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Variation of the Transmembrane Potential Level as a Basic Mechanism of Mitosis Control

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Abstract. A range of experimental observations suggests that a significant correlation exists between the level of the electrical transmembrane potential difference in somatic cells and the degree 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 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 DNA synthesis and other essential preparations for mitosis. 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 [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, senescence, and malignancy.

In a recent paper, CONE [1969a] 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 electroosmotic 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 and TONGIER, in press] 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 to substantially expand and elaborate the basic concepts of the original paper [CONE, 1969a] 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. 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. General 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)[1], 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 devoid of mitoses [WEISS, 1956][2]. This mitotic quiescence has generallly 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 —40 to —50 mV, and mitotic activity is very low (mitotic coefficients $\simeq 0.03$). 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

[1] 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.

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

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. nontumorigenic) 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 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 demonstrated by nuclear transplantation experiments [Gurdon and 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 and Schanne, 1956; Tokuoka and Morioka, 1957; Johnstone, 1959], this decrease being accompained 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 nondividing muscle cells was found to have undergone a decrease to only —10 mV in the actively proliferating homologous sarcoma cells [Balitsky and 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.[3] In malignant transformation a prime alteration is the decreased adhesive binding of the transformed cells [COMAN, 1944], thus leading to invasiveness and metastasis, the primary pathological characteristics of medical malignancy, and indicating a pronounced 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 (—30 mV to —60 mV) and intermediate proliferation rates, thus further reenforcing 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 endpoints 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 polarization in nerve and muscle cells,

[3] 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.

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. Theoretical Precepts of 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 appreciable magnitude. In addition to these general observations, a number of more quantitative, specific indications (to be discussed in section 4) exist which strongly imply a definite functional relationship between E_m level and mitotic activity. Of primary importance among these is the demonstration [Cone and Tongier, in press] that imposition of intracellular ionic conditions approximating those at an E_m level of —70 mV (equivalent to the case of nondividing nerve) *reversibly* blocks DNA synthesis and mitosis *in vitro*.

On the basis of such general 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[1],

[1] 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 and Kallsen, 1969; Kushmerick and Podolsky, 1969] and other observed electroosmotic characteristics of cells. The present theoretical development of mitosis control is based in its major perspectives on the precepts of conventional membrane 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, which may be taken as a generalized theory of electroosmotic regulation of somatic cell mitosis, is subsequently applied in section 4 to the interpretation of a series of additional mitotic observations.

3.1 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 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)[5], 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 purpose of the present development is 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 inorganic ions (in terms of conventional theory). The result of this latter alteration is that for osmotic balance, the total particle content of nonpermeating 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, 1969a]. 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

[5] 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.

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 3.4).

3.2 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 [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, 1969b] 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 possiblities 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^{++}) could exert an influence at the most basic level, possibly by regulation of enzyme m-RNA transcription. Such action might take place through influence of the ionic environment directly on the release or binding of repressor 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 $[K^+]_i/[Na^+]_i$ ratios) which surround specific large enzyme molecules [Overbeek, 1952a] might exert considerable influence on the relative activity of 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 consequent alteration of enzyme activity. The effects of ionic environment on the steric conformation of various macromolecules are well known [Mysels, 1969; Balazs and 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, 1952b], 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^{++} and Mg^{++} 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 [TOBEY *et al.*, 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.

3.3 Possible Mechanisms for E_m-Mediated Control of Prophase Initiation (G_1 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, 1969a]. 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 electroosmotic 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.

3.4 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 aspects of cellular metabolism, the question of how the E_m level is itself 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 E_m level then follows directly as a consequence of these interactions.

A proposed system of such feedback interactions which illustrates the essential relationships involved is outlined in figure 1. For clarity, only the primary feedback loop is shown, although a host of secondary

interactions obviously exists. This model, while hypothetical, is constructed on the basis of a wide range of suggestive experimental evidence, particularly in regard to the relation between cell surface conditions and mitosis.

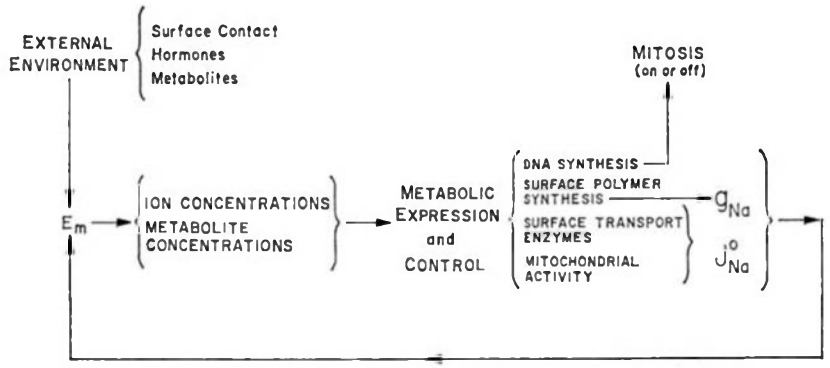

Fig. 1. Schematic flow chart illustrating the proposed system of feedback interactions whereby the intracellular ion hierarchy associated with E_m acts to influence cellular metabolic activity concerned with DNA synthesis, surface polymer production and mitochondrial activity. The latter two factors,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

Considering first E_m in the proposed system of figure 1, this factor (or, more correctly, 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 enzymatic 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 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 early G_1. If the E_m level is sufficiently high, DNA synthesis and mitosis will not take place and the cell will remain in G_1 as long as the high E_m level is maintained. Upon reduction and 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 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 and intercellular associations of the cell surface polymers, properties which in turn are determined by the particular metabolic expression of the cell and the immediate molecular environment. Of particular interest here are the mucopolysaccharides and similar saccharide polymers (collectively denoted herein as MPS) known to be associated with the cell surface [RINALDINI, 1958; BRANDT, 1958; BELL, 1962; DORFMAN, 1963]. For example, the surface MPS appear to be a primary functional agent in immunological expression [MOSCONA and MOSCONA, 1952; DAVIES, 1959], and it is well established that immunologically active changes in the cell surface accompany malignant transformation in cells [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 are accompained by the previously described drop in E_m level, with simultaneous increase in proliferative activity. The E_m changes presumably are associated with changes in the effective values of g_{Na} (and/or J^o_{Na}) produced by the surface alterations, either directly or indirectly; increased permeabilities of malignant cells have been recorded [ABERCROMBIE and AMBROSE, 1962]. J^o_{Na} as well as g_{Na} may be effectively altered by such surface changes, as evidence exists that 'active transport' is associated with pumping systems situated within the cell surface complex [TOSTESON, 1963]. As will be discussed, a drop in E_m due to an increase in g_{Na} might also influence J^o_{Na} through alteration of the energetics machinery of the cell [CONE, 1969a], 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 J^o_{Na}) depend not only upon the direct chemical structure of the surface polymers, but also on their conformational arrangement in the surface

complex and their interaction with the surface polymers of adjacent cells.

At this point, it is instructive to consider some basic aspects of malignancy in regard to the involvement of the cell surface in mitogenesis, and to examine some basic potential mechanisms of the cytogenetic etiology of carcinogenesis. Of pertinent interest 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 virus types are carcinogenic and produce surface antigenic changes in transformed somatic cells, it appears entirely possible (by analogy with the bacterial case) that their action could be due to a redirection of MPS or other surface polymer metabolism, with a resultant altered surface composition producing decreased surface adhesion and lowered E_m, with the characteristic attendant increase in mitotic activity.

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 carcinogenic viruses, the viral coat appears to possess constituents very much like those of the somatic cell surface. This fact suggests that those aspects of cellular metabolism concerned specifically with surface polymer synthesis are, among others, altered or redirected by these viruses, thus preventing normal surface polymer production and/or superposing foreign polymer forms which 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 could result in alteration of the E_m level. For example, in the case of the Rous Sarcoma Virus (RSV), the viral genome is unable to produce a functional viral coat, presumably due to viral-directed synthesis of abnormal coat polymers. Such abnormal polymers, together with alteration of the normal surface polymer synthetic pathways, could 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 (4 or 5), 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 and 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 such a 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 acts to stably maintain the new metabolic pathways, which will then continue to produce the initial altered polymer or subsequent deviate forms [Cone, 1969a]. 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.[6] 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 nonlysogenic viral, carcinogenesis thus providing a unifying explanation of carcinogenesis 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,

[6] 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.

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[7] 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. 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 figure 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

[7] 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 envisioned as undergoing a complex train of metabolic oscillations which gradually dampen out as the new stable state is reached.

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, 1969a], mitochondria 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^o_{Na} and E_m level.

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 another possible 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 mitochondrial membrane surfaces, thereby accomplishing a reduction in oxidation phosphorylation, J^o_{Na}, and consequently E_m. 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 figure 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 on 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 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 cytogenetically induced in such a manner that it becomes selfsustaining.

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°_{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°_{Na}), and hence in the E_m level. Also, as is well known in colloid science [OVERBEEK, 1952a], 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 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°_{Na}. For example, the local concentration of such divalent cations as Ca^{++} 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 $[Ca^{++}]_o$ exert a pronounced effect on the g_{Na} level in nerve; specifically, higher $[Ca^{++}]_o$ 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 $[Ca^{++}]_o$ 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 and 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.[8] The characteristic surface antigenicity and other surface abnormalities of malignant cells are again of particular interest 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 metabolically-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.

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, 1969b], one cell must divide for each cell which dies, for mitotic homeostasis. For maintenance of mature tissue morphology, it would

[8] 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.

appear particularly desirable for division 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].

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 and that even the various mitotic preparatory pathways proceed independently in many respects [MAZIA, 1961].

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

In addition to the general observations of Section 2, several sources of more specific results exist which demonstrate or imply a relationship between mitotic activity and E_m level. A few of the more pertinent of these are cited here.

4.1 E_m-Level Simulation Experiments

The results obtained in E_m simulation experiments with Chinese hamster somatic cells *in vitro* [CONE and TONGIER, in press] constitute one of the most conclusive sources of experimental evidence demonstrating a direct E_m-mitotic activity relationship. It was found in these tests that culture medium compositions designed to impose intracellular ionic conditions simulating high E_m levels ($\sim$—70 mV) reversibly blocked DNA synthesis and mitosis, while lower levels (in the neighborhood of those which normally exist for these cells in culture $E_m \cong$ —10 mV) produced maximum proliferation rates. Intermediate levels (—40 to —60 mV) produced intermediate proliferation rates. The results of these experiments, specifically designed to test the precepts of the present theory in regard to an arbitrary, mitotically representative somatic cell type, demonstrate clearly that conditions approximating those of high E_m levels do in fact block DNA synthesis and mitosis (equivalent to a G_1 mitotic block *in vivo*), and that mitosis actively resumes when the E_m level is again lowered sufficiently. These findings that high E_m levels introduce reversible G_1 mitotic blockage in somatic cells *in vitro* lend considerable credence to the proposition developed herein that natural modulation of cellular E_m levels *in vivo* may constitute a basic mechanism for mitosis control *in vivo*.

4.2 Mitotic Stimulation by High $[Na^+]_o$

Several sources of experimental data imply that Na^+ concentrations in excess of those normally used in various culture media have a stimulating effect on mitotic activity *in vitro*. Although it has not been proven that the sodium ion *per se* is the primary active agent in all of these cases (since other cation and anion concentrations were often simultaneously varied), it appears significant that mitotic activity is stimulated by an increased $[Na^+]_o$, in accordance with the predictions of the present theory (wherein a high $[Na^+]_i$ and an increased intracellular particle content accompany low E_m levels). Hypotonic pulsation of synchronized, monolayered mouse cells in a variety of salt solutions [CONE, 1969a] produced a striking increase in the mitotic synchronization and a shortening of interphase when NaCl was used, but no mitotic effect when KCl, $CaCl_2$, or H_2O alone were used. Likewise, continuous growth of naturally synchronized CHO cells grown in spinner culture in normal F-10 culture medium supplemented with 20% additional Na^+ (as NaCl) showed a pronounced shortening of interphase. Following attainment of improved synchronization

with the Na^+ pulsation technique in mouse cells, an attempt was made [Olson, 1969] to improve the temperature-cycling mitotic synchronization of the aquatic phycomycete *allomyces cystogenes*, by a short, pulsed exposure to concentrated NaCl solution (1 M) in interphase. The result was an increase in synchrony from 20% (obtained with temperature cycling) to 82% (Percentage of cells in a given mitotic state at a given instant). Other studies have shown that culture media having tonicity levels considerably above normal (obtained by use of balanced higher concentrations of salts, particularly NaCl) increase the rate of DNA synthesis and decrease the length of interphase in grasshopper neuroblasts [Gaulden, 1956].

It should be noted, however, that in the case of naturally-generated E_m level changes, a change in $[Na^+]_i$ is presumably always accompanied by a change in $[K^+]_i$, so that in some cases it may really be the decrease in the latter rather than an increase in the former which is the mitotically active agent. Since the existence of K^+ pumps has been documented [Tosteson, 1963], it is possible that a primary mitogenic role may be played by K^+ (or even Cl^-) in some systems.

4.3 Mitotic Stimulation by Cell Surface Treatments

Trypsinization of living tissue has long been used as a method for preparation of free cell suspensions [Rous and Jones, 1916; Rinaldini, 1958]. Trypsin digestion apparently breaks protein-associated bonds in the cell surface molecular complex which are directly or indirectly responsible for binding the cells together. As noted previously, a primary result of such dissociation is a pronounced drop in E_m level and an initiation of active proliferation. In addition, however, a number of investigators have noted an acceleration of cell proliferation associated with the use of trypsin *per se* in the normal culturing medium [Simms and Stillman, 1937a, b; Medawar, 1941; Pace, Aftenomos and Arthur, 1959]. Interestingly, it has been found by direct E_m measurement [Cone, 1969a] that trypsin treatment of monolayer cells *in vitro* produces an increase in cell volume and an additional depolarization beyond that accompanying initial dissociation during adaptation to culture, and that use of trypsin in culture media leads to an increase in the degree of mitotic synchrony of L-cells much like the effect observed with Na^+ pulsations. Presumably, this depolarization results from trypsin action on the cell surface, since trypsin does not normally penetrate into the cell [Northrop, 1926]. On the basis of the present theory, depolarization should lead to an increase in cell volume

as well as an increase in $[Na^+]_i$, so that the observed effects of trypsin in promoting proliferative activity may be due, at least in part, to depolarization resulting from trypsin action on the (free) cell surface. PACE *et al.* [1959] found that pretreatment of culture serum alone with trypsin was practically as effective mitotically as direct addition to the culture medium, thus implying an effect due to serum modification by the trypsin. However, other interpretations of this result are possible; e.g. the available Na^+ content of serum may vary widely depending upon its source and state and additional Na^+ may be added by the trypsin itself, so that the observed effect may have been one of increased $[Na^+]$ in the test medium.

4.4 Mitotic Blockage by Cell Surface Treatments

There is appreciable evidence that mucopolysaccharides and allied compounds (e.g. glycoproteins) are constituents of cell surfaces in general (see references previously cited), and since these polymers are also a basic component of most intercellular matrix materials it seems reasonable to assume that they are situated at the outermost region of the surface molecular hierarchy. This premise is certainly true if, as appears to be the case, the glyoproteins of somatic cells play the same immunological role that they do in bacteria [DAVIES, 1959]. The well documented ion-exchange properties of these polymers [KATCHALSKY, 1964], along with their surface location on the cell, suggests their possible basic involvement in E_m generation mechanics and regulation. Evidence does in fact exist that such natural polymers intimately influence the electrical properties of excitable cells, presumably by action at the cell surface. For example, heparin is capable of inducing cardiac arrest [REGELSON and HOLLAND, 1958], and the glycoproteins are intimately associated with CNS nerve [BOGOCH, 1968]. It thus appears significant, in light of the present theory, that heparin (and other polysaccharides) are also potent inhibitors of cell division [REGELSON, 1968; LIPPMAN, 1955]. Although the actual molecular mechanism(s) by which heparin blocks division has apparently not been investigated, it is reasonable to speculate that blockage is associated in some way with the alteration of the electroosmotic balance of the cell. Another example of a mucopolysaccharide having antimitotic activity is Shear's polysaccharide [SHEAR, 1947]. In view of the antigenic nature of somatic cell surfaces, it might be expected that a given polysaccharide would be quite selective in its mitotic action. In addition, other classes of compounds having known effects on mem-

brane electrical properties (e.g. diphenylhydantoin) also possess mitotic influencing activity [BOGOCH, 1969].

4.5 E_m-Division Correlations in Oogenesis

A comprehensive experimental study of the E_m levels in maturing oocytes of the toad [MAÉNO, 1959] yields a picture quite in agreement with the precepts of the present theory. It was found, for example, that resting oocytes maintain an E_m level of —70 mV (significantly, the same high level as in nerve). Detailed experiments demonstrated that the ionic hierarchy of the oocytes was essentially the same as found in nerve and muscle, viz. that Na^+ was the actively transported ion while K^+ was passively distributed. Upon hormonal stimulation, and passage of the oocyte on through the maturation process, the E_m level continuously decreased, ultimately reaching a stable level of —12 mV in the mature egg *in vivo*. (Presumably, a similar condition exists in frog eggs and may explain the source of GURDON and WOODLAND's [1968] neuron nucleus reactivation, following transplantation and egg activation.)

Interestingly, the E_m level of the mature egg, unlike that of the oocyte, was found to be maintained by an apparently equal participation of Na^+ and K^+ in the active transport system, with Cl^- being the primary passively distributed ion. This change in E_m properties was associated with a number of morphological changes in the cell surface including development of the vitelline layer and an increased plasticity of the cell membrane. The membrane electrical resistance of the egg was some eight times that of the resting oocyte. Upon activation of the egg in fresh water (as in fertilization or initiation of parthenogenesis), the E_m rose to a level of +90 mV over a period of a few minutes. This increase in positive potential in fresh water (essentially free of Cl^-) presumably demonstrates that the egg, following activation, is acting to concentrate Na^+ and K^+ so as to substantially raise their intracellular levels; this rise in intracellular cation levels is accompanied by the initiation of mitosis in the egg. It is interesting to note in this regard that exposure of such eggs to hypertonic saline solutions can also stimulate parthenogenesis, implying an elevation in the $[Na^+]_i$. This same stimulatory effect of Na^+ increase (or K^+ decrease) on division of somatic cells *in vitro* has already been cited. These results appear to demonstrate a particular specialization of the toad egg (which will have to undergo activation in fresh water) for active transport of Na^+ (and K^+) *into* the cell. The ability of frog

skin to actively transport Na^+ from fresh water into the animal's blood system is well known.

4.6 Activation of DNA Synthesis in Nuclei of Mature Neurons

Somatic cells of most developing tissues undergo a pronounced decrease in mitotic activity as the state of full differentiation and maturation is reached. In the case of central nervous system neurons, mitosis ceases completely in the fully differentiated cell. This cessation of all mitotic activity in nerve has raised the question of whether the blockage is truly permanent, being the result of some irreversible aspect of differentiation, or whether it is maintainned by some potentially reversible mechanism (such as the E_m level change of the present theory) which could be activated under proper conditions. Experiments using nuclear transplantation techniques have shown conclusively that the latter alternative is true [GURDON, 1968]. Nuclei of mature frog neurons, upon transplantation to enucleated frog eggs, were found to undergo full resumption of DNA synthesis, preparatory for mitosis. The interpretation of this result, in terms of the present theory, is that DNA synthesis in the neuron nucleus is continuously blocked by the intracellular conditions accompanying the high E_m level, but is fully activated upon exposure to the changed ionic conditions accompanying the equivalent low E_m level of the (activated) egg. The absolute constancy with which the high E_m level of the neuron is maintained thus appears to be a key reason for the absence of mitotic activity. The apparent control of DNA synthesis by E_m level as demonstrated in this experiment is, significantly, in full accord with the results of the E_m-simulation experiments previously described [CONE and TONGIER, in press].

4.7 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. As already discussed, the $[K^+]_i$ and the $[K^+]_i/[Na^+]_i$ are no doubt also very important (perhaps even dominant in some systems), although 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 $[Na^+]_o$ and $[K^+]_o$ being very closely maintained at constant values by regulatory mechanisms, *in vivo*. Some nondividing cell systems are known in which

K^+ is the primary actively transported ion [Tosteson, 1963]. Ca^{++} has long been considered a key ion in mitogenesis by numerous investigators; in terms of the present theory Ca^{++} may play an essential role 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, 1969a].

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^+ (and the associated K^+ level). 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 $[K^+]_i$ correspondingly low), 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 (extracellularly) much the same 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 surmised 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 similarities between nerve and muscle function, and mitogenesis in regard to electroosmotic phenomena. In this connection, it may also be significant that much experimental evidence exists for a relationship between proliferation and nervous activity [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 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.

5. Experimental Approaches for Determining the Generalized Applicability of the Theory

Although a variety of general and specific experimental data are available which demonstrate the existence in a reasonably wide range of cell forms of a functional relation between E_m level and mitotic activity, much additional investigation is required in order to determine the generality of applicability of the concept and the extent to which it is actually operative as a basic mechanism of mitosis control in somatic cell systems *in vivo*. Two types of additional data are desirable. The first involves a much more comprehensive and systematic determination of natural *in vivo* E_m levels in dividing cells (e.g. villus crypt, basal epithelium, and hematopoietic blast cells) and in mitotically quiescent cells (e.g. squamous epithelium), as well as for types with intermediate mitotic activity levels (e.g. connective tissue, liver). The second involves detailed experimental determinations of the precise E_m-mitotic activity relationship during periods in which naturally quiescent cells (or cells with a low mitotic index) are induced to enter division by application of proper stimuli, and in which naturally dividing cells are induced, by externally imposed or natural means, to stop dividing. Some examples of suitable systems of the former type are viral and chemical induction of carcinogenesis in any of a range of somatic tissues, hormone induced proliferation (e.g. estrogen-induced mammary and uterine proliferation), adaptation of somatic tissue cells

to proliferation in culture, wounding and healing (e.g. liver regeneration, skin cuts), and mitosis induction in mature CNS neurons. A prime example of the latter type is the E_m-mitotic activity correlation for various cell types over the development period from the early embryonic form to the mature, fully differentiated state.

In addition to these tests for determining the general applicability of the theory, the viral gene-mapping studies suggested in Section 3.4 for determining if one or more genes of carcinogenic viruses are specifically concerned with alteration of elements of the cell surface, and whether such alterations are responsible for the decreased surface adhesion and E_m decrease of the transformed cells, would be most worthwhile from the carcinogenic standpoint.

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 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 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 theory [CONE and TONGIER, in press] 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 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°_{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 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 of 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.

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