J. theor. Biol. (1971) **30**, 151–181

Unified Theory on the Basic Mechanism of Normal Mitotic Control and Oncogenesis

CLARENCE D. CONE, JR

Molecular Biophysics Laboratory, Langley Research Center, National Aeronautics and Space Administration, Hampton, Va. 23365, U.S.A.

(*Received* 10 *February* 1970, *and in revised form* 10 *June* 1970)

A large number of experimental observations suggests that a significant correlation may exist between the level of the electrical transmembrane potential difference in somatic cells and the intensity of their mitotic activity. The present paper, after review of pertinent experimental background data, assumes that a functional relationship between potential level and mitotic activity does in fact exist and, invoking the precepts of classical membrane potential theory, proceeds with the formulation of a basic theory of mitosis control wherein the intracellular ionic conditions associated with various levels of the potential difference act to regulate preparation for DNA synthesis and other essential mitotic preparations. The theory links the activity of the potential-generation mechanisms of the cell surface complex, and hence mitogenic activity, with cellular metabolism and with external environmental influences through an explicit system of interacting feedback circuits. Inherent in the overall theoretical development is the formulation of a unified theory of the cytogenetic etiology and maintenance of the malignant state. Additional specific experimental evidence is cited in support of the theoretical concepts developed.

1. Introduction

Experimental studies covering a variety of mature somatic cell types *in vivo* have shown that the great majority of such cells reside in the G_1 period and must first pass through the S period of DNA synthesis before entering mitosis in response to a mitotic stimulus (e.g. Baserga, 1965), although a small fraction may be arrested in the G_2 period (Gelfant, 1958). These observations suggest that maintenance of natural mitotic homeostasis is accomplished primarily by the arrest of cells in the G_1 period, presumably by the reversible blockage of one or more essential preparative events for DNA synthesis, with controlled release of this blockage as cell proliferation is required for growth or replacement of dead cells. Elucidation of the fundamental nature of the blockage and release aspects of such control mechanisms is a matter

of central importance in all biological phenomena involving mitotic regulation and balance, e.g. morphogenesis, development, wound healing and regeneration, systemic mitotic homeostasis, sensescence, and malignancy.

In a recent paper, Cone (1969*a*) presented experimental data demonstrating a pronounced variation (relative to the basic G_1 level) of the transmembrane electrical potential difference (E_m) accompanying the initiation of mitosis. Although this paper was concerned primarily with electro-osmotic events of prophase and the G_2 period, it was proposed therein that the changes in intracellular ionic concentrations associated with substantial variations of the basic G_1 E_m level itself might be a key factor in the much more prevalent G_1 blockage of mitosis. A theoretical model of a possible system of E_m mediated metabolic feedback circuits whereby G_1 mitotic control might be accomplished was briefly outlined, and a potential role for the operation of altered feedback circuits in malignancy was proposed. Significantly, recent experiments (Cone & Tongier, 1970) designed to test this basic premise by establishing whether intracellular ionic conditions simulating those which theoretically would occur with various natural E_m levels could effect a reversible G_1 mitotic block, have yielded conclusive results in full accord with the precepts of the theory. Both DNA synthesis and mitosis were found to be reversibly blocked in a mitotically representative somatic cell line *in vitro* by negative E_m levels corresponding to those of mitotically quiescent cells *in vivo* (e.g. nerve).

The purpose of the present paper is substantially to expand and elaborate the basic concepts of the original paper (Cone, 1969*a*) by developing a more formal and complete model, within the limitations imposed by existing experimental evidence, demonstrating how the intracellular ionic balance associated with different levels of E_m in somatic cells might provide a fundamental mechanism for natural mitotic control. The essential results of a recent critical experimental challenge of the basic theoretical precepts are also summarized. As might be expected in any generalized study of mitotic control mechanisms, the many pertinent aspects of the fundamental problems of malignancy arise repeatedly. The resulting considerations of these aspects, in light of the precepts of the general theory, have been formulated herein into a "unified" theory of the cytogenetic development and maintenance of the malignant state.

2. Basic Observations Suggesting a Potential Level–Mitotic Activity Relationship

Among somatic cell types, nerve and muscle cells are characterized by their exceptionally high (interphase) transmembrane potential difference levels

(E_m),† referred to hereafter simply as "membrane potential" or E_m. Equally characteristic though less often cited is the fact that these cells exhibit an extremely low degree of mitotic activity, mature neurons of the central nervous system being permanently devoid of mitoses (e.g. Weiss, 1956).‡ This mitotic quiescence has generally been attributed simply to the fact that these cells are "highly differentiated". It appears significant, however, in view of the substantial differences in form and function between these cells, that maintenance of a very high E_m level is accompanied by an almost complete absence of mitotic activity. This apparent correlation suggests, *a priori*, that some functional relation between E_m level and mitotic activity may perhaps exist.

This suggestion prompts consideration of the mitotic activity of cells maintaining other E_m levels and, although desirably comprehensive and systematic data on E_m levels of various somatic cell types under various mitotic conditions are not presently available, a number of highly interesting E_m level–mitosis correlations do in fact exist. A primary example is the pronounced decrease in E_m level which accompanies the onset of active proliferation in somatic cells during adaptation from *in vivo* conditions to growth *in vitro*. The interphase (G_1) E_m level of mature somatic cells (e.g. liver, lung, connective tissue) is generally found to be in the range of -50 to -60 mv, and mitotic activity is very low (mitotic coefficients $\cong$ 0·03). Upon dissociation from the explant *in vitro* and adaptation to continuous proliferation in culture, the cells undergo a decrease in the basic interphase (G_1) E_m to the vicinity of -10 mv, where this basic level remains as long as active proliferation continues. This characteristic decrease in G_1 E_m level appears to be a general phenomenon, occurring as it does for widely different cell types, and demonstrates the existence of the inverse of the high E_m situation, viz. that a low value of the E_m level is associated with very active cell proliferation. Furthermore, the ability of cells to switch effectively from a high E_m state with relative mitotic quiescence to a low E_m state with high mitotic activity upon imposition of a proper stimulus is also demonstrated by adaptation to culture. In the case of normal (i.e. non-tumorigenic) cells, the adaptation process is apparently reversible.

An interesting observation in this regard, involving mature neurons in tissue explants from rat brain, suggests that cells which are able to maintain

† Since essentially all somatic cells exhibit negative E_m values, reference will be made herein only to the absolute magnitude of the E_m level; thus a "high" E_m level designates a large negative value (e.g. -70 mv), while a "low" E_m level refers to a small negative E_m value (e.g. -10 mv). Also, it should be emphasized that reference herein to "E_m effects" on mitogenesis is in actuality but a reference to the integrated effects of the associated intracellular ion hierarchy, of which E_m is a convenient experimental index.

‡ Other divisionally quiescent cell types which possess equally high E_m levels are also known, as will be discussed subsequently.

their original *in vivo* E_m level after explantation and maintenance *in vitro* will not increase their proliferation rate beyond the *in vivo* value. Mature neurons have been maintained for months *in vitro* under proper conditions and, despite some rearrangement of the explant's cellular aggregation due to glial cell migrations (the neurons themselves demonstrated no mobility), the neurons maintained a constant E_m level of -70 mv with total absence of mitosis. The fact that the capacity of the nucleus of mature, fully differentiated neurons for resuming DNA synthesis and mitotic preparations is not irreversibly blocked has been clearly demonstated by nuclear transplantation experiments (Gurdon & Woodland 1968). Interestingly, the E_m level associated with the nuclear reactivation described in this reference was most probably in the mitotically active region of -10 to -20 mv, as will be discussed subsequently.

A second, and perhaps even more significant, example of an apparently general correlation of E_m level with mitotic activity lies in the pronounced cellular depolarization which accompanies malignant transformation of somatic cells *in vivo*. Although adequately comprehensive, systematic data are again lacking, the available data suggest that a basic characteristic of malignant transformation is a significant decrease in the E_m level from that of the normal homologous cell (Shaefer & Schanne, 1956; Tokuoka & Morioka, 1957; Johnstone, 1959), this decrease being accompanied by the gross increase in proliferation activity characteristic of the malignant state. In many cases the drop in E_m level is extreme. For example, in the case of a myosarcoma, the -90 mv potential exhibited by adjacent normal non-dividing muscle cells was found to have undergone a decrease to only -10 mv in the actively proliferating homologous sarcoma cells (Balitsky & Shuba, 1964). In regard to the E_m level–proliferation activity relationship, the similarity between adaptation of somatic cells to culture and the *in vivo* transformation to the malignant state is indeed noteworthy.

An additional similarity between culture adaptation *in vitro* and malignant transformation *in vivo* also appears highly significant in regard to the proposed mitotic relationship. A primary change during adaptation of normal cells to culture is the dissociation of the original tissue into individual cells, with attendant changes in the molecular constitution and immediate molecular environment of the individual cell surfaces.† In malignant transformation a prime alteration is the decreased adhesive binding of the transformed cells (e.g. Coman, 1944), thus leading to invasiveness and metastasis, a primary pathological characteristic of medical malignancy, and indicating a pro-

† The term "cell surface" is used herein to denote the entire molecular complex of the cell boundary, including the conventional pericellular lipoprotein membrane and the various surface polymer systems (protein, glycoprotein, lipopolysaccharide, mucopolysaccharide, and the like) as integral parts.

nounced functional change in the nature of the cell surface. It appears very much as though malignant cells during transformation acquire properties which make them behave much like normal cells which have become adapted to culture. This similarity seems quite important in the present context in that it suggests that a primary factor which has changed in both cases is the functional nature and molecular environment of the cell surface and, in conventional membrane theory, the cell surface plays an intimate role in determining the E_m level and its variations. It is thus quite possible that the same types of cell surface alterations which lead to invasion and metastasis in malignant cells are also the source of the lowered E_m level and active proliferation of these cells.

Although *in vivo* nerve and muscle, on the one hand, and somatic cells *in vitro* on the other, represent the extremes in E_m level and associated proliferation rates, most mature somatic cells (*in vivo*) apparently maintain intermediate E_m levels (-50 to -60 mv) and intermediate proliferation rates, thus further reinforcing the possibility that a general E_m level–mitotic activity relationship exists. The precise correlation of the E_m level with the actual degree of mitotic activity, although known for representative cell types at the end-points of the E_m spectrum, is uncertain in the intermediate E_m region since data of the required detail do not exist. The major questions in this regard are whether an E_m level exists for each cell type above which mitosis is fully blocked, and whether mitotic activity is maximal at all E_m values below this level, or increases continuously as the E_m level decreases.

One final observation is of interest here. The supposedly primary purpose of the high degree of membrane polarization in nerve and muscle cells, coupled with their membrane excitability, is readily understandable in terms of the basic functions of these cells. The reason as to why apparently *all* somatic cells, however, embracing a great variety of forms and functions, should possess a (negative) membrane potential of appreciable magnitude is not so obvious. In view of the above observations, it seems reasonable to suspect that, since continuous and precise maintenance of mitotic homeostasis is imperative in all somatic cell systems, the omnipresent potential of such cells may in some way be functionally related to mitosis control.

3. Fundamental Precepts of a Theory on Mitosis Control by Variation of the Transmembrane Potential Level

The foregoing generalized observations suggest a positive correlation between the degree of mitotic activity of somatic cells and the transmembrane potential level, a very high level being associated with essentially zero mitotic activity and a very low level with maximum proliferation. As pointed out, it

appears significant that all somatic cells wherein control of division is required possess E_m levels of appeciable magnitude. In addition to these general observations, a number of more quantitative, specific indications (to be discussed in sections 4 and 5) exist which strongly imply a definite functional relationship between E_m level and mitotic activity. Of primary importance among these is the demonstration (Cone & Tongier, 1970) that imposition of intracellular ionic conditions approximating those at an E_m level of -70 mv (equivalent to the case of non-dividing nerve) *reversibly* blocks DNA synthesis and mitosis *in vitro* (section 4).

On the basis of such observational implications, we shall in the present section assume the position that a functional relationship between E_m level and mitotic activity does in fact exist and, invoking the general precepts of conventional membrane potential theory,† proceed to elucidate possible ways in which various aspects of the resulting intracellular electroosmotic regime might act to exert a controlling influence on mitosis initiation in the cell. The resulting development may be taken as a generalized theory of electroosmotic regulation of somatic cell mitosis.

(A) MEMBRANE POTENTIAL THEORY AND ITS IMPLICATIONS FOR CONTROL OF INTRACELLULAR IONIC AND OSMOTIC CONDITIONS

In terms of conventional membrane potential theory, the E_m is simply a consequence of the ion concentration balance of the cell, brought about by "active transport" mechanisms and differential permeability of the membrane for the various ion species. Hence, the E_m level of the cell can be taken as an experimentally convenient, representative index of the ion balance, with the obvious understanding that reference to "E_m effects" on mitosis is but reference in actuality to the integrated effects of the associated ion hierarchy. Considering the cell as a freely conducting body, the $\hat{E}$ field associated with the pericellular E_m is confined essentially within the cell surface (i.e. membrane) thickness and consequently can exert no *direct* influence on ion mobilities and distributions within the cell interior proper. It is possible, however, that

† Conventional or "classical" membrane potential theory is based upon the concept of "active transport" and the "passive" distribution of nontransported ion species according to the Nernst equation. A great amount of experimental data exists, derived principally from studies in nerve and muscle physiology, which fully supports the precepts of conventional theory. Other theoretical explanations of E_m generation have been proposed (Ling, 1962; Cope, 1967) based upon the concept of a semicrystalline structure for intracellular water; such explanations appear, however, to be at odds with results from basic ionic diffusional experiments (Bunch & Kallsen, 1969; Kushmerick & Podolsky, 1969) and other observed electro-osmotic characteristics of cells. The present theoretical development of mitosis control is based in its major perspectives on the precepts of conventional membrane theory.

the large potential gradients within the cell membrane caused by the E_m associated $\hat{E}$ field may have indirect secondary effects on intracellular conditions by producing steric modifications of the cell surface structure which in turn may alter membrane permeabilities for various ionic and molecular species.

In classical membrane potential theory (i.e. as developed for nerve and muscle), the generation of E_m is ascribed to the relatively low conductivity or permeability of the cell membrane for Na^+. Thus, when Na^+ is actively transported out of the cell, $[Na^+]_i$ decreases and the E_m level (numerically) increases accordingly. Simultaneously, K^+ enters and Cl^- leaves the cell passively under the drive of the potential gradient, both of these movements acting to decrease the E_m level generated initially by the Na^+ exit. Ultimately, the steady state condition is reached where Na^+ influx from leakage exactly equals the actively transported efflux, and K^+ and Cl^- become passively equilibrated across the membrane. Under these conditions, the higher the efflux of Na^+, the smaller will be the $[Na^+]_i$, the larger will be the $[K^+]_i$, and the larger will be the E_m level at the steady-state condition. Although active transport of a number of ions in addition to Na^+ has been postulated to exist under various conditions (in order to account for experimental electrochemical potential differences presumably different from zero),† it appears that Na^+ is the primary cation generally involved in active transport, so far as E_m generation in somatic cells is concerned. Since Na^+ is by far the most abundant inorganic cation in (mammalian) interstitial fluid, it is only reasonable to expect that it, along with the second most abundant cation K^+, should play the same major role in E_m generation in most somatic cells as it does in nerve and muscle. In any event, so far as the immediate purposes of the present development are concerned, it is the variation in the relative and absolute concentrations of the various ions (particularly Na^+ and K^+) that accompany E_m variations which is of primary interest, rather than the precise mechanisms of the transport involved in the E_m generation.

The major results of the active Na^+ transport regime and its associated E_m level are that the ion balance of the cell (primarily the $[K^+]_i$ and $[Na^+]_i$) and the overall intracellular particle concentration (which governs the osmotic balance $\pi_i = \pi_o$; π denotes osmotic pressure) can be significantly varied through changes in the E_m level. In particular, high E_m levels produce large values of $[K^+]_i/[Na^+]_i$ and a reduced total intracellular particle content of

† The basic Nernst Equation is strictly applicable only to free ions in solution. Unfortunately, this equation is often applied to data based on the total content of a given ion in cells. If an appreciable percentage of the "ion" is in the bound form, this can lead to considerable error in the calculated value of the electrochemical potential and to the erroneous conclusion that the ion is being actively transported.

inorganic ions (in terms of conventional theory). The result of this latter alteration is that for osmotic balance, the total particle content of non-permeating intracellular organic molecules, A, for a given cell volume can (and must) be larger with a higher E_m level, since the cell membrane cannot support appreciable hydrostatic pressure differences and hence A will be more concentrated than at a lower potential (Cone, 1969*a*). The reverse conditions apply for a low E_m level. As will be discussed, both of these factors can possibly exert a substantial influence on mitosis preparations and execution.

It should be noted here that at the present stage of our theoretical development the proposed involvement of the intracellular ionic hierarchy in mitogenesis control is inferential, following on the basis of classical E_m theory from the experimentally observed differences in E_m levels. Whether the relative absence of Na^+, abundance of K^+, their concentration ratio, or all three intracellular factors are of major importance in mitotic blockage at high E_m levels is unknown at present. Evidence to be cited later indicates, however, that the absolute concentration of Na^+ exerts a definite control on DNA synthesis activity. In any event, changes in E_m level which are mediated by the mechanisms of classical membrane potential theory would result in a set pattern of variations among $[Na^+]_i$, $[K^+]_i$, $[Cl^-]_i$ and $[K^+]_i/[Na^+]_i$ for fixed values of $[Na^+]_o$, $[K^+]_o$, and $[Cl^-]_o$, so that only the value of $[Na^+]_i$ need be considered explicitly.

Since the pericellular membrane and its complex hierarchy of associated surface molecules (i.e. the cell surface) plays a major role in classical theory in determining selective ion permeabilities and (presumably) Na^+ active efflux rates the capability for controlling intracellular conditions is intimately associated with the cell surface. Thus, classical membrane potential theory as applied to the present problem of mitogenesis predicts that the cell surface (which depends directly on the state of cellular metabolism as well as on the local external physicochemical environment, including all surface contacts, for its functional state at any given time) can play a central role in governing the expression of mitotic activity under various conditions. Some of the more important features of this complex of interactions involving the cellular surface, metabolism, and external environmental conditions are discussed in some detail subsequently [section 3(D)].

(B) POSSIBLE MECHANISMS FOR E_m MEDIATED CONTROL OF DNA SYNTHESIS (G_1 MITOTIC BLOCKAGE)

Although mitosis of cells with tetra- and higher ploidy levels can take place at the expense of a reduction in ploidy without preceding or intervening DNA synthesis (e.g. Lindner, 1959), it is a general fact that mitosis of normal

diploid somatic cells must be preceded by DNA replication and chromosome duplication (to produce mitotic chromatids). In accord with this fact, it has been demonstrated for a large number of somatic cell types *in vivo* (Baserga, 1965; Gelfant, 1958) that the vast majority of cells remains arrested in the G_1 period presumably until either natural death (Cone, 1969*b*) occurs or some natural mitotic stimulation takes place (whence DNA synthesis commences and the cell moves on through the complete division process). Since DNA synthesis is thus an essential prerequisite for normal mitosis, any mechanism which acts to prevent DNA synthesis can constitute an effective block to mitosis; since the cell remains in the G_1 period during such an arrest, the blocking agent or mechanism may appropriately be referred to as a G_1 mitotic block. During such a naturally imposed mitotic block *in vivo*, operation of the specific, overall G_1 metabolic regime characteristic of the particular cell type must remain undisturbed by the blockage and, consequently, any natural blockage mechanism must be fully compatible with these metabolic requirements, thus greatly restricting the range of potential G_1 blocking mechanisms.

There appears to exist a number of ways in which the intracellular ionic and accompanying osmotic environments associated with a given E_m level could act to regulate various osmotically associated aspects of G_1 metabolism, particularly those connected with DNA synthesis, and hence to regulate mitosis initiation itself. The most obvious of these means are concerned with regulation of the synthesis and activity level of various enzymes associated specifically with synthesis of DNA or its precursors, and with regulation of general metabolite concentration levels. A few of these possitibilities are cited briefly here.

In regard to ionic effects on DNA-associated enzyme activity, it seems reasonable to expect that the relative concentration balance among intracellular cations (particularly Na^+ and K^+, and Ca^{2+}) could exert an influence at the most basic level, possibly by regulation of enzyme mRNA transcription. Such action might take place through influence of the ionic environment directly on the release or binding of repressor molecules with the genome or indirectly by activation of inducer molecules in the cell. On a higher cytogenetic level, the ionic environment could act by activation or repression of the activity of already-formed enzymes. For example, the very specific electrical diffuse double layers (accompanying various $[K^+]_i/[Na^+]$ ratios) which surround specific large enzyme molecules (Overbeek, 1952*a*) might exert considerable influence on the relative activity of the various DNA-associated G_1 enzymes. Variation of the E_m level could result in double layer changes on both free and bound enzymes of such magnitude as to cause functionally significant alterations of the steric conformation of the molecule, with conse-

quent alteration of enzyme activity. The effects of ionic environment on the steric conformation of various macromolecules are well known (e.g. Mysels, 1959; Balazs & Laurent, 1951; Mathews, 1953). The double layer might also influence enzyme effectiveness by shielding active sites of enzymes from sufficiently close approach of substrate molecules, thereby blocking specific reactions. Since Na^+ has a different flocculation power from K^+ (Overbeek, 1952*b*), the variation in ionic double layer composition, potential distribution and thickness with different $[K^+]_i/[Na^+]_i$ could be significant.

In addition to such steric conformational and shielding effects, the ionic environment might also influence enzyme activity directly by ion replacement or exchange mechanisms. Several enzyme systems are known which are particularly sensitive to the $[K^+]/[Na^+]$. Indirect effects of Na^+ and K^+ could also exist, wherein the intracellular release of such enzyme-activating ions as Ca^{2+} and Mg^{2+} might be mediated through ion exchange reactions sensitive to variations in the Na^+–K^+ environment, and hence to the E_m level of the cell.

In regard to effects on DNA synthesis of intracellular metabolite concentration levels associated with E_m induced cell volume variations, it is well known that relative and absolute concentrations of metabolites play a central role in determining which systems of cellular cytogenetic and biosynthetic pathways will be active, and in determining reaction rate kinetics in these pathways. Since E_m associated volume changes can effectively vary intracellular metabolite concentrations (for given initial weights of metabolites), with subsequent feedback alteration of further metabolism, E_m level variation appears to offer an additional potential means for controlling DNA synthesis by regulation of metabolic pathways involving concentration-sensitive metabolites. For example, excessive intracellular concentrations of thymidine constitute an effective block to DNA synthesis and are commonly used to obtain synchronized cell populations (e.g. Tobey, Petersen, Anderson & Puck, 1966). High E_m levels could conceivably concentrate certain cellular metabolites to such an extent that, ultimately, specific DNA precursor synthesis, or DNA synthesis itself, is blocked.

In summary, there appears to be a number of plausible mechanisms by which the ionic and osmotic changes associated with variations in the E_m level could act to block DNA synthesis and/or other aspects of mitosis preparations, and hence mitosis initiation itself.

(c) POSSIBLE MECHANISMS FOR E_m MEDIATED CONTROL OF PROPHASE INITIATION (G_2 MITOTIC BLOCKAGE)

Although this paper is concerned only with G_1 mitotic blockage, it should be noted that the same mechanisms whereby conditions associated with the

E_m level might impose a G_1 mitotic block by preventing DNA synthesis could also be active in principle, in imposing mitotic blockage by preventing mitotic preparatory events during the G_2 period. Following completion of DNA synthesis, a number of additional metabolic preparations must be completed, primary among these being synthesis or elaboration of the mitotic spindle structural precursors, blockage of which could prevent progress into mitosis. Presumably, proper metabolite and ion distributions and concentration levels are essential to these preparatory activities. Significant, characteristic variations in E_m from the basic interphase level have been found to be associated with mitosis initiation *in vitro*, and experimentally imposed osmotic changes simulating high E_m levels have been shown to effectively block prophase initiation in synchronized G_2 cells (Cone, 1969*a*). A detailed examination of possible ionic involvements in G_2 and prophase initiation has been given in this same reference and will not be considered further here. The main point of this present section is that, although G_2 blockage of mitosis is relatively rare compared to G_1 arrest *in vivo*, the same electro-osmotic mechanisms might be the active factors in both cases, with the high E_m condition of the G_2 blockage presumably developing after completion of DNA synthesis; that of the G_1 blockage before DNA synthesis. At present, no experimental data on the E_m level of G_2 blocked cells *in vivo* are available.

(D) E_m METABOLIC FEEDBACK CIRCUITS AND MITOSIS CONTROL

Since the foregoing observations and considerations imply that changes in ionic and osmotic balance associated with variations in E_m level can exert a regulatory influence over DNA-synthesis preparations and other mitotically important aspects of cellular metabolism, the question of how the E_m level is itself generated and regulated becomes one of central importance in mitosis control. In terms of classical membrane potential theory, the two primary factors involved in E_m level determination are g_{Na}, the Na^+ conductivity of the cell surface, and J^o_{Na}, the active efflux rate for Na^+. Since each of these parameters is intimately affected in turn by the state of cellular metabolism, a complex pattern of feedback interactions between E_m level and cell metabolism can be envisioned; the degree of stimulation or repression of mitotic activity which accompanies the resulting "steady-state" E_m level then follows directly as a consequence of these interactions.

The essential relationships of the present proposed system of primary feedback interactions governing mitotic control is outlined in Fig. 1. For clarity, only the primary feedback loop is shown, although a host of secondary interactions obviously exists. The model presented is constructed on the basis of a wide range of experimental evidence particularly in regard to the relation between the cell surface condition and mitosis.

Considering first E_m in the proposed system of Fig. 1, this potential difference (or, more precisely, the associated intracellular ionic and osmotic regime) acts to influence the metabolic expression and activities of the cell, presumably by means of the basic cytogenetic and enzymic mechanisms considered previously. The influence of this regime on two specific aspects of cell metabolism are of primary concern here, the effects on activation or repression of DNA synthesis, and the effects on those metabolic pathways which feed back directly or indirectly to control the E_m level itself.

In regard to DNA synthesis in the present scheme, the decision of whether the cell will begin preparations for and actually synthesize DNA, and hence enter division, is governed by the E_m level which exists during G_1. If the

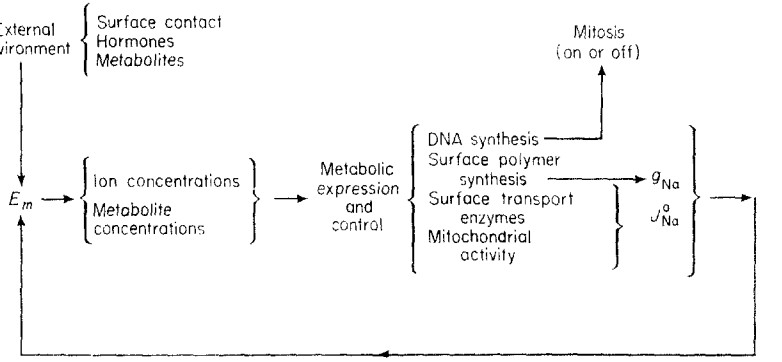

FIG. 1. Schematic flow chart illustrating the proposed system of feedback interactions whereby the intracellular ion hierarchy associated with E_m acts to influence cellular metabolic activity concerned with DNA synthesis, surface polymer production and mitochondrial activity. The latter two factors along with the external environment, in turn feed back to determine the E_m level.

E_m level is sufficiently high, DNA synthesis and mitosis will not take place and the cell will remain in G_1 (or G_0) as long as the high E_m level is maintained. Upon reduction and subsequent maintenance of the E_m at a sufficiently low level for a time adequate to permit full metabolic readjustment and initiation of mitotic preparatory activity under the new ionic and osmotic conditions, DNA synthesis and ultimately mitosis will take place. Thus mitotic preparatory pathways are assumed to undergo activation rather automatically in response to ionic conditions of a low E_m level and to undergo repression when a sufficiently high E_m level is maintained. This direct dependence of mitotic activation on E_m level places great importance on the second area of metabolism cited above, viz. those elements which are involved in determination of the E_m level.

The two primary factors which determine the E_m level, g_{Na} and J^o_{Na}, are both intimately associated with the metabolic state of the cell, which in turn is influenced by the E_m level, thus leading to a dynamic feedback circuit of relationships. The first factor, the effective Na^+ conductivity of the surface, g_{Na}, appears to be quite sensitive to the specific nature, chemical structure, surface steric conformation and disposition, and intercellular associations of the cell surface polymers, properties which in turn are determined by the particular metabolic expression and the immediate external molecular environment of the cell. Of particular interest here are the mucopolysaccharides and similar saccharide polymers (e.g. lipopolysaccharides, glycoproteins, and the like, collectively denoted herein as MPS) known to be associated with the cell surface (e.g. Rinaldini, 1958; Brandt, 1958; Bell, 1962; Dorfman, 1963). For example, the surface MPS appear to be a primary functional agent in immunological expression (Moscona & Moscona, 1952; Davies, 1959), and it is well established that immunologically active changes in the cell surface accompany malignant transformation in cells (e.g. Alexander, 1966; Ambrose, 1966); this surface change accompanying malignant transformation has also been demonstrated by electrophoretic mobility studies (Ambrose, 1966). These immunologically active surface changes during transformation are accompanied by the previously described drop in E_m level, with simultaneous increase in proliferative activity (Cone, 1969*a*). These E_m changes are most probably associated with changes in the effective values of g_{Na} (and/or J^o_{Na}) resulting, either directly or indirectly, from the surface alterations; increased permeabilities of malignant cells have been recorded (e.g. Abercrombie & Ambrose, 1962).

J^o_{Na} (as well as g_{Na}) may also effectively be altered by such surface changes; evidence exists that "active transport" is associated with pumping systems situated within the cell surface complex (Tosteson, 1963). As will be discussed later in regard to malignant transformation, a drop in E_m due to an increase in g_{Na} might also influence J^o_{Na} significantly through alteration of the energetics machinery of the cell (Cone, 1969*a*), leading to a coupling interaction between g_{Na} and J^o_{Na}. It is important to note that the effective values of g_{Na} (and perhaps of J^o_{Na}) depend not only upon the specific chemical structure of the surface polymers, but also on their steric conformational arrangement in the surface complex and their contact interaction with the surface polymers of adjacent cells or basement membranes.

In light of this general theory of E_m level control of mitogenesis, it is particularly appropriate to introduce at this point the present theory of oncogenesis, with emphasis on the central involvement of the cell surface. Of fundamental interest in this regard is the well documented ability of certain viruses, after undergoing lysogeny in host bacteria, to alter in a characteristic and highly

specific manner the structure of particular surface lipopolysaccharides of the bacteria, by redirection of the metabolic pathways involved in the synthesis of the surface polymers (Losick, 1969). Since a number of oncogenic viruses exist, all of which also produce surface antigenic changes in the somatic cells they transform, it is quite possible (by analogy with the foregoing bacterial example) that their oncogenic action, with regard to both proliferation and invasiveness and metastasis, is in essence entirely due to an alteration or redirection of MPS and other surface-associated polymer metabolism, resulting in an altered surface composition which produces decreased surface adhesion (thus allowing invasion and metastasis) and lowered E_m level (which allows the characteristic attendant increase in mitotic activity, as previously described) (Cone, 1970*a*).

A prime part of the redirected metabolic activity induced by the viral genome in a somatic cell is concerned with synthesis of specific structural polymers for the viral coat. In a number of oncogenic viruses, the viral coat appears to possess constituents very much like those of the somatic cell surface. This fact suggests that in all probability those aspects of cellular metabolism concerned with cell surface polymer synthesis are specifically altered or redirected by these viruses, resulting in prevention of normal cell surface polymer production and/or superposing altered polymer forms which then serve to disrupt the cell surface functionality as regards surface adhesion and E_m maintenance; as will be discussed, the alteration of surface adhesion alone in somatic cells by changes in the surface polymers can result in alteration of the E_m level, and thus produce continuous proliferation as a consequence. In the case of the Rous sarcoma virus (RSV), for example, the viral genome is unable to produce a functional viral coat, presumably due to mutant viral-directed synthesis of abnormal viral coat polymers. Such abnormal polymers, together with possible alteration of the normal cell surface polymer synthetic pathways, may be the basic source of the malignant properties induced by RSV. There are indications that the functional coat formed by RSV with the aid of its helper virus actually contains elements from the surface of the host cell. Since the genomes of a number of carcinogenic viruses contain only a few genes at most (four or five), it should prove feasible to determine explicitly for some of these viruses if one or more genes is acting to alter or redirect cellular metabolic pathways associated specifically with surface-polymer production.

It should be emphasized that the required change in normal surface polymer structure necessary to maintain the malignant state might be quite subtle indeed, such as the replacement of α with β glycosidic bonds at a few key points. Such seemingly slight changes have been observed to completely block ε^{15} virus entry into ε^{15}-lysogenic *S. anatum* bacteria (Losick & Robbins, 1969). Such subtle changes in the surface polymer structure of somatic cells

could by analogy introduce major alterations in cell surface adhesion specificity with proportionately large changes in the effective values of g_{Na} and consequently in the E_m and proliferative activity levels.

It is quite important to note in the mechanism of virus-induced malignant transformation as proposed here that continuous lysogeny is not necessarily required for maintenance of the malignant state if it occurs that once the entire complement of normal cell surface polymers has become replaced (or superposed) with the altered form, the accompanying low E_m level conditions act to stably maintain the new metabolic pathways, which will then continue to produce the initial altered polymer or subsequent deviate forms (Cone, 1969*a*). In this case, the virus merely serves as an intermediate agent for altering the cell metabolism for a period sufficient that a new self-sustaining metabolic state can be attained.† The virus can thus be looked upon as a sort of initial "activation agent" allowing the cell to shift from one (stable) "metabolic well" to another; in this sense the "well" constitutes a particular state of differentiation or stable pattern of genome expression, characterized by a highly specific pattern of interacting metabolic pathways. In the absence of specific external influences capable of restoring the original metabolic state, the cell would remain in the "redifferentiated", malignant form, with attendant "abnormal" mitotic activity. Such a mechanism of malignant transformation would obviously apply equally well in principle to explain chemical and physical, as well as non-lysogenic viral, carcinogenesis thus providing a unifying explanation of oncogenesis in general. Of course, in the case of permanent lysogeny the viral genome could remain the agent for forced maintenance of the surface aberrancy. In any event, the key feature of the resultant change of metabolic state is that the cell surface is now "permanently" altered, thus leading to the invasive, metastatic, and proliferative pathology of malignancy.

Returning now to the problem of mitogenic control in general, if once a cell has been cytogenetically induced‡ by factors of external origin to initiate synthesis of surface polymers resulting in a low E_m level, the intracellular ionic conditions associated with this level favor the continued synthesis of the low effective g_{Na} (or J^{o}_{Na}) surface polymer hierarchy (as in malignancy), the low E_m level will be maintained and mitosis will continue on a sustained basis.

† In such a case, it is important to note that "viral-specific" antigens would continue to be detected in the transformed cells even though no actual viral genome (or segment thereof) was present.

‡ By "cytogenetic induction" of new polymer forms is meant the subsequent interactions and sequential alteration of the balanced, steady state metabolic hierarchy of the cell following the initial disturbance of the external factor. Following initiation of the disturbance, the cell can be envisaged as undergoing a complex train of metabolic oscillations which gradually dampen out as the new stable state is reached.

If, on the other hand, a *temporary* lowering of the E_m level is produced by some external factor (e.g. hormones, temporary detachment of cell surfaces) in such a manner that the sustained production of low-E_m-favoring surface polymers is not induced (as in initial adaptation of normal cells to *in vitro* proliferation), then the low E_m level and associated mitotic activity will exist only so long as the low E_m effecting agent is present, and will revert to the original condition upon removal of the agent. For example, in wound healing mitoses cease once proliferation has replaced dead cells to the extent that intimate surface contact again maintains. Apparently, however, the sustained imposition of low E_m conditions on normal cells is itself conducive ultimately to the induction of self-sustaining low E_m surface polymer production, as is evidenced by the fact that most primary cultures of normal cells, and even normal cells in abnormal contact environments *in vivo*, eventually undergo spontaneous malignant transformation.

Without the intervention of external factors, an existing high E_m level would favor continued synthesis of polymer forms leading to the maintenance of the high level, with associated blockage of mitosis. As will be discussed in more detail, a number of external factors can act to alter g_{Na} and/or J^o_{Na} either directly or indirectly, and hence lead to a corresponding activation or suppression of mitosis. Thus the $\{g_{Na}/J^o_{Na} \rightarrow E_m \rightarrow$ surface polymer metabolism $\rightarrow g_{Na}/J^o_{Na}\}$ feedback circuit outlined in Fig. 1 constitutes a potentially effective mechanism for regulation of mitosis.

J^o_{Na}, in addition to its dependency upon surface conditions, is closely associated with the energetics aspect of cell metabolism through the energy requirements of active transport, and hence is dependant upon E_m level effects on such activities as glycolysis and oxidative phosphorylation. As noted in a previous paper (Cone, 1969*a*), mitochrondria appear to be quite sensitive to changes in their ionic and osmotic environment and presumably could respond to a decreased E_m level in somatic cells by decreasing ATP production, with a subsequent lowering of energy availability for sustained active transport. This lowered energy availability could, in the absence of factors acting to increase E_m, feed back to keep J^o_{Na} and hence E_m at a low level. It is well known that metabolic inhibitors which block oxidative phosphorylation lead to a reduction in J^0_{Na} and E_m level. In addition to the effects of the changed ionic environment (due to the lowered E_m level) on mitochondrial activity, it should be noted that the same basic polymer alterations appearing in the pericellular membrane might also appear in the mitochondrial membrane, thus altering their functionality as regards ATP generation.

An interesting observation in this regard is the apparently characteristic glycolytic production of ATP in malignant cells (Warburg, 1924). In view of the foregoing considerations, the existence of such glycolytic metabolism may

have as its basis the lowered E_m level of the malignant cell, and may in turn help to maintain and stabilize the lowered E_m level through small values of J^o_{Na}. These relationships also suggest an associated means for accomplishment of malignant transformation by viral, chemical, or physical agents, wherein the changes in surface polymer metabolism affect in addition to the cell surface the mitchondrial membrane surfaces, thereby accomplishing a reduction in oxidation phosphorylation, J^o_{Na}, and consequently E_m. Thus, the present "membrane" theory of oncogenesis provides a unified picture, rationally relating Warburg's observations with many other prominent features of malignancy. There is also some suggestive evidence that the active transport rate of Na^+ (i.e. J^o_{Na}) may be influenced by Na^+/K^+-sensitive ATPase activity in the cell surface (Tosteson, 1963), thus again implying the possibility of g_{Na} and J^o_{Na} coupling through structural changes in the cell surface.

To summarize the proposed system of internal E_m metabolic feedback interactions outlined in Fig. 1, E_m acts to set the intracellular ionic and osmotic (metabolite concentration) environment, which in turn exerts an influence on metabolic pathways specifically concerned with: (1) DNA synthesis and mitosis preparations; (2) surface polymer structural specificity; and (3) cell energetics. These three aspects of metabolism subsequently act to set the effective values of g_{Na} and J^o_{Na}, the two primary factors controlling the E_m level; the resultant E_m level then determines the rate and direction of the pertinent aspects of metabolic activity, thus completing the feedback circuit. Presumably, in the absence of specific external influences, this feedback circuit can exist in stable states (basic G_1 E_m level constant with time) at both high and low E_m levels. At the higher levels, DNA synthesis and mitosis are blocked; at the lower levels active mitosis maintains.

If the proposed stable states exist at high and low E_m levels in the feedback circuit they provide the basis for an effective means for mitosis control: provided suitable external switching mechanisms are available, repression or activation of mitosis can be obtained merely by metabolic switching from a high E_m level to a low one, or *vice versa*. A wide range of potential switching mechanisms is available, in principle, since any agent or condition which would alter the E_m level either by direct action on the cell surface or through alteration of one or more aspects of metabolism *specifically* concerned with E_m maintenance could serve as an effective mitotic switch. Thus, such chemical factors as hormones, mitogenic metabolites, and viruses on the one hand and such physical factors as wound induction and cell contact modulation on the other, could all serve as effective mitotic switches between the blocked and unblocked states (Fig. 1). Rather than speculate on the specific modes of action of the many known mitogenic factors, only two primary ones will be considered by way of example, hormonal action and cell contact.

In terms of the present theory, mitogenic hormonal action in tissues could take place either by direct effect on the cell surface (e.g. induced g_{Na} or permeability changes) or by internal action of E_m related metabolic pathways. Known mitogenic effects of hormones and other such agents are usually quite specific (e.g. estrogen level effects on mammary and uterine tissue), a given hormone stimulating primarily one (or at most a few) tissue types. This fact would suggest a high degree of specificity in hormone action as a "mitotic switching" agent. In particular, if direct changes in the cell surface are involved, a high degree of specificity of the surface polymers of different cell types is implied; the existence of such specificity is of course well established. The action of mitogenic hormones normally is entirely reversible, i.e. their continued presence is required for sustained mitotic action. In terms of our hypothetical switching system, the cell remains in the "low E_m state" only so long as the hormone is present, and reverts to the stable "high E_m state" upon its disappearance since metabolically-sustained surface changes have not occurred. The analogy with a spring-loaded electrical switch which remains "on" only so long as pressure is maintained is appropriate here. Sustained hyperactive hormonal sources may, however, lead to continuous mitotic stimulation and ultimately malignancy. Other mitogenic agents, such as some of the chemical carcinogens, may act by similar mechanisms, only their switching action is "permanent" in the sense that the low E_m state is crytogenetically induced in such a manner that it becomes self-sustaining.

In regard to cell contact effects, mature cells in most somatic tissues are always in intimate contact (with each other or an appropriate basement membrane), and under such conditions normally exhibit a very low degree of mitotic activity; consequently, the question of how surface contact *per se* might act to suppress mitosis is of great importance. Although little knowledge exists at the molecular level on the nature of cell surface interactions or on the structure and functioning of surface polymers in intercellular "space", some interesting justifications do exist, in terms of the present theory, for expecting an influence of contact on mitogenesis.

When cell surfaces come into sufficiently close contact to actually form intercellular bonds, the surface polymers extending into intercellular space must be in intimate proximity and their ionic double layers must interact significantly. If the physical state (i.e. aggregation and bonding) of the specific surface polymers is active in setting the level of g_{Na} (and also J^o_{Na}), then the alteration of this state which must occur when the cell surfaces vary their degree of contact could conceivably induce correspondingly significant changes in the effective value of g_{Na} (and/or J^o_{Na}), and hence in the E_m level. Also, as is well known in colloid science (Overbeek, 1952*a*), the specific ion concentration and electrical potential distributions existing in the ionic double

layer at charged surfaces such as those of cells extends an appreciable distance (on an Angstrom basis) into the bathing medium. With intercellular spacings of the order of 100 Å, which includes most somatic cells, a significant overlap of the electrical double layers of the two surfaces would occur, thus leading to a pronounced alteration of the distribution of ionic species and concentrations relative to those of free cells, with a corresponding change in the ionic environment "seen" by the cell and hence in the effective values of g_{Na} and J^{o}_{Na}. For example, the local concentration of such divalent cations as Ca^{2+} in the immediate vicinity of the surface polymer layer would be expected to rise under such contact conditions. Phenomenologically, it is well known that changes in $[Ca^{2+}]_o$ exert a pronounced effect on the g_{Na} level in nerve; specifically, higher $[Ca^{2+}]_o$ levels (as would presumably be obtained as a result of intimate cell contact in the present case) are required for maintenance of high E_m levels, while low $[Ca^{2+}]_o$ levels lead to depolarization. Thus, through this and similar ion-surface polymer interactions in the intercellular space, it appears that intimate cell contact could lead to increased E_m levels with associated suppression of mitosis. As already cited, aggregation of (normal) cells *in vivo* is attended by relatively high values of E_m, while decreased bonding in malignancy and the disaggregation *in vitro* are accompanied by a significant drop in E_m level, with attendant mitotic activity increase.

The closeness to which cell surfaces can approach, and the tenacity with which they bond are factors which depend almost entirely upon the nature and specificity of the surface polymers. The great specificity with which various cell types aggregate into tissue form (Moscona & Moscona, 1952) demonstrates clearly the exacting surface immunological compatibility involved in cell surface contacts. In view of such specificity, it is understandable how even slight changes in surface polymer composition can lead to alteration of the required contact intimacy, with ensuing effects on E_m and mitogenesis.† The characteristic surface antigenicity and other surface abnormalities of malignant cells are again highly significant in this regard. The foregoing considerations indicate that the uncontrolled division of such cells is directly related to the cell's altered surface characteristics. It is most probable that the modified antigenicity of the malignant cell's surface is in fact due to a stable, metaboli-

† Although the totality of possible enzymes available to a cell is contained in the cell's genome, any cell is probably capable of making a range of biochemically different species of a *derived* product, such as surface protein and MPS, in response to the effects of the environment upon the expression of the available enzyme patterns. Thus, for example, a given cell may conceivably produce the same basic MPS surface polymer, but with a wide variation in specific features, say, in the degree of sulfation (and hence in antigenic specificity) in response to different ionic concentration levels in the cell, and in response to the particular pathway of metabolic alterations by which it was induced to progress from its initial to its (new) final state.

cally-sustained alteration of the surface polymer hierarchy which in turn prevents sufficiently close contact and/or adequate bonding between such cells thus permitting abnormally low E_m levels with continuous division and, simultaneously, invasiveness and metastasis. Thus, it is proposed that the two major pathological features of malignancy, invasiveness and metastasis and uncontrolled proliferation, are intimately related and result from the same fundamental source, a basic aberrancy in the molecular architecture of the cell surface hierarchy. This basic theory of oncogenesis has been discussed in some detail previously (Cone, 1970*a*).

Contact control of mitosis as outlined above suggests an interesting mechanism for maintaining normal mitotic homeostasis in somatic tissues. As is obvious from constant tissue-mass considerations (Cone, 1969*b*), one cell must divide for each cell which dies, for mitotic homeostasis. For maintenancy of mature tissue morphology, it would appear particularly desirable for divisions to occur directly at the sites of cell death This could be most simply accomplished if a local cell death were to act as a stimulus for division of an adjacent cell thus effecting cell-for-cell replacement. Death of a given cell would certainly result in the breaking of functional surface contact with neighboring cells, and in view of our foregoing discussion would presumably allow a decrease in E_m and hence stimulate DNA synthesis and division in at least one adjacent cell. Preliminary experiments with mitotically inhibited confluent monolayers *in vitro* have indicated the operational feasibility of such a mechanism (Cone, 1967, unpublished data). A detailed consideration of mitotic homeostasis mechanisms based on the present theory of E_m level control of mitogenesis has been presented by Cone (1970*b*).

In conclusion of this section, the proposed system outlined herein for mitotic control by E_m modulated metabolic feedback circuits and switching mechanisms, appears to be compatible with a range of experimental observations, and provides a unifying picture of some heretofore apparently unrelated mitogenic phenomena. Although necessarily based in some areas on meager and incomplete experimental data, the theory provides an effective basis for the design of further experiments to investigate its specific and general validity. Of particular significance are the prediction of a fundamental involvement of the cell surface in mitogenesis, and the unifying explanation resulting therefrom of the relationships among the primary pathological features (invasiveness, metastasis, and unchecked proliferation) of malignant cells, all aspects of which are open to critical experimental investigation. If the present theory is to possess general applicability, it appears a necessary requirement that DNA synthesis and mitotic preparatory pathways and their regulation by ionic conditions, represented by E_m, be capable of relative dissociation (during G_1 and S at least) from the more specific aspects of cell metabolism, since this

latter factor is so grossly different in different cell types. There is good evidence that such is the case that even many of the various mitotic preparatory pathways proceed independently in several respects (Mazia, 1961).

4. Results of an Experimental Challenge of the Theoretical Precepts

The precepts of the foregoing theory are of course open to direct experimental challenge and such a challenge has in fact already been carried out (Cone & Tongier, 1970). Since this investigation produced results of primary significance regarding the present theory, the experimental plan and pertinent results are briefly outlined here. In essence, the experimental procedure consisted in varying the E_m level of typical somatic cells *in vitro* and recording the resulting effects on the progress of DNA synthesis and subsequent mitosis. Unfortunately, determination of the effects of E_m level and its associated ion balance on the mitotic activity of somatic cells by electrode-imposed variations in the physical membrane potential *per se* is not experimentally meaningful or feasible. From a practical standpoint, however, such difficulties can be overcome and reasonable simulations of desired E_m levels obtained by exposing test cells to external media of such compositions that, upon equilibrium, the intracellular concentrations of the various ions approximate those which would exist if the cells were actually able to generate and maintain the E_m level being simulated. Design of the ionic compositions of such "potential simulation" media can be based either upon existing experimental concentration data for cells of known E_m level (e.g. nerve, muscle, or upon estimated values obtained from conventional membrane transport theory using appropriate assumptions regarding permeabilities and whether active or passive transport is occurring for the various ion species.

Utilizing this potential-simulation technique, tests were conducted to establish the effect of E_m level variation on the mitotic activity of a typical and mitotically representative mammalian cell line *in vitro*. Of primary interest was the question of whether sufficiently negative E_m levels could *reversibly* block mitosis in cells which, at their normal low E_m level *in vitro* (-10 mv), exhibit vigorous and continuous proliferation, and whether such blockage is mediated through prevention of DNA synthesis as is the case *in vivo*.

(A) PLAN OF EXPERIMENTS

Using monolayer populations of *naturally* synchronized cells† so as to preclude introduction of metabolic and mitotic abnormalities by the synchronization process itself, the minimum E_m level (i.e. the corresponding simulation

† Obtained by shaking rounded metaphase cells free from the surface of large monolayer cultures.

medium composition) which blocked all mitosis was determined in preliminary tests using a graded series of E_m simulation media covering the maximum E_m range normally found *in vivo* (-10 to -90 mv). The quantitative effects of this minimum E_m medium on mitotic activity and DNA synthesis were then determined by monitoring the time increase of both total and [^{3}H]thymidine pulse-labeled cells during continuous incubation of newly synchronized cell populations for a period of approximately $2T$ (T = normal cell cycle period *in vitro*), followed by a mitotic recovery period of one T during which the test medium was replaced with normal culture medium. The data of the first period established the mitosis and DNA synthesis blocking effectiveness of the (simulated) "high E_m" condition. Those of the second period established the time and degree of mitosis and DNA synthesis recovery upon return to normal E_m conditions, thus demonstrating the relative reversibility of the E_m imposed mitotic block. A "long-term" test in which newly synchronized populations were monitored for cell number increase during continuous maintenance in the high E_m medium for a period of approximately $6T$ was also run to ascertain that spontaneous recovery of mitotic activity did not occur as long as the cells were maintained at the high E_m condition, and also to determine the rate of cell death during prolonged mitotic blockage. All experiments were performed with Chinese hamster cells (diploid CHO cell line).

(B) RESULTS

The experimental results are summarized in Figs 2 to 5. The effects of simulated E_m, level on the mitotic activity of naturally synchronized CHO cells over a 96-hour period (Fig. 2) show that immediate mitotic suppression (i.e. within 24 hours) begins at about $E_m = -45$ mv, and reaches the condition of complete arrest at $E_m = -75$ mv. Interestingly, this latter level is essentially the same as that maintained *in vivo* by non-dividing neurons. Above -75 mv cell division is completely arrested. As ascertained by direct observation, no cell death above the normal control value of 4% of a given population per 24-hour period, occurred at $E_m = -75$ mv and below during the 96-hour period. To insure complete mitotic blockage in the ensuing tests, -75 mv was taken as the minimum E_m for mitotic arrest.

As expected from these preliminary tests, an E_m level of -75 mv entirely blocks cell division during the 33-hour treatment of the short-term reversibility test (Fig. 3), whereas the control cells in normal culture medium proceed to divide in a synchronous wave with a cycle time of 20 hours. Direct visual observation revealed no cell death beyond control values during the 33-hour blockage period. The data of this figure clearly demonstrate that the E_m-induced mitotic blockage is fully reversible. The percentage of the initially

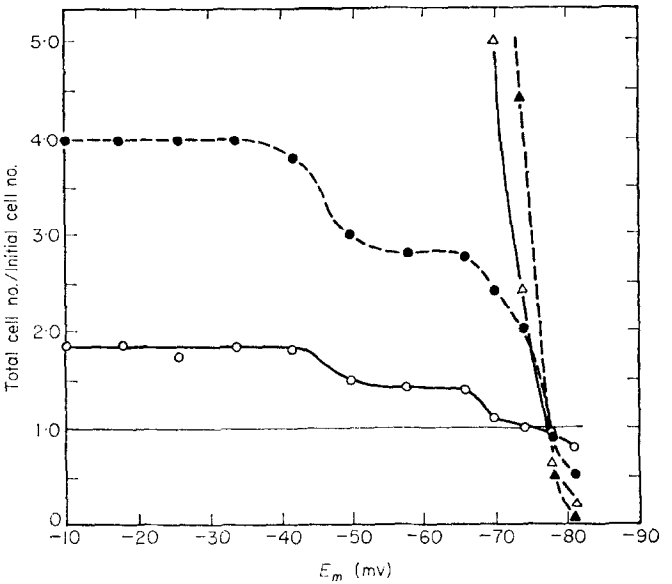

FIG. 2. Effect of simulated E_m level on mitotic activity in naturally synchronized CHO cells at 24 hr intervals. ——, 0 hr; ○—○, 24 hr; ●----●, 48 hr; △——△, 72 hr; ▲---▲, 96 hr.

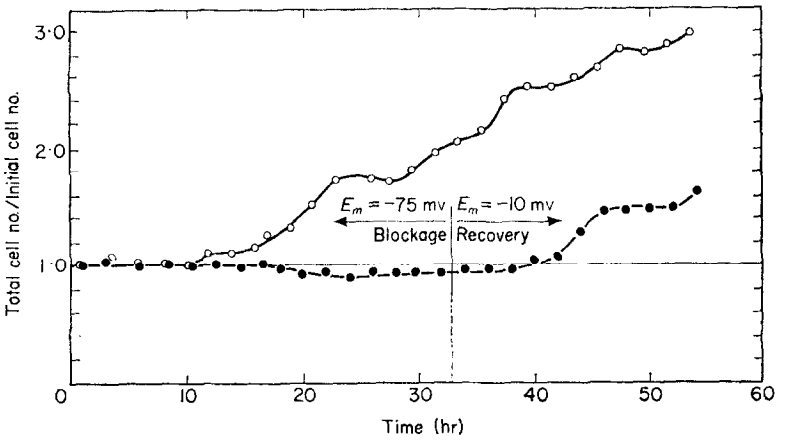

FIG. 3. Effect of simulated E_m level of −75 mv on mitotic activity in naturally synchronized CHO cells. Control cells maintained in normal culture medium. ○——○, Control; ●——●, test.

blocked cells which divided upon E_m reduction was essentially the same as for the first mitotic wave of the unblocked controls.

The results of the tests to ascertain the long-term blocking effect of $E_m = -75$ mv are shown in Fig. 4. It is seen that mitosis is in fact completely blocked so long as the cells are maintained at the high E_m level. At $t = 120$ hours (6 T) some 26% of the initial population were still viable and morphologically normal in appearance except for slight rounding. Following return to normal medium ($E_m = -10$ mv), mitotic activity resumed in the remaining cells such that 40% had divided within 26 hours. Also shown for comparison are the results of a long-term mitotic blockage test with 5-aminouracil (5-AU). The 5-AU concentration used was the minimum required for blocking all

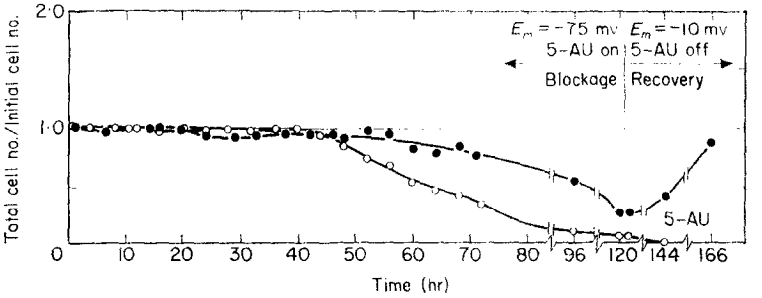

FIG. 4. Long-term effect of continuous simulated E_m level of -75 mv, and treatment with 5-AU (0·4 mg/ml.) on mitotic activity and cell death in naturally synchronized CHO cells. ○———○, 5-AU; ●———●, -75 mv simulation test.

mitosis in CHO cells. As is evident, long-term cell death with 5-AU appreciably exceeds that accompanying high E_m level blockage, beyond 50 hours. In addition, the 5-AU blocked cells underwent grotesque morphological changes, forming into multinucleate giant cells; these cells subsequently died without division following replacement of the 5-AU medium with F-10. It appears that while the cell death rates during long-term mitotic blockage by high E_m level and 5-AU are roughly comparable, such mitotic blockage is reversible only in the case of the E_m simulation treatment.

The effect of $E_m = -75$ mv on DNA synthesis in CHO cells is shown in Fig. 5; these data correspond to the mitotic blockage and recovery test results presented in Fig. 3. The data clearly demonstrate that the simulated high E_m level fully (but reversibly) blocks DNA synthesis, and clearly implies that the mitotic blockage shown in Fig. 3 is a consequence of the prior blockage of DNA synthesis. From a consideration of the time required for resumption of DNA synthesis after high E_m level reduction, it appears that the mitotic block occurs at a point in the last quarter of the G_1 period. Subsequent tests

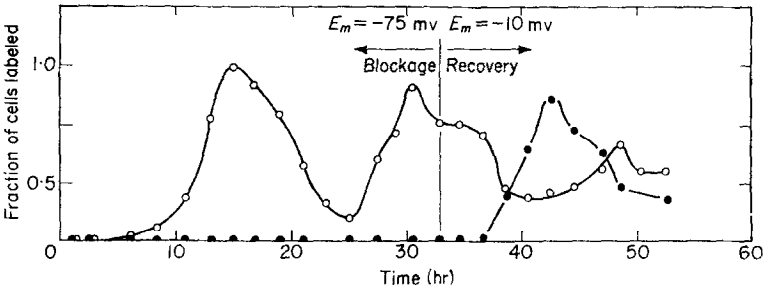

FIG. 5. Effect of simulated E_m level of -75 mv on DNA synthesis activity in naturally synchronized CHO cells as determined by [^{3}H]thymidine pulse-labeling. Control cells maintained in normal culture medium. ○———○, Control; ●———●, test.

have also revealed that it is specifically the decreased intracellular concentration of the Na^+ associated with the high E_m level that is the active agent in effecting the mitotic blockage, rather than either $[K^+]_i$ or $[Cl^-]_i$.

(C) CONCLUSIONS

In view of the foregoing results, it can be concluded that the premise that mitotic activity and associated DNA synthesis might be blocked by intracellular ionic conditions accompanying high E_m levels is in fact valid for the CHO cell line. Since the CHO line is generally representative mitotically of a wide range of somatic cell types in culture, it is quite reasonable to expect that all other such cell lines will exhibit the same mitotic blockage in response to imposition of simulated high E_m conditions. The positive results of the experimental study with the CHO line in culture therefore give full credence to the present theoretical concepts regarding E_m level variations as a fundamental mechanism of mitosis control, and provide a basis for designing extended investigations of *in vivo* systems. In view of the results obtained for cells *in vitro*, it seems entirely feasible that natural variation of the E_m level of somatic cells could serve as an effective mechanism of mitosis control *in vivo*. The fact that E_m level variation *in vitro* blocks mitosis by prevention of prior DNA synthesis indeed suggests that this blockage mechanism is the same as that naturally occurring *in vivo*, where DNA synthesis prevention is well known to be the mechanism of mitotic blockage.

(D) E_m LEVEL CHANGES ASSOCIATED WITH MITOTIC CONTACT INHIBITION OF CONFLUENT CELL MONOLAYERS

In other recent studies (Cone, 1970, unpublished data) with CHO cells, it has been found that the E_m level of -15 mv of the free cells in monolayer culture increases steadily as the cells approach the mitotically contact inhibited

condition of full confluency, where an E_m of approximately -55 mv maintains. These observations are in full accord with the predictions of the present theory, and again demonstrate the significant dependency of E_m level on surface contact conditions. The possibility is thus clearly suggested that the basis of the mitotic contact inhibition commonly observed in monolayer cell populations *in vitro* at confluency is the result of E_m level mediation through cell surface contact. Similar observations on the resumption of mitotic activity in CHO cells moving into a cleared swath within a previously confluent monolayer have revealed in accord with the present theory, that the reverse condition maintains, viz. E_m levels decrease from -55 mv in the mitotically quiescent cells in the still confluent region to -15 mv in the free dividing cells which have migrated into the swath. In view of these *in vitro* results with normal cells, the central importance of the cell surface aberrations in malignant cells (which exhibit contact inhibition of neither motility nor mitosis *in vitro*), as emphasized in the foregoing theory of oncogenesis is made quite clear.

5. Some Additional Observations Relating Mitotic Activity with the Transmembrane Potential Level

In addition to the specific experimental results just outlined, a wide range of other observations exists which clearly demonstrate or imply a relationship between mitotic activity and E_m level. A few of the most pertinent of these are briefly cited here.

(A) EXPERIMENTAL CORRELATIONS OF E_m LEVEL WITH MITOTIC ACTIVITY

Mitotic stimulation by high $[Na^+]_0$

Several sources of experimental data imply that Na^+ concentrations in excess of those normally used in various culture media have a pronounced mitotic stimulatory effect *in vitro*. Hypotonic pulsation of synchronized, monolayered mouse fibroblasts using a variety of salt solutions (Cone, 1969*a*) produced a striking increase in mitotic synchronization and shortening of interphase when NaCl was used, but no effect when KCl, $CaCl_2$ or H_2O were used. Similar results were obtained with pulsed exposure to concentrated NaCl solutions of an aquatic phycomycete (Olson, 1969, personal communication). Gaulden (1956) also found an increased rate of DNA synthesis induced in grasshopper neuroblasts by media made hypertonic by extra salt addition.

Mitotic stimulation by cell surface treatments

Trypsin, which acts upon the cell surface to disrupt intercellular bonds (Rous & Jones, 1916; Northrop, 1926; Rinaldini, 1958), has been noted to produce resulting acceleration of mitotic activity (Simms & Stillman, 1936*a*,*b*;

Medawar, 1941; Pace, Aftonomos & Arthur, 1959). Interestingly, it has been found by direct E_m measurement (Cone, 1969*a*) that trypsin treatment of monolayered fibroblasts *in vitro* produces an increase in cell volume and a further decrease in the E_m level (from -10 to -3 mv), followed by a shortening of interphase and an increase in the degree of mitotic synchrony in populations previously synchronized with 5-aminouracil.

Mitotic blockage by cell surface treatments

Mucopolysaccharides and allied compounds (e.g. glycoproteins) are constituents of cell surfaces in general, and appear to have an immunological role in somatic cells similar to that in bacteria (Davies, 1959). The ion-exchange properties of these surface polymers (Katchalsky, 1964) suggests their possible involvement in E_m generation and regulation mechanics, and such activity has been observed. Heparin, for example is capable of inducing cardiac arrest (Regelson & Holland, 1958) and the glycoproteins are intimately associated with nerve function (Bogoch, 1968). Significantly, heparin, and similar polysaccharides are potent inhibitors of mitosis (Regelson, 1968; Lippman, 1955; Shear, 1947). Other E_m influencing agents (e.g. diphenylhydantoin) also possess mitotic alteration capabilities (Bogoch, 1969).

E_m *mitosis correlations in oogenesis*

Results of a study by Maéno (1959) on E_m changes in the oocyte of the toad yields a picture quite in agreement with the present theory. During the resting period, the oocyte maintains an E_m level of -70 mv (the same as in nerve). Upon reaching the mature egg stage, the E_m drops to only -12 mv.

Activation of DNA synthesis in nuclei of mature neurons

Gurdon (1968), by nuclear transplantation techniques, was able to stimulate DNA synthesis in the nucleus of a mature frog neuron by transplantation to an enucleated frog egg. In view of the E_m level of toad eggs found by Maéno (1959) and other foregoing results, the DNA synthesis stimulating agent might well have been the increased $[Na^+]_i$ of the egg relative to that in the nerve cell from which the nucleus came.

(B) EVOLUTIONAL IMPLICATIONS FOR E_m CONTROL OF MITOGENESIS

The experimental evidence and precepts of classical membrane theory on which the present concept of mitogenesis control is based, wherein Na^+ is the primary actively transported ion, imply that Na^+ plays a central role in the mitogenic process. $[K^+]_i$ and $[K^+]_i/[Na^+]_i$ are no doubt also important (perhaps even dominant in some systems). They do not appear explicitly in the theory because their value is assumed to follow as a direct consequence of

the E_m and $[Na^+]_i$ levels, the extracellular $[K^+]_o$ and $[Na^+]_o$ being very closely maintained at constant values by regulatory mechanisms, *in vivo*. One non-dividing cell system is in fact known in which K^+ is the primary actively transported ion (Tosteson, 1963). Ca^{2+} has long been considered a key ion in mitogenesis by numerous investigators; in terms of the present theory Ca^{2+} may still play an essential role, but primarily through its influence on the membrane's permeability to Na^+ (and hence on E_m level) or more directly by intracellular action following its release, or binding, by ion-exchange reactions with Na^+ or K^+ (Cone, 1969*a*).

From an evolutional point of view, it appears only logical to expect that Na^+ might play a key role in mitogenesis. If we postulate that life originated as replicative unicellular entities in the primeval oceans, where Na^+ presumably was by far the cation present in greatest abundance (as is true today), it would be a situation of the highest evolutional and survival value if the division and multiplication of such entities were to be positively stimulated by the omnipresent Na^+. Under such free-cell conditions, the E_m level would presumably have been low (as it is for free somatic cells in culture today), and hence the $[Na^+]_i$ relatively high, with consequent stimulation of DNA synthesis and division. As these primitive entities differentiated and it became possible (and evolutionally advantageous) for functional aggregation into multicellular forms, the need arose for specific morphogenesis with its attendant requirement for precise mitotic control. Consequently, the cell surface specialization required for formation of specific functional aggregates was presumably accompanied by the ability to generate substantial E_m levels by active Na^+ transport and thus to regulate $[Na^+]_i$ and division or mitosis accordingly. In this manner, the multicellular organism evolved the ability to control (locally and generally) its mitotic activity while maintaining (extra cellularly) much the same ionic environment as existed during the basic morphological and metabolic evolution of the original cell in sea water.

Extending this supposition to its conclusion, it might be surmized that the highly differentiated and functionally specialized nerve and muscle cells ultimately arose from the E_m generation capability initially developed for mitosis control. There are, in fact, many interesting electroosmotic similarities between nerve and muscle function, and mitogenesis. In this connection, it may also be significant that much experimental evidence exists for a relationship between proliferation and nervous activity (e.g. Singer, 1952; Overton, 1950).

Although existing experimental evidence and the present theory imply a central role for E_m and, more specifically, for the relative and absolute $[Na^+]_i$ and $[K^+]_i$ in mitogenesis control, it is not intended to suggest herein that E_m variation is the *only* mechanism of somatic mitotic control operative.

Indeed, many physical and chemical factors are either mitoinductive or mitorepressive and in specific instances it may be the activity of such agents which is involved in the immediate and specific control of mitosis. For example, organic agents or metabolites of external origin could conceivably act to block essential metabolic pathways even when the cell E_m level was low, or to bypass mitotic blockage mechanisms at high E_m levels. However, there is much evidence indicating that, under *natural* somatic conditions, E_m level and mitotic activity are related in a wide range of cell types and in many cases the activity of many natural (e.g. hormones, wound healing) and pathological (carcinogenic viruses and chemicals) mitotic agents may actually be mediated through direct or indirect influence on the E_m level.

(c) Other experimental approaches for determining the generalized applicability of the theory

A number of excellent cell systems exist for further investigation of the validity and generalized applicability of the theory. These fall into two classes. cells which are naturally mitotically quiescent and which can be induced to divide by suitable stimuli (e.g. uterine and mammary proliferation by estrogenic action, carcinogenic action in many somatic tissues, liver regeneration, wound-healing and the like) and naturally proliferative cells which stop dividing by natural means (e.g. cessation of division in regeneration and wound healing, and in maturation of cells of various developing embryonic tissues). By measurement of the E_m level (and associated ionic concentrations) and correlation with the mitotic activity in a range of such cell sytems it should be possible to clearly define the degree of general applicability of the present theory.

6. Concluding Remarks

The primary theoretical concept of mitogenic control advanced herein is based on the experimental observation that a correlation exists between the electrical transmembrane potential level and the degree of mitotic activity for a substantial range of somatic cell types. The precepts of conventional membrane potential theory invoked in the present considerations state that variations in the E_m level are but a consequence of corresponding shifts in the steady-state ionic balance of the cell, thus implying that the observed mitotic effects accompanying E_m level changes are mediated through changes in the intracellular ionic hierarchy (principally in $[Na^+]_i$ and $[K^+]_i$). The role proposed herein for ionic shifts as a basis for mitogenic regulation thus stems from conventional membrane potential theory, and its validity rests to this extent upon the validity of the conventional theory in regard to overall ion

balance mechanics. The fact that E_m simulation experiments designed on the basis of conventional membrane theory (Cone & Tongier, 1970) have yielded positive results may in fact be interpreted as experimental support of the validity of the latter. Although little is presently known experimentally regarding the actual molecular mechanisms by which blockage or stimulation of DNA synthesis and mitosis by such ionic changes (particularly those of Na^+) are mediated, some generalized conceptual biophysical and biochemical possibilities have been proposed herein.

The present theory places prime emphasis upon the cell surface because of the apparently central importance of the surface in E_m generation mechanics. Unfortunately, relatively little is known at the fundamental molecular level about the factors determining the effective values of g_{Na} and J^o_{Na}, and hence the E_m level. This is particularly true of contact phenomena of cell surfaces and their involvement in E_m generation. If the precepts of the present theory prove to be generally valid, they place a high priority upon gaining a comprehensive functional understanding of E_m generation and level-determination mechanics at the molecular level, an understanding from which effective means for controlling normal and abnormal mitogenesis might ultimately be developed.

The theory of malignancy developed herein appears to offer some interesting and potentially important insights into the functional relationships between immunological-electrophysical surface aberrations, and the excessive mitotic activity characteristic of malignant cells, and leads to a unifying interpretation of viral, chemical, and physical carcinogenesis.

REFERENCES

ABERCROMBIE, M. & AMBROSE, E. J. (1962). *Cancer Res.* **22,** 525.
ALEXANDER, P. (1966). In *Biology of Cancer*, Chap. 6. (E. J. Ambrose & F. J. C. Roe, eds). London: D. Van Nostrand Co. Ltd.
AMBROSE, E. J. (1966). In *Biology of Cancer*, Chap. 4. (E. J. Ambrose & F. J. C. Roe, eds). London: D. Van Nostrand Co. Ltd.
BALAZS, E. A. & LAURENT, T. C. (1951). *J. Polymer Sci.* **6,** 665.
BALITSKY, K. P. & SHUBA, E. P. (1964). *Acta Un. int. Canc.* **20,** 1393.
BASERGA, R. (1965). *Cancer Res.* **25,** 581.
BELL, L. G. E. (1962). *J. theor. Biol.* **3,** 132.
BOGOCH, S. (1968). *The Biochemistry of Memory*. New York: Oxford University Press.
BOGOCH, S. (1969). *Bibliography on Biological Effects of Diphenylhydantoin*. New York: Dreyfus Medical Foundation.
BRANDT, P. W. (1958). *Expl. Cell Res.* **15,** 300.
BUNCH, W. H. & KALLSEN, G. (1969). *Science, N.Y.* **164,** 1178.
COMAN, D. R. (1944). *Cancer Res.* **4,** 625.
CONE, C. D., JR. (1969*a*). *Trans. N.Y. Acad, Sci.* **31,** 404.
CONE, C. D., JR. (1969*b*). *J. theor. Biol.* **22,** 365.
CONE, C. D., JR. (1970*a*). *Twelfth Science Writers Seminar, A. Cancer Soc.* San Antonio, Texas.

CONE, C. D., JR. (1970*b*). *J. theor. Biol.* **30,** 183.
CONE, C. D., JR. & TONGIER, M., JR. (1970). *Oncology.* **24,** (in the press).
COPE, F. W. (1967). *J. gen. Physiol.* **50,** 1353.
DAVIES, D. A. L. (1959). *Proc. R. phys. Soc.* Edinb. **28,** 79.
DORFMAN, A. (1963). *J. Histochem. Cytochem.* **1,** 2.
GAULDEN, M. E. (1956). In *Mitogenesis,* p. 44. Chicago: University of Chicago Press.
GURDON, J. B. & WOODLAND, H. R. (1968). *Biol. Rev.* **43,** 233.
GURDON, J. B. (1968). *Sci. Am.* **219** (6), 24.
JOHNSTONE, B. M. (1959). *Nature, Lond.* **183,** 411.
KATCHALSKY, A. (1964). *Biophys. J. Pt.* 2 Suppl. **4,** 9.
KUSHMERICK, M. J. & PODOLSKY, R. J. (1969). *Science, N.Y.* **166,** 1297.
LINDNER, A. (1959). *Cancer Res.* **19,** 189.
LING, G. N. (1962). *A Physical Theory of the Living State.* New York: Blaisdell Publishing Co.
LIPPMAN, M. (1955). M.Sc. Thesis, University of Pennsylvannia.
LOSICK, R. (1969). *J. molec. Biol.* **42,** 237.
LOSICK, R. & ROBBINS, E. (1969). *Sci. Am.* **221** (5), 121.
MAÉNO, T. (1959). *J. gen. Physiol.* **43,** 139.
MATHEWS, M. (1953). *Arch. Biochem. Biophys.* **43,** 181.
MAZIA, D. (1961). In *The Cell,* Vol. 3. (J. Brachet & A. E. Mirsky, eds). Chap. 2. New York: Academic Press.
MEDAWAR, P. B. (1941). *Nature, Lond.* **148,** 783.
MOSCONA, A. & MOSCONA, H. (1952). *J. Anat.* **86,** 287.
MYSELS, K. J. (1959). *Introduction to Colloid Chemistry.* New York: Interscience.
NORTHROP, J. H. (1926). *J. gen. Physiol.* **9,** 497.
OVERBEEK, J. T. G. (1952*a*). In *Colloid Science,* Vol. 1. (H. R. Kruyt, ed.). New York: Elsevier.
OVERBEEK, J. T. G. (1952*b*). In *Colloid Science,* Vol. 1. (H. R. Kruyt, ed.). New York: Elsevier.
OVERTON, J. (1950). *J. exp. Zool.* **115,** 521.
PACE, D. M., AFTONOMOS, L. & ARTHUR, W. M. (1959). *J. natn. Cancer Inst.* **23,** 655.
REGELSON, W. & HOLLAND, J. F. (1958). *Nature, Lond.* **181,** 46.
REGELSON, W. (1968). *The Antimitotic Activity of Polyanions (Heparin and Heparinoids).* Richmond, Va.: Medical College of Virginia.
RINALDINI, L. M. J. (1958). *Int. Rev. Cytol.* **7,** 587.
ROUS, P. & JONES, F. S. (1916). *J. exp. Med.* **23,** 549.
SHAEFER, H. & SCHANNE, O. (1956). *Naturwissenschafften* **43,** 445.
SHEAR, M. J. (1947). In *Approaches to Tumor Chemotherapy.* (F. R. Moulton, ed.) p. 236. Washington, D.C.: American Association for the Advancement of Science.
SIMMS, H. S. & STILLMAN, N. P. (1936*a*). *J. gen. Physiol.* **20,** 603.
SIMMS, H. S. & STILLMAN, N. P. (1936*b*). *J. gen. Physiol.* **20,** 621.
SINGER, M. (1952). *Q. Rev. Biol.* **27,** 169.
TOKUOKA, S. & MORIOKA, H. (1957). *Gann* **48,** 353.
TOBEY, R. A., PETERSEN, D. F., ANDERSON, E. C. & PUCK, T. T. (1966). *Biophys. J.* **6,** 567.
TOSTESON, D. C. (1963). *Fedn Proc. Fedn Am. Socs exp. Biol.* **22,** 19.
WARBURG, O. (1924). *Biochem. Z.* **152,** 309.
WEISS, P. (1956). In *Mitogenesis,* p. 44. Chicago: University of Chicago Press.