, which have been heated, frozen or dehydrated; there is an interface between the vacuole and the rest of the cytoplasm, but no membrane

Section E

Indices

Key References

Notes on bibliography

References have been arranged alphabetically from the first authors, except when an author has written a coherent sequence of papers, where they may appear in date order. The complete cast of *et al* has been listed in the bibliography, but not, alas, in the text. I apologise to them for the inadequate recognition that this represents, especially in view of my belief that all co-authors share intellectual responsibility for publications headed by their names.

It seemed undesirable to contribute to the labour of typing, while increasing the world consumption of ink, so that I have minimised the use of punctuation. I must apologise to Scandinavian and German readers for ignoring the diacritics and treating letters as if they followed the nearest English letters they resemble in the native languages. The present practice represents a continuous weakness of publications in English, until the responsible academics provide authors with an accurate agreed alphabetical order.

Apologies are also due to the authors of important publications I have omitted, and those whose names I have misspelt accidentally. In the future, I will be pleased to correct errors to whose attention I have been drawn.

The pioneers of findings and the views cited in the latest editions of textbooks, have been quoted, in an attempt to deal with the current consensus about cell biology.

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