ELECTROOSMOTIC INTERACTIONS ACCOMPANYING MITOSIS INITIATION IN SARCOMA CELLS *IN VITRO**

Clarence D. Cone, Jr., Ph.D.

Head, Laboratory of Molecular Biophysics
Langley Research Center
National Aeronautics and Space Administration
Langley Station, Hampton, Va.

INTRODUCTION

The complex hierarchy of molecular activities and interactions that constitutes the metabolic generation cycle of the somatic cell is primarily chemical in nature. Its particular sequencing of events follows innately as a consequence of the chemical cytogenetic programming dictated by that portion of the genome that is active under the particular physiological environment of the cell. The extent, direction, and temporal scheduling of this program, however, are subject also to the regulatory action of factors that are primarily physical in their nature. These physical (or physicochemical) aspects of metabolic regulation can, in particular, affect metabolic pathways and events specifically associated with essential preparations for mitosis, and thus can significantly influence whether or not a cell will divide, i.e., physical factors can serve as effective blocks or stimulants to mitosis. The present paper outlines the results of some recent theoretical and experimental studies which demonstrate a pronounced involvement of electroosmotic activity during the initiation of mitosis in mammalian somatic cells *in vitro* and discusses the interpretation of these results and their suggestive implication that electroosmotic feedback modulation of cytogenetic activity may constitute a mechanism of mitotic control. The implications of the theoretical and experimental results for the possible involvement of electroosmotic abnormalities in malignant proliferation are also discussed, in light of certain characteristic aberrancies of malignant cells.

All experimental results in this paper are based on studies of the L-strain mouse fibroblast, clone L-929, maintained *in vitro*.[1] This particular cell line, although originally derived by Earle[2] from normal tissue, subsequently underwent malignant transformation in culture, and hence is now regarded as a representative sarcoma strain. Although based primarily on observations of the malignant L-strain *in vitro*, the fundamental hypotheses and conclusions presented in this paper in regard to the involvement of electroosmotic mechanisms in the initiation of mitosis are drawn in general terms and have relevancy to normal as well as malignant cells, both *in vivo* and *in vitro*.

*This paper, illustrated with slides and time-lapse films, was presented at a meeting of the Section on December 9, 1968.

THE "ROUNDING-UP" PHENOMENON OF MONOLAYERED CELLS

Description of the Rounding Process

The subject investigation of this paper had its origin in certain time-lapse cinephotographic observations concerning premitotic shape changes of L-strain fibroblasts in monolayer culture. It was observed in these film recordings that, some hours before any intracellular morphological indications of prophase initiation became apparent, a clearly discernible change in cell shape began to occur, with the cell slowly evolving over a period of 2 to 3 hours from its characteristically flat interphase shape to a more thickened, rounded form. This gradual thickening ultimately culminated at prophase in a strikingly abrupt "rounding up" of the cell into a fully spherical form (FIGURE 1).[1] This rapid spheralization of L-strain cells prior to division was also observed in a wide variety of other somatic cell types in monolayer culture and was found to be a characteristic indicator of incipient mitosis. The generality of occurrence of cell rounding *in vitro* and its direct association with the onset of mitosis suggested that the pronounced changes in cell shape might perhaps be indicative of significant alterations occurring generally in the internal physicochemical regime of cells preceding and accompanying

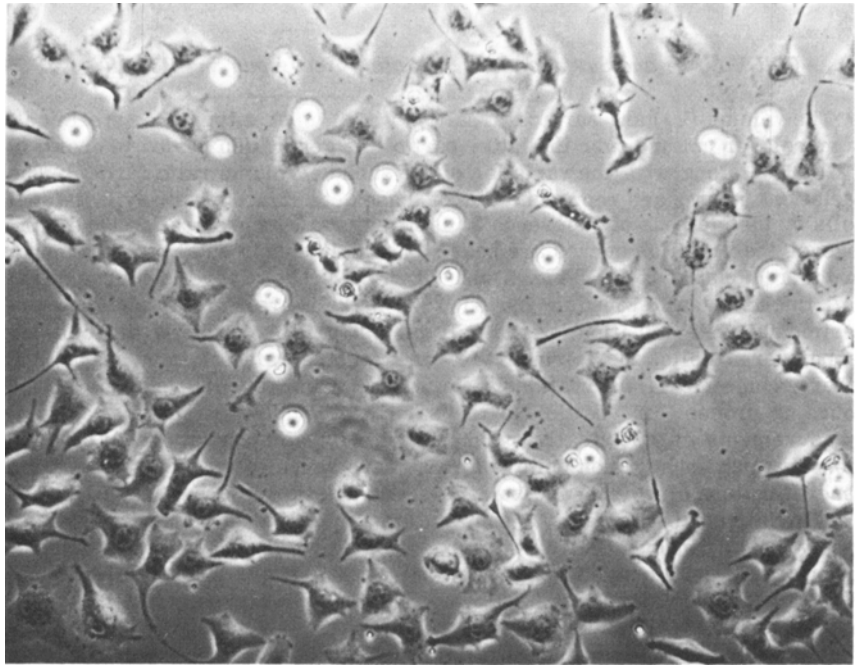


FIGURE 1. A typical monolayer culture of L-strain fibroblasts showing interphase (flat) and mitotic (rounded) cells.

active prophase and, hence, that a study of the phenomenon might shed some light on the processes of mitosis initiation.

The general appearance in time-lapse observations of the thickening and rounding-up process was that the cell was swelling, slowly at first and then at an increasingly rapid rate as the final spherical form was approached. These observations suggested that a decrease in the cellular surface/volume ratio was taking place, leading ultimately to the generation of tension in the pericellular membrane with consequent rounding of the cell. This decrease could be brought about by either a decrease in membrane area or an increase in cell volume, or both; preliminary observations indicated that the cell volume was in fact increasing prior to and during the roundup. A rapid volume increase at cell roundup could, of course, be an indication of significant intracellular reactions tending to increase the osmotic pressure of the cell, reflecting, in turn, a rapid increase in the total free-particle content of molecular and/or ionic species immediately preceding division. To define more precisely the nature and possible mitotic implications of the rounding-up process, a determination of the cell volume and surface-area variation over the *in vitro* generation cycle of the L-strain was carried out. Since theoretical considerations (to be discussed) indicated that cell volume is intimately associated with the transmembrane electrical potential and active ion-transport processes, a determination of the membrane potential variation over the generation cycle was also undertaken to provide information for use in interpreting the volume changes.

Cell Volume and Surface-Area Variation Accompanying Rounding

The volume (V_C) and surface-area (S_C) variation of a typical L-strain cell in monolayer culture over the latter part of the generation period is shown in FIGURE 2, along with the variation of the surface/volume ratio (S_C/V_C). The variations shown are representative of those determined for many individual cells by the methods of Reference 3, using natural synchronization. The fairings of the variation curves are based on actual experimental measurements in the regions of 15–21.5 hours and 23.25–25 hours. Over the course of the cell thickening and rounding period (21.5–23.25 hours), where accurate experimental determination of the cell geometry is exceedingly difficult for monolayered cells, the fairings are based on the rates of form change and other geometrical observations determined from time-lapse films of the rounding process. In addition to the natural-cell determinations, extensive measurements on chemically synchronized L-strain populations in both the monolayer and suspension culture forms[3] have revealed essentially the same general pattern of volume and surface-area variation as shown in FIGURE 2. The variations thus appear to constitute a general mitotic characteristic of *in vitro* cells, independent of the mode of culture. Furthermore, studies of mitotic cells within fixed tissue sections in which the *in vivo* architecture of the tissue is maintained, have indicated that the V_C increase or swelling seen in cultured cells at division also occurs during mitosis *in vivo*.

The salient features of the variations of FIGURE 2 may be summarized as follows. The onset of the cell thickening seen in time-lapse films commences at t = 21.5 hours and is associated with an increasing V_C. S_C remains essentially constant during the thickening period, and thus S_C/V_C decreases. Over

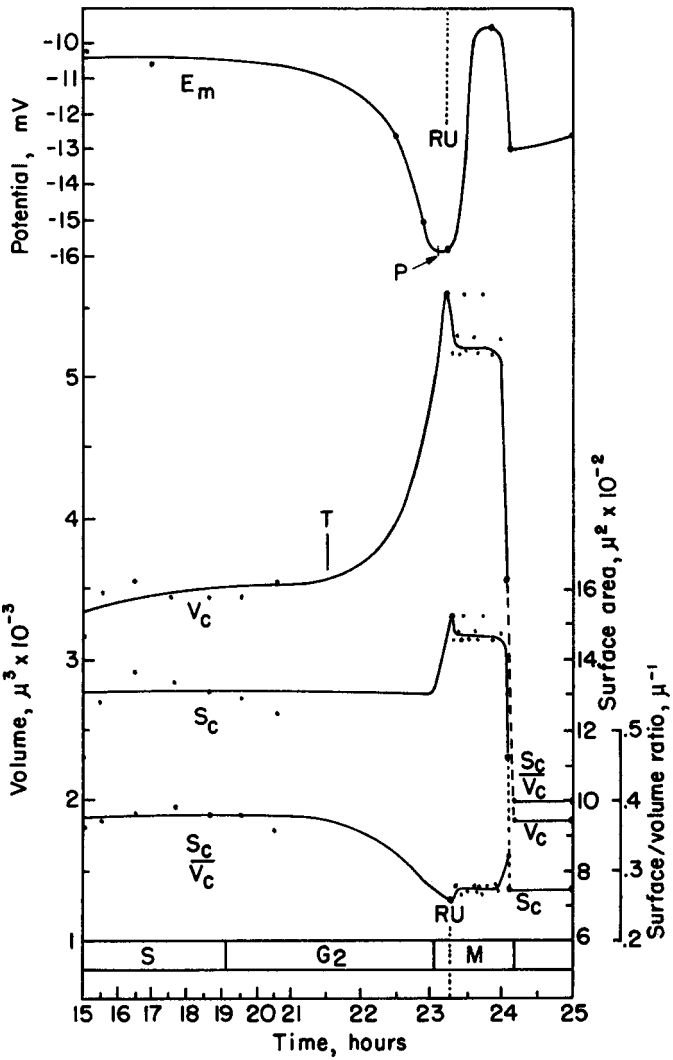


FIGURE 2. Time variations of cell volume (V_C), surface area (S_C), surface/volume ratio (S_C/V_C), and membrane potential (E_m) over the latter part of the generation cycle of the L-strain fibroblast in monolayer culture. Solid symbols are experimentally determined data points. Fairings of the V_C and S_C variations in the interval 21.5 to 23.25 hours, where measurement is difficult, are based upon the rates of form change determined from time-lapse films; that of E_m is based on actual measurements of cells in known states relative to prophase. T denotes the time of thickening onset, P the time of observable prophase initiation, and RU the time of cell spheralization or roundup. Note the expanded time scale in the interval 21–25 hours. The G_1 period of this particular cell was slightly longer than the average for large populations of L-strain cells.

the preceding part of the cycle, S_c/V_c has remained essentially constant; in fact, S_c/V_c maintains a practically constant level over the entire generation cycle, except during the thickening and mitotic periods. As the cell approaches mitosis, V_c swells at an increasingly rapid rate and ultimately the cell rounds into a fully spherical form, completing the roundup by early prophase (t = 23.25 hours). After a slight decrease following initial roundup, V_c remains essentially constant on through the mitosis period up to telophase. S_c begins to increase, and S_c/V_c to decrease even further, after the cell has assumed a fully spherical form. This S_c increase appears to be primarily the result of stretching of the membrane due to continued V_c increase after the spherical form is attained, since the mitotic sphere can be appreciably shrunken by hypertonic treatment before folding or wrinkling of the membrane (indicating the disappearance of membrane tensile stresses) is observed. The S_c/V_c decrease follows from the geometry of the rounding cell, since the sphere has the smallest S_c/V_c value of all possible shapes of given volume, and since S_c/V_c of the sphere also decreases with increasing sphere size.

Transmembrane Potential Variation Accompanying Rounding

The variation of the membrane potential (E_m) over the latter part of the generation cycle, as determined for naturally synchronized L-strain cells,[4] is also presented in FIGURE 2. The fine definition of this curve in the vicinity of roundup was obtained by E_m measurement of selected cells in known mitotic states relative to roundup. This E_m variation shown is typical for both monolayer and suspension cultured cells. Although E_m oscillates slightly about a mean value of approximately -10.5 mv over most of interphase, at t = 21.5 hours a pronounced and steady increase in the (negative) polarization of the cell commences, coincident with the onset of the cell thickening and volume increase cited above. This polarization continues to increase up to several minutes before visible prophase, at which time E_m begins to level off and approach its minimum value (-16 mv). During this leveling-off period, the first evidence of prophase is observed and the cell volume increase accelerates. By the time the minimum E_m level is reached, the cell has attained the fully spherical form and is in early prophase. Almost immediately after the cell becomes spherical, a rapid depolarization trend is initiated which reduces the negative E_m of the cell to slightly less than its initial interphase level in approximately 10 minutes, so that depolarization appears to be completed by mid metaphase. The close chronological correlation of the V_c and S_c variations (and attendant changes in cell shape) with the E_m variation is evident.

These geometrical and electrical variations characterize the mitotic roundup phenomenon in the L-strain cell *in vitro*. In view of other evidence to be cited herein, they appear to constitute fundamental, perhaps essential, features of the mitosis initiation process for cells in general. Particularly significant is the direct correlation of the V_c and E_m variations, which immediately suggests the involvement of electroosmotic interactions in the roundup process. Also of interest are the facts that the onset of the various changes associated with roundup and mitosis initiation, such as increasing electrical polarization of the cell, commences long before any morphologically visible signs of prophase are evident and that initiation of prophase follows soon after the polarization trend begins to level off. Before considering pos-

sible interpretations of these variations in regard to the mechanisms underlying roundup, and the potential role and implications of roundup in the mitotic initiation process itself, a brief presentation of the essential precepts of a theory covering the basic mechanism of free volume and concentration regulation in the cell by electroosmotic interactions is given.

THEORY OF ELECTROOSMOTIC REGULATION OF CELL VOLUME AND CONCENTRATION

The reason for the maintenance of a membrane potential by excitable cells such as nerve and muscle is understandable in view of their particular specialized functions. The answer as to why apparently all somatic cells possess a membrane potential is not so obvious. However, the foregoing correlation of the V_c and E_m variations suggests one potentially important function, viz., the regulation of cell volume and, consequently, of intracellular concentration. To provide a picture of the functional relationship between V_c and E_m for later use in interpreting the roundup process, and to serve as a quantitative basis for estimating the effect of E_m changes on V_c, the following sketch of the pertinent precepts of an electroosmotic theory of cell volume regulation is outlined for a generalized model of the somatic cell *in vitro*.

Electroosmotic Model of the Somatic Cell In Vitro†

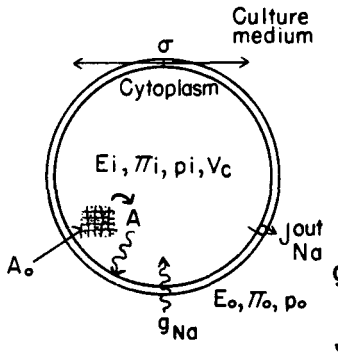
FIGURE 3 presents a sectional view of a generalized somatic cell which, for concreteness, is assumed to possess the same electrical properties as mammalian muscle for which the potential and ion distributions at equilibrium are relatively accurately known. The intra- and extracellular ion concentrations ($[\ ]_i$ and $[\ ]_o$) listed are typical for the steady-state condition in mammalian muscle at a resting potential of -90 mv. It is assumed that only Na^+, K^+, Cl^-, and A^- are significant in determining the potential, A^- representing organic anions that are wholly confined to the interior of the cell. It is further assumed that Na^+ alone is actively transported, K^+ and Cl^- being passively distributed. In addition to A^-, the cell also contains osmotically active neutral organic molecules designated by A. Since neither A^- nor A can penetrate the membrane, these species can be considered collectively so far as direct osmotic effects are concerned.

When the cell possesses a negative potential, as in the present case, electrical forces act within the membrane to oppose the movement of negative ions into the cell (and to resist the exit of positive ions). Thus, electrical retarding forces exist which are precisely analogous to the physical "pore-size" barrier forces of nonpermeable membranes and which are equivalently effective in preventing a net movement of negative ions into the cell (or positive ions out). Although ions do actually pass through the membrane in each direction, the net flux is zero if total pressure balance $\pi_i + p_i = \pi_o + p_o$ is main-

†The model developed here for the *in vitro* cell also applies directly to the *in vivo* cell with one possible exception. The *in vivo* cell (except in the malignant state) generally has firm surface attachments with neighboring cells and hence may be able to sustain a detectable internal hydrostatic pressure, i.e., $p_i > p_o$ (see FIGURE 3).

tained, so that the transmembrane concentration difference of each ion species remains constant. The fact that an electrically maintained concentration difference exists for a given ion species thus leads to the existence of a "partial" osmotic pressure due to this species, which is greater on the side of the membrane where the species exists in higher concentration.

From the steady-state data of FIGURE 3, $[Na^+]_i$ is seen to be a small fraction of $[Na^+]_o$. K^+ moves into the cell and replaces most of the Na^+ deficit at



E Electric potential
Em= $E_o - E_i$
π Osmotic pressure
p Hydrostatic pressure
σ Membrane tension
V_c Volume of cell
A_o Permeating molecules
A Nonpermeating molecules
g_{Na} Na conductivity of membrane
J^{out}_{Na} Na pumping rate of membrane

Particle Distribution in Mammalian Muscle Cells*

Ion	Intracellular $[]_i$	Extracellular $[]_o$
Na^+	12	145
K^+	155	4
Cl^-	4	120
$(others)^-$	8	34
$A+A^-$	155	57

*Concentrations in $\mu M/cm^3$; Em = -90mV

FIGURE 3. Schematic view of a typical somatic cell illustrating basic parameters involved in electroosmotic regulation of cell volume. The intra- and intercellular concentrations of ions listed are representative of mammalian muscle at a membrane potential of -90 mv.

equilibrium, but the replacement is not complete due to the resulting high concentration gradient of K^+ within the membrane. The net negative intracellular charge left by these two ion movements drives Cl^- from the cell, simultaneously decreasing the interior charge and the negativity of E_m until the steady-state condition is reached. At equilibrium, $[Cl^-]_i$ is seen to be only a small fraction of $[Cl^-]_o$. The result of active Na^+ transport in the cell, for

the equilibrium conditions listed in FIGURE 3, is that the cell loses 249 μmol/cm^3 of ($Na^+ + Cl^-$) and gains 151 μmol/cm^3 of K^+, for a net particle deficit of 98 μmol/cm^3. Thus, assuming that the internal and external osmotic pressures (π_i and π_o) were equal before Na^+ pumping was commenced by the cell, the cytoplasm would be left with a decreased osmotic pressure at electrochemical equilibrium if the volume were maintained constant. To reestablish osmotic equilibrium ($\pi_i = \pi_o$), the cell must balance this $Na^+ + Cl^-$ deficit by synthesis of 98 μmol/cm^3 of osmotically active organic molecules A.

It should be noted that it is not simply the particle equivalent of the net charge deficiency of the cell that is providing the osmotically significant ion concentration difference. The particle equivalent of the net amount of charge q which is separated by the membrane is exceedingly small. However, almost every Na^+ pumped from the cell is accompanied by a Cl^-, so that this pair-exit, while leading to an osmotically significant total ion concentration difference across the membrane, produces only a small net deficit of positively charged particles. This deficit, however, is sufficient to produce the observed E_m with its attendant osmotic effect.

A significant result of active Na^+ transport in the somatic cell, therefore, is that, with this potential-generating capability, a cell of given volume and shape can maintain osmotic equilibrium ($\pi_i = \pi_o$) with a greater internal concentration of organic molecules. Alternately, for a given total content of intracellular organic molecules, the cell volume will be smaller and the organic contents more concentrated, when the cell maintains an electrical potential difference across the membrane. As is discussed subsequently, changes in S_c/V_c can also lead to variations in E_m, even with a constant value of $[Na^+]_o - [Na^+]_i$.

The effect of variation of E_m on V_c due to changes in $[Na^+]_i$ can be quite pronounced. By way of illustration, the increase in V_c that would accompany complete depolarization of the model cell of FIGURE 3 can be calculated. Assuming the membrane to be completely elastic and capable of undergoing sufficient stretch to accommodate the volume increase without generation of significant hydrostatic pressure within the cell (a condition which clearly applied for *in vitro* cells, but which may not be true for the *in vivo* case), the increase in intracellular particle content upon complete depolarization would produce a corresponding maximum volume some 300% of the initial volume at osmotic equilibrium $\pi_i = \pi_o$. In practical situations, of course, before this volume is reached, either the membrane might rupture or else become so permeable (as in stretched-membrane hemolysis) as to lose sufficient A that swelling would cease. Additionally, most somatic cells for which E_m values have been determined possess less negative E_m levels than nerve or muscle, so smaller volume changes would accompany complete depolarization. The presence of A in the extracellular medium would also lower the maximum depolarization volume. Nevertheless, this example illustrates the fact that variation of the membrane potential, through modulation of the active Na^+ pumping rate (J_{Na}^{out}) and/or Na^+ conductivity (g_{Na}) of the membrane, can effectively regulate the cell volume and, hence, intracellular ionic and molecular concentrations. The manifestation of this electroosmotic effect on volume is demonstrated even in the case of nerve cells with their high Na^+ pumping capacity, wherein swelling of the entire neuron is discernible following long periods of sustained transmission. It should be noted that cell swelling in practical situations *in vivo* due to depolarization (i.e., $[Na^+]_i$ increase) may

be quite different from that *in vitro* where the volume of extracellular fluid is relatively infinite. Rapid V_c increases *in vivo* due to depolarization may be limited by the volume of interstitial fluid in the immediate vicinity of the cell. Rapid removal of water from this fluid may so concentrate it as to raise its osmotic pressure sufficiently to restore osmotic equilibrium.

Hypothetical Involvement of Electroosmotic Mechanisms in the Regulation of Cell Metabolism

The variation of A with E_m required for osmotic equilibrium at constant V_c appears potentially to offer a sensitive means for regulation of the basic molecular and ionic concentration levels of the cytoplasm and, through these, regulation of cellular metabolism. By cytogenetic/metabolic modulation of J_{Na}^{out} and g_{Na} of the membrane‡ in conjunction with the total synthetic rate of A, E_m and, hence, the concentration of A could be closely controlled. Thus E_m-modulated changes in metabolite concentrations could conceivably exert a regulating influence on metabolic kinetics in general, as well as on specific pathways through activation or suppression of particular reactions. For example, the existence of a large negative value of E_m for a cell of given V_c might so concentrate the cytoplasm with respect to a particular metabolite species A_j as to inhibit further synthesis or metabolic reactions involving this species. The relative concentration of water itself is of course quite important in the kinetics and equilibria of many cellular reactions. Changes in intracellular water content could also produce significant metabolic changes through secondary osmotic effects on intracellular subsystems involving membranes, such as the mitochondria, nucleus, and ribosome-endoplasmic reticulum complex. The nucleus, for example, maintains its own intranuclear potential, the level of which is no doubt dependent upon cytoplasmic conditions. In addition, the basic changes in the relative and absolute intracellular concentrations of Na^+ and K^+ accompanying E_m variation could in themselves possibly lead to activation of enzymatically important ion-exchange reactions with consequent alteration of specific metabolic pathways.

Although the actual extent to which such electroosmotic interactions are involved in the regulation of cellular metabolism or particular aspects thereof is unknown at present, the potential for such involvement is evident. Experimental results to be discussed subsequently strongly suggest such an involvement exists, at least in regard to those aspects controlling mitosis initiation. Since the variation of E_m is governed (exclusive of imposed extracellular factors) by various aspects of cellular metabolism, and since variation of E_m may, in turn, act to alter the cellular metabolism, the regulation of metabolic activity, at least in part, by the operation of a complex system of electroosmotically modulated metabolic feedback circuits can be readily envisioned. This paper is concerned particularly with indications that such

‡Throughout this paper, Na^+ is taken as the actively transported ion. For many aspects of the theoretical treatment, this is a matter of convenience only and the conclusions would not be altered if another cation were substituted. In other cases, the presence specifically of Na^+ may be required. From experimental evidence to be cited, it appears that Na^+ is actively transported in the L-strain cell, although complete electrochemical potential data for this cell are not yet available.

circuits and the associated E_m level may be involved in mitosis regulation in the somatic cell.

POTENTIAL INVOLVEMENT OF ELECTROOSMOTIC MECHANISMS IN MITOSIS INITIATION

Electroosmotic Interpretation of the Mitotic Rounding Process

The correlation of the experimental V_c and E_m variations of FIGURE 2 clearly suggests that electroosmotic interactions are involved in the mitotic rounding-up process. The fact that mitotic roundup (or its equivalent in V_c and E_m changes in suspension cultured cells) is a phenomenon common to apparently all types of somatic cells in culture indicates that these variations are a general, perhaps essential, feature of the initiation of cell division *in vitro*. Consequently, it appears worthwhile, even if necessarily on a somewhat hypothetical basis, to attempt an interpretation of the variations of FIGURE 2 in terms of the precepts of the foregoing theory, and to examine briefly the implications of this interpretation in regard to the potential involvement of electroosmotic factors in the initiation and regulation of mitosis in general.

Commencement of the characteristic V_c and E_m variation trends which lead to mitosis begins at approximately t = 21 hours, soon after completion of DNA synthesis, but some 2 hours before any intracellular morphological evidence of prophase can be discerned.[§] Prior to this time, the value of E_m, although oscillating slightly, has maintained an essentially constant average over the entire interphase period, as has S_c/V_c. S_c remains initially constant as V_c continues to increase, thus leading to a continuous decrease in S_c/V_c. Simultaneously, E_m increases negatively. The simultaneous decrease of S_c/V_c and increase in polarization of the cell prior to the onset of prophase is particularly interesting. Although it is possible that the increase in negative E_m may be the result of an increase in the Na^+ pumping rate of the cell during this period, the polarization can be explained simply in terms of the observed decrease in S_c/V_c, with one reasonable assumption.

In general, if it is assumed that the $[Na^+]_i$ of a cell is maintained constant (as in nerve[5]) by the Na^+ pump during a period over which V_c is increasing due to cell growth or production of A, an increasing amount of negative charge q will accumulate in the cell ($[Na^+]_i < [Na^+]_o$). The effect of this charge on E_m depends upon the change in S_c/V_c accompanying the V_c increase. The increase in q is proportional to the increase in V_c, and since E_m is proportional to q/S_c, E_m will vary as $(S_c/V_c)^{-1}$. The change in S_c/V_c, and hence in E_m, for a given increase in V_c will, therefore, depend upon the *relative* change in S_c. If S_c increases in the same proportion as V_c, S_c/V_c (and E_m) will remain constant; if S_c increases proportionately more than V_c, E_m will become less negative; and if S_c increases proportionately less than V_c, E_m will become more negative. Thus, under the condition that $[Na^+]_i$ is maintained essentially constant, increased polarization of a cell is to be expected as a natural consequence of a decrease in S_c/V_c. Such a decrease can come

[§]The discussion here relates to monolayered cells. However, the same types of variations in V_c and E_m are observed in suspension cultures, where the cells maintain a spherical form.[3,4]

from one or a combination of three changes: (1) an increase in absolute cell size (i.e., volume) while geometrical similarity of form is maintained; (2) a change in cell form such that the volume increases while S_c is maintained constant; and (3) a decrease in the absolute value of S_c while the volume is maintained constant. Thus, cell size and shape can exert a significant influence in determining the actual E_m level which will result from a given Na^+ concentration difference $[Na^+]_o - [Na^+]_i$.

The polarization increase of FIGURE 2 can be interpreted in terms of the decrease in S_c/V_c if maintenance of constant $[Na^+]_i$ is assumed. The validity of this assumption is in fact supported by the essential constancy of both S_c/V_c and E_m over the entire interphase period (i.e., 0–21.5 hours), despite the appreciable increase in V_c over this period. At t = 21.5 hours, V_c begins to increase significantly, presumably because of a cytogenetically induced increase in A; S_c remains essentially constant. This condition results in the onset of the S_c/V_c decrease, and in time-lapse films it can be seen that the cell commences thickening at this time, gradually changing from its initially flat interphase shape to the rounded form. The implication is that during the 1.5-hour polarization period, V_c is increasing to fill the closed membrane area S_c to maximum capacity; thus the cell is slowly "inflating" and S_c/V_c is decreasing to the minimum value of the spherical form. Simultaneously, as S_c/V_c decreases, the cell becomes increasingly polarized for the reasons previously discussed. Although polarization would presumably occur just from an increase in cell size (with the cell maintaining its flat, monolayer form), polarization in the present case is more pronounced since S_c remains essentially constant during the polarization period and the S_c/V_c decrease is due to *both* size increase and form change. (In reality, there are indications that S_c decreases somewhat over the thickening period prior to roundup, probably due to contraction of the slightly stretched membrane as it pulls free of its surface attachments.) The fact that S_c remains constant (or decreases) over this period is thus significant in that it results in an increased rate of polarization for a given rate of volume increase. The reason for the relative constancy of S_c while V_c continues to increase is unknown; however, the changes could be cytogenetically mediated and the two variations metabolically coupled. The increase in V_c associated with the polarization period may result from an increase in either general or specific metabolic activity following DNA replication and in particular may involve production of osmotically active peptides and similar subunits[6] for use in construction of the mitotic spindle, possibly through lysis of larger polymers.[7] In any event, the important feature in the polarization is that V_c increases faster, relatively, than S_c so that the ratio S_c/V_c decreases continuously following DNA synthesis.

It should be noted that the fairing of the S_c/V_c curve of FIGURE 2 in the interval 21.5–23.25 hours is based upon the relation $E_m \cdot S_c/V_c$ = constant, where the constant must satisfy the experimental values of E_m and S_c/V_c for each end of the thickening interval and for the postmitotic daughter cells. The fact that a single constant exists which not only satisfies the experimental E_m and S_c/V_c data, but also gives a V_c variation in full accord with quantitative time-lapse film observations of the rate of roundup implies that the assumption of a constant value of $[Na^+]_i$ over the interphase and early roundup periods is valid. This constancy of $[Na^+]_i$ has important implications, for

then the E_m level becomes a function only of the cell size and shape, i.e., of S_c/V_c.

The polarization continues to increase until just before visible prophase (i.e., up to 22.75 hours) when E_m begins to level off, reaching its maximum negative value of -16 mv at 23.25 hours. The trend then reverses and a relatively rapid depolarization ensues. Just before the onset of full roundup and depolarization, the cell begins to thicken at a rapidly increasing rate, and the first changes of prophase, such as granulation of the nuclear sap, become visible (c. 23 hours). The rounding process accelerates and just prior to the final rapid spheralization, the nucleoli are observed to disintegrate and well-formed chromonemata can be discerned, although the nuclear membrane is still intact as the cell becomes spherical. The cell is thus in early prophase at the time it first reaches the spherical form. Depolarization appears to commence just after the cell becomes spherical, i.e., in midprophase.

The proposed interpretation, on the basis of the available data,[¶] of this chain of events associated with termination of the polarization trend involves the supposition that the cell is incapable of sustaining a negative E_m beyond a certain "threshold" level, whence $[Na^+]_i$ begins to increase. The failure of the cell to continue polarization as osmotically induced swelling continues is assumed to be a result of the inability of the membrane (under the existing physiological and environmental conditions) to sustain an increase in the transmembrane electrochemical potential of Na^+, $\Delta\mu_{Na}$, beyond a certain maximum level. When E_m reaches the value at which the polarization trend starts to level off (-15 mv), $\Delta\mu_{Na}$ apparently becomes sufficiently large that the Na^+ leakage current brings Na^+ into the cell as fast as the Na^+ pump can remove it; a further increase in V_c can then only result in a net flow of Na^+ into the cell with a consequent increase in $[Na^+]_i$ and leveling off of the E_m toward its minimum value. Thus Na^+ enters the cell at an increasing rate and $[Na^+]_i$ rises correspondingly over the period from 22.75 to 23.25 hours. Such an influx of Na^+ will itself, however, generate an increase in V_c because of increased Na^+ osmotic activity within the cell. Thus the rate of V_c increase will be even greater once the E_m value at which the Na^+ influx commences is reached (22.75 hours). The rapid increase in V_c swelling rate expected on the basis of this "limiting E_m" supposition following the attainment of the "Na^+ - influx" E_m level is clearly observed experimentally, and terminates in full roundup of the cell within a short time. The most obvious intracellular change thus brought about by the hypothesized inability of the *in vitro* L-strain cell to sustain increasing polarization beyond the -15 mv level is that $[Na^+]_i$ increases steadily above the (constant) interphase level. That the onset of this proposed increase in $[Na^+]_i$ is soon followed by the visible initiation of prophase (23 hours) suggests a possible role for $[Na^+]_i$ in basic prophase activities such as nucleoli disintegration, chromonemata condensation and coiling.

A pertinent observation in regard to the postulated entry of Na^+ into the cell at mitotic roundup is the characteristic rounding induced by treatment of interphase monolayered cells with trypsin or versene (EDTA) for culture

[¶] Although actual experimental data on Na^+ fluxes in the vicinity of prophase are not yet available, experimental data to be presented subsequently indicate that Na^+ exerts a pronounced influence on the initiation of mitosis in the L-strain once DNA synthesis has occurred.

transfer purposes. The rounding up of the L-strain cell under such treatment is almost identical in appearance and nature to the mitotic roundup. Periodic measurements of the V_c and E_m of L-strain cells following addition of trypsin have shown that the roundup is characterized by an appreciable V_c increase and simultaneous electrical depolarization analogous to the case of natural mitotic roundup, and implications are that the same is true for roundup induced by versene. It thus appears that rounding induced by trypsin and versene occurs primarily as a result of cellular swelling due to depolarization below the normal interphase level of E_m, i.e., cell swelling resulting from the net entry of Na^+ into the cell through the altered membrane. The tension created in the membrane upon swelling of the cells apparently pulls the membrane free of its peripheral surface attachments and, hence, is primarily responsible for cell detachment with these agents. Trypsin-induced roundup probably results from a Na^+ influx generated by an enzymatic degradation of cell surface proteins essential in maintaining g_{Na} and J_{Na}^{out} at their normal *in vitro* levels. The roundup action of versene is most probably a result of the depolarization following Ca^{++} removal from the cell surface by chelation. Electrical depolarization as a result of Ca^{++} deficiency is a well-documented source of nervous disorder involving tetany. These treatments may be of potential value in shedding some light on the particular moieties of the cell surface which are involved with E_m maintenance (or lack thereof) and, consequently, with mitosis initiation.

Once the cell has reached a fully spherical form, the continued increase in V_c results in a stretching of the membrane (as indicated by hypertonic shrinking tests). This stretching presumably increases g_{Na} (or decreases J_{Na}^{out}) sufficiently that the associated Na^+ influx initiates a relatively rapid depolarization of the cell and produces a correspondingly rapid increase in V_c. It is possible that, for the monolayered cell, some stretching of the membrane may occur just prior to full roundup due to the membrane stresses generated as the cell is pulled free of its peripheral surface attachments. Once membrane stretching commences in the fully spherical cell, the V_c increase becomes divergently unstable and the initial swelling leads to further stretching and swelling. Ostensibly, this instability should lead to an accelerated swelling of the cell and ultimately to complete depolarization or rupture. However, time-lapse film observations indicate that condensation of the mitotic spindle begins at essentially the same time that the cell reaches its spherical form, and presumably the condensation of the initially osmotically active peptides and other small molecules[6,7] into the spindle system produces a countering osmotic effect which balances that of the Na^+ influx such that further volume increase and membrane stretching is arrested. Depolarization proceeds rapidly and appears to be complete by the time the spindle has fully formed (mid metaphase), E_m having reached its smallest negative level (-9.8 mv). Thus, for most of the mitotic period, the cell has a large volume but relatively small negative E_m due to the presumably high $[Na^+]_i$. Possibly the membrane stretch maintained during the mitosis period increases g_{Na} to such an extent that the cell cannot restore the interphase $[Na^+]_i$ level. At late telophase, V_c of the dividing cell begins to decrease rapidly and E_m simultaneously decreases to -13 mv, the initial interphase level. Since S_c/V_c for the daughter cells is larger than for the mitotic sphere, the Na^+ pump has presumably reduced the $[Na^+]_i$ to the interphase level during the period of V_c decrease.

An apparent failure of the osmotic countering mechanism (whereby spindle protein condensation balances depolarization-induced swelling) has been observed occasionally in time-lapse films; the cell, after mitotic roundup fails to check its V_c increase and continues to swell until the membrane ruptures. Failure of the spindle to condense in such cases would lead to greater stretching of the membrane upon initiation of depolarization and would, in turn, lead to complete depolarization and possible rupture. In the aberrant cells it was in fact noted that no alignment of metaphase chromosomes occurred. It is possible that the rising value of $[Na^+]_i$ during early depolarization is instrumental either directly or indirectly in activating the condensation of the spindle tubules under normal conditions and hence provides an automatic self-balancing mechanism for preventing the volume increases to be expected from depolarization alone.

It appears on the basis of the foregoing correlations that electroosmotic mechanisms are intimately involved in all phases of the mitosis initiation process *in vitro*. The proposed interpretation of the variations, although somewhat hypothetical, does provide a satisfactory explanation and correlation of the major experimentally observed features in terms of the precepts of electroosmotic theory. It appears that the V_c and E_m variations preceding somatic cell mitosis *in vitro* constitute primarily a mechanism for initiating an increase in $[Na^+]_i$ at the proper time and at a proper rate. The polarizing V_c increase is initiated apparently by cytogenetic activity and, perhaps significantly, follows closely the completion of DNA replication. This V_c increase, in turn, generates an increasingly negative level of E_m through the decrease in S_c/V_c associated with the resultant cell size and shape changes preceding prophase. The inability of the membrane of the *in vitro* cell to sustain the increasingly large $\Delta\mu_{Na}$ ultimately leads to increasing $[Na^+]_i$ and prophase initiation. Thus, the factors underlying the V_c increase (while preventing a proportionate increase in S_c) may be regarded as the mitotic "trigger." In this proposed system of interactions, S_c/V_c acts to set the general size limit which the particular cell type can attain and guarantees the onset of division when this size level is reached.** That the polarizing V_c increase may be the (osmotic) result of spindle monomer production would further guarantee that the cell is fully prepared for mitosis before the prophase initiation; presumably, termination of the polarization increase would in itself occur only after a sufficient supply of spindle monomers had formed, as signaled by the cell reaching the proper V_c (and E_m) level for Na^+ entry and prophase to begin. In view of similar volume changes associated with mitosis in somatic cells *in vivo*, it appears reasonable to assume that the same basic processes as outlined above for the *in vitro* cell are active in *in vivo* mitosis, once DNA replication has been completed.

The results of the foregoing interpretation immediately pose two important questions in regard to mitosis regulation. First, since the increase in $[Na^+]_i$ due to premitotic polarization is supposedly instrumental in initiating prophase and division, once proper metabolic preparations are completed, is it possible to block mitosis *in vitro* by preventing the polarizing V_c increase of the G_2

**In this regard, it is interesting to note that L-strain cells in both monolayer and suspension cultures appear to maintain essentially the same values of S_c/V_c and of E_m, despite gross differences in cell shape and size for the two culture modes. This again suggests that $[Na^+]_i$ is a constant of the cell system and that S_c/V_c thus acts to assure division when this ratio begins to decrease significantly.

period and, more generally, is the division of somatic cells *in vivo* (where much more negative E_m levels generally exist than in culture) naturally blocked or regulated by the maintenance of sufficiently negative E_m levels (which would presumably arrest mitosis preparations at some stage prior to the onset of the polarizing V_c increase)? The results of an investigation designed to provide at least a partial answer to this question are dealt with in the following section. The second question involves the possible specific role of Na^+ in prophase initiation and other aspects of mitosis, such as spindle condensation, either directly or through ion-exchange reactions involving other ions such as K^+ and Ca^{++}. Results (to be presented in a following section) of an experimental investigation on mitotic stimulation have shown Na^+ to be uniquely active among several ions in inducing early initiation of mitosis in the L-strain fibroblast.

Mitotic Blockage by Simulated E_m Variation

As discussed in the previous theoretical considerations, the primary effect of E_m is to allow a larger $[A]_i$ for a given V_c and S_c/V_c than would otherwise be possible without a rigid or inelastic cell surface; as E_m becomes more negative for a given V_c and S_c/V_c, $[A]_i$ can (and must) be correspondingly larger at osmotic equilibrium $\pi_i = \pi_o$. As mentioned, $[A]_i$ might potentially be of importance in the regulation of metabolic activity associated with mitotic preparations and events. Thus, in the present study, it was desired to ascertain what effect regulation of the E_m level would have on the ability of a cell to enter mitosis. Since it is not feasible (or usually possible) to regulate satisfactorily the E_m of small somatic cells directly by microelectrodes, and since the primary expected result of altering the E_m under natural conditions would be to increase $[A]_i$ at a given V_c, E_m variation was simulated by a controlled variation of the osmotic pressure of the external culture medium by addition of nonpermeating mannitol. Identical tests using sucrose were also run, with the same results as for mannitol. In this way, by increasing the tonicity of the culture medium sufficiently, the cell volume, and hence $[A]_i$, could be regulated to a desired level, equivalent to what in the normal case would be associated with a particular value of E_m. Thus, for example, maintenance of the cells in a relatively concentrated mannitol medium would be osmotically equivalent to the natural maintenance of a relatively negative E_m level by the cells. The simulation is not exact, of course, since the ion distributions which would exist in the case of a natural E_m variation obtained by Na^+ pumping may not be the same as in the hypertonic equilibrium; however, the regulation of $[A]_i$, the factor of primary interest, can be precisely accomplished with this technique. Although cultured cells are apparently unable to sustain any appreciable polarization beyond the characteristic depolarization level, the hypertonic-treatment technique allows the simulation of the major osmotic and concentration changes that would occur if the cells could naturally maintain larger negative potentials and hence allows first order simulation by use of *in vitro* cells of E_m-level effects on mitosis which would exist in somatic cells *in vivo*.

By use of this simulation, the effect on subsequent mitotic activity of imposing and maintaining a series of increasingly negative E_m levels at different times in the cell cycle relative to the time of normal prophase initiation

has been determined for the L-strain cell. The experimental cell cultures used were synchronized with 5-aminouracil (5-AU); the peak onset of mitosis in these cells normally commences some 8 hours after removal of the 5-AU block. The treated cells were monitored and scored for mitotic activity by use of a time-lapse recording procedure.[1] Representative results of these tests are illustrated in FIGURE 4, which shows the effects of three concentration levels of mannitol on subsequent mitosis initiation, when applied 2 hours after 5-AU removal (i.e., 6 hours before normal division). An appreciable reduction in the degree of synchrony[1] is produced by the lowest concentration used (0.01 M), although no actual blockage of division results. The

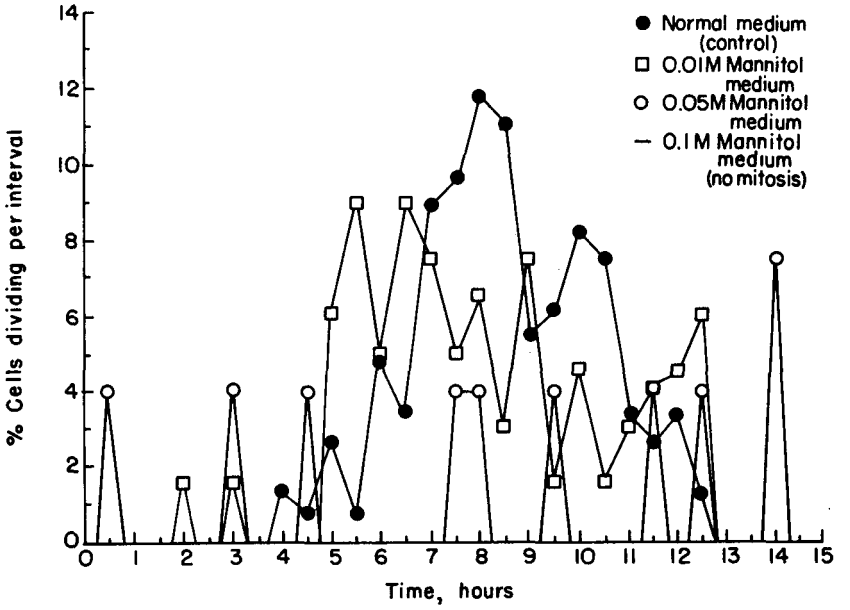


FIGURE 4. Mitotic frequency distributions for L-strain cell populations synchronized with 5-aminouracil (5-AU) illustrating the effect on mitosis of simulating E_m changes by application of hypertonic mannitol media. Time t = 0 represents the time of 5-AU removal.

number of dividing cells is significantly reduced by the 0.05 M medium, with a gross reduction in synchrony, and with the 0.1 M medium a complete blockage of division is produced. Despite this pronounced effect on mitosis, the cells treated with the 0.01 M and 0.05 M concentrations were fully viable, and appeared quite normal by morphological and mobility criteria, throughout the test period. The cells treated with 0.1 M mannitol medium were viable through the test period, but had a slightly rounded form and lacked the usual mobility seen in cells in time-lapse films. In addition to the effects of concentration (simulating E_m level), a pronounced effect of the time of application of the test medium (simulating time of E_m level change) was also observed. Appli-

cation of lower concentrations just before normal division time was considerably less effective in blocking mitosis than when applied very soon after 5-AU removal (i.e., presumably before or during early DNA synthesis); 0.1 M mannitol (corresponding to a highly negative E_m) blocked mitosis completely, regardless of the time of application.

These results indicate that high intracellular concentration levels, presumably equivalent to those which would be produced naturally in the cell by relatively negative E_m levels (such as are apparently maintained in normal cells *in vivo*), are effective in blocking the mitosis of L-strain cells. Although the actual mechanisms by which such blockage is effected are undetermined, a logical assumption is that the high intracellular concentrations induced act to inhibit or block metabolic pathways essential in preparations for mitosis, at least for mannitol applications made prior to the normal onset of the polarization trend. The effects of such increased concentrations on other aspects of cellular metabolism are apparently not sufficient to cause a noticeable change in cell morphology or mobility. The suggestive implication of these results is that if the value of E_m in the somatic cell (either *in vitro* or *in vivo*) were naturally maintained at a sufficiently negative level by the Na^+ pumping system starting in early interphase, division of the cell would be blocked; thus modulation of the cell E_m level could constitute a means for regulation of mitosis. On the basis of the experimental observations, it is conceivable that if a cell in the late G_2 period (*in vitro* or *in vivo*) were able to lower its E_m-level (without exceeding the depolarization $\Delta\mu_{Na}$ value) by active Na^+ pumping so as to prevent the increase in V_c which would normally occur, the $[A]_i$ might rise sufficiently before prophase to become self-inhibiting and hence the V_c would level off as production of the osmotically active molecular species ceased, without the cell entering mitosis. Such a situation would constitute a natural "G_2 blocking mechanism" for mitosis. More importantly, the fact that very early applications of hypertonic medium provide maximum effectiveness in producing mitotic blockage suggests that the natural existence of more negative values of E_m in early interphase might serve as an effective block of mitosis through metabolic preparatory inhibitions, possibly even of DNA synthesis. Such a condition would constitute a "G_1 mitotic blocking mechanism"; most evidence indicates that natural mitotic blockage *in vivo* occurs in the G_1 period. The ability of a cell to generate and maintain a sufficiently negative E_m for such hypothetical blockage would, of course, depend in large measure upon the basic capability of the cell to maintain adequate values of J_{Na}^{out} and g_{Na} and, hence, upon the immediate chemical and physical environment of the cell, as well as upon the cell's own basic cytogenetic properties. For example, existence of a highly negative E_m could conceivably induce (through [A] and $[Na^+]$, $[K^+]$ changes) metabolic production of surface polymers yielding a small value of g_{Na} (and hence guaranteeing maintenance of the E_m at its high negative value), thereby blocking mitosis. Alteration of the polymer complex by external agents acting either directly on the cell surface or through induced metabolic changes could increase g_{Na} and decrease the negativity of E_m, thereby creating a condition similar to that of the cultured cell and allowing mitosis to proceed. Similar feedback circuits involving J_{Na}^{out} can likewise be envisioned.

In regard to the foregoing experimental results, a seemingly general correlation between E_m level and mitotic activity *in vivo* is of particular interest. Cells which characteristically maintain exceedingly negative values of E_m

(e.g., CNS nerve and muscle) seldom, if ever, divide under normal conditions, while cells which maintain unusually small negative values of E_m (e.g., malignant cells) are the most highly active mitotically. Moreover, cells in culture possess the least negative E_m levels of all and correspondingly demonstrate the highest degree of mitotic activity. Under appropriately altered physiological conditions, which perhaps result in temporarily reduced polarization, it is possible, of course, that usually highly polarized somatic cells may enter mitosis. That this apparently general relationship between E_m level and relative mitotic activity as observed *in vivo* is in accord with the implications of the experimental hypertonic-blockage results cited above lends support to the plausibility of the latter. Malignant cells in particular appear to offer some significant correlations of E_m level with mitotic activity, the possible implications of which are discussed in a following section.

An interesting, and perhaps not altogether coincidental, observation in regard to E_m level and mitotic activity is the almost exact agreement between the mannitol concentration which completely blocks mitosis in cells *in vitro* and the increase in the $[A]_i$ which results from the -90 mv E_m value of (nondividing) muscle cells. A mannitol concentration of 0.1 M is sufficient to block all mitoses in L-strain cells in culture; in a previous example it was shown that an E_m of -90 mv (representative of nondividing muscle and nerve cells) corresponds to an increase of 0.098 M in the intracellular concentration of osmotically active molecular species within the cell.

Many obvious experimental approaches exist for investigating the generality and validity of the premise, suggested by the foregoing results, that the E_m level and mitotic activity of a cell are functionally related. For example, on the basis of this premise, it would be expected *a priori* that the E_m level of somatic cells that will ultimately attain a state of relative mitotic quiescence (e.g., nerve, muscle) should become more negative as growth and differentiation of the cells proceed from the fertilized egg on through the various embryonic stages to full differentiation in the neonatal or adult organism. Cells which will remain continuously mitotically active (e.g., villus crypt cells) should remain at a relatively less negative potential. The validation of a general correlation of E_m level with mitotic activity would, of course, have important implications for such fields as differentiation, morphogenesis, and growth and development, as well as general systemic homeostasis.

Mitotic Stimulation by Pulsed Infusion of Sodium Ions

Studies of the mitotic frequency distributions of 5-AU synchronized L-strain cells,[1] and of shape-change rates at roundup, early suggested that some relatively sudden triggering action was involved in the initiation of mitosis following the period of gradual form thickening. (These studies were conducted prior to the investigation of the E_m variation; the roundup "triggering" event was subsequently correlated with the onset of depolarization.) It was hypothesized that prophase was possibly initiated by a buildup in the intracellular concentration of some essential ion, presumed at the time to be Ca^{++}, to a precipitating threshold value and, therefore, that an infusion of Ca^{++} into synchronized cells prior to the normal prophase time might result in an increase in the degree of mitotic synchrony, since all cells would be synchronously "precharged" with Ca^{++}. In an attempt to obtain a sharply defined and minimal

period of exposure to abnormal medium conditions, the cells were hypotonically pulsed with a 0.1 M $CaCl_2$ water solution (the normal culture medium was only 0.001 M in Ca^{++}). The pulsation technique was similar to that used for coalescing L-strain cells[8] and consisted of a brief hypotonic swelling of the cells to approximately 1.5 times their normal volume during which Ca^{++}, under the strong concentration gradient, supposedly entered the cell through the stretched membrane (reversed "hemolysis").[8] The pulsation was performed 3 hours after removal of 5-AU. The normal culture medium was immediately replaced and the subsequent mitotic activity followed by time-lapse recording.

Surprisingly, the results of the Ca^{++}-pulsation tests using 5-AU synchro-

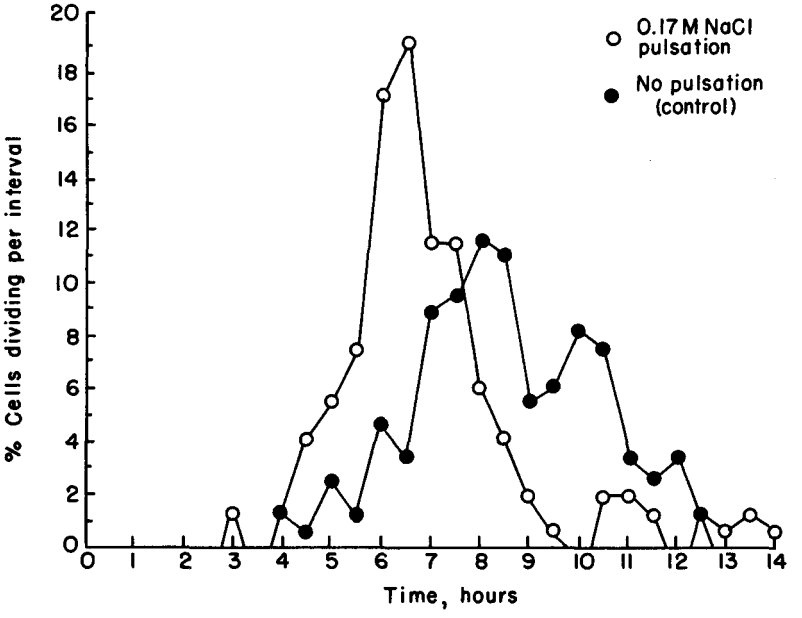


FIGURE 5. Mitotic frequency distributions for L-strain cell populations synchronized with 5-aminouracil illustrating the stimulating effect on mitosis of Na^+ hypotonic pulsations at t = 3 hours. Time t = 0 represents the time of 5-AU removal.

nized L-strain cells showed no effect at all either on mitosis or degree of mitotic synchrony, when compared to unpulsed 5-AU synchronized controls. The pulsation experiment was subsequently repeated using, respectively, 0.170 M NaCl and 0.10 M KCl solution, and simply H_2O. Again, no discernible effect was observed with either K^+ or H_2O, but the results of the Na^+ pulsation were striking (FIGURE 5). Not only was the degree of synchrony markedly improved by the treatment, but the time required for mitosis initiation after synchronization was significantly reduced, corresponding to a decrease of approximately 3 hours, supposedly in the S, and possibly G_2, periods.

Although it has not yet been shown analytically that ions of any type actu-

ally enter the cell during such hypotonic "infusion" treatment, the unique and striking results obtained with Na^+ suggest that such entry does in effect occur and that it is the sodium ion which is primarily involved in the active transport system of the L-strain. In view of the foregoing E_m implications that prophase initiation and roundup are manifestations of Na^+ entry into the cell, the present results on Na^+ infusion can be interpreted as indicating that the infusion treatment effectively accomplishes what otherwise would require the advent of the natural polarization-depolarization process, viz., a significant increase in $[Na^+]_i$. The fact that the cells used in this experiment had undergone 5-AU synchronization treatment may be significant in these results in that such cells metabolize actively during the 5-AU treatment period[1,3] and apparently have already completed many mitotic preparations which untreated cells normally would commence only in the G_2 period. Thus, if Na^+ is actually an essential element in the prophase initiation mechanism, its entry into the cell upon pulsation could reasonably be expected to precipitate mitosis "prematurely," as is observed experimentally.

POTENTIAL INVOLVEMENT OF ELECTROOSMOTIC FACTORS IN MALIGNANCY

The foregoing implication that electroosmotic factors are involved in mitosis initiation naturally suggests that the electroosmotic behavior of the (*in vivo*) cancer cell, whose basic pathological property is autonomous division, should be carefully examined. Two basic characteristics which apparently are possessed by malignant cells in general are of particular interest in this regard: both the E_m level and the Ca^{++} content are highly abnormal. The E_m level in various cancer cell types *in vivo* is strikingly less negative than that of homologous normal cells. For example, normal muscle cells maintain an E_m level of approximately -90 mv and very seldom divide. In homologous cells of a myosarcoma, however, the negative E_m level drops to only -15 mv, and vigorous malignant proliferation maintains. Observation of this E_m abnormality of cancer cells has been mentioned by numerous investigators,[9–12] but without suggestion as to its possible significance in regard to cancerous proliferation. However, in view of the foregoing results suggesting blockage of mitosis by sufficiently negative values of E_m, the implication is that the abnormally small negative E_m of the cancer cell is basically inadequate to block essential mitotic preparation of the G_1 and S period and that the cell membrane is unable to sustain a sufficiently negative E_m during the ensuing polarization period to block mitosis in the G_2 period. Periodic division of the cancer cell would thus follow naturally upon completion of the essential mitotic preparations, much like cells in culture.

If natural metabolic feedback control of the cellular E_m level is indeed a basic mechanism in mitosis regulation, as is suggested by the foregoing theoretical and experimental results, an understanding of the principal factors underlying the cancer cell's inability to maintain a sufficiently negative value of E_m becomes a matter of considerable importance. Since the cell membrane plays the fundamental role in maintenance of E_m, the membrane (including its surface molecular complex) constitutes a logical first source of potential E_m aberrancies, with the realization, of course, that such surface

aberrancies are, ultimately, but a manifestation of an abnormal†† but stable cytogenetic equilibrium within the cell. In regard to possible surface aberrancy in cancer cells, the second general characteristic cited above, viz., the well-documented Ca^{++} deficiency (presumably of the cell surface and also, perhaps, of the mitochondrial surface), is of considerable interest.[13,14] The deficiency of Ca^{++} and its associated causes may underlie such characteristic properties of the cancer cell as lack of cellular adhesion, metastasis, lack of contact inhibition in culture, and increased negativity of the cell surface,[15–18] all of which suggest the molecular abnormality of some moiety of the cell surface complex. Particularly pertinent is the fact that lack of Ca^{++} in the case of nerve is accompanied by tetany, with inability of the cell membrane to stably maintain its highly negative potential. Both J_{Na}^{out} and g_{Na} of the cell membrane are involved in determining the equilibrium value of E_m and, in view of the effects of Ca^{++} deficiency on nerve, each of these parameters might be adversely affected by the inability of the cancer cell surface to incorporate Ca^{++} in functionally normal amounts. In particular, an increase in g_{Na}, associated with an aberrant surface complex incapable of the necessary Ca^{++} association, would be in a direction to give the decreased negative E_m level found in the cancer cell. On the other hand, a sufficient decrease in J_{Na}^{out}, whether the result of a specific surface abnormality or more general metabolic aberrancies or both, could also cause the observed decrease in E_m, independently of g_{Na}. For example, since J_{Na}^{out} is intimately associated with the energetics regime of the cell, a decrease in the Na^+ pumping rate could result from the existence of low energy production in the cell due to anaerobic glycolysis of the type discussed by Warburg; such glycolysis might in turn be stably maintained by the effect on mitochondria or metabolic activity involved in oxidative phosphorylation of the higher intracellular water content accompanying the less negative E_m level.

It is conceivable, if the E_m level is truly capable of mediating the metabolic expression of the cell through regulation of $[A]_i$, that the maintenance of the malignant state could be the result of a dynamic, self-sustaining metabolic feedback system. For example, the autonomous division characteristic of malignancy could be envisioned as the result of a stable feedback circuit wherein ("abnormal") surface polymers (e.g., proteins, mucopolysaccharides) produced by the cell as a result of induced malignant transformation cause a low negative E_m (through a high value of g_{Na} or low value of J_{Na}^{out}) which, in turn, insures continued activation of metabolic pathways involved in the production of the particular polymer forms necessary for maintaining the low negative E_m and associated mitosis. Since these polymers would presumably remain on the cell surface during mitosis, they would institute the same small negative E_m level at early interphase of the daughter cells and thus induce continued production of the same polymer complex leading to the same low negative E_m level and mitosis; hence, continued autonomous division would be insured. It is interesting that, in this proposed mechanism of autonomous proliferation, the "information" that will guarantee continued "high g_{Na}" or "low J_{Na}^{out}" polymer production and mitosis in the daughter cells is transmitted to the daughters via the *cell surface* in the form of the polymer itself

††Although the malignant state may be "abnormal" with respect to the state of normal homologous somatic cells, it may be regarded as a perfectly normal consequence of redirected genome expression (or "redifferentiation").

and is translated into operational cytogenetic activity by the resultant E_m level in the daughter cells. This form of feedback relationship thus offers a mechanism for insuring the reactivation of the same phenotypic expression in the daughter cells, at least as regards surface characteristics and mitosis, as existed in the parent cell, despite the supposed "shutdown" of the cytogenetic apparatus during the period in which the DNA is condensed into the chromosomes for division. Interestingly, such a mechanism carries the inherent implication that its attendant state of malignancy is potentially reversible.

A final observation pertinent to the possible involvement of electroosmotic factors in malignancy concerns the change in E_m that accompanies the transfer of normal *in vivo* somatic cells to tissue culture. *In vivo,* somatic cells of various types are found to maintain E_m values of the order of -40 to -50 mv and to possess a very low mitotic index. Upon transfer and accommodation to tissue culture, however, the interphase E_m rises to a level of only -10 to -15 mv, and simultaneously the cells enter sustained, periodic division. The changes which occur in E_m level and mitotic activity as a result of such transfer of normal cells to culture are precisely analogous to those which apparently occur *in vivo* as a result of malignant transformation. The similarity is further emphasized by the fact that many, if not all, initially normal cell lines eventually become malignant when maintained in culture. Interestingly, the E_m level of many malignant cell types becomes even less negative when the cells are transferred from the *in vivo* state to monolayer culture. Since one of the primary changes effected by the transfer is the full dissociation of the cells, and alteration of their contact environment, the involvement of the cell surface is again suspect.

The significant implication of the foregoing considerations in regard to cancer is that the autonomous division of the malignant cell could be primarily the manifestation of a stable abnormality (i.e., a self-sustaining "redifferentiation") in the electroosmotic balance of the cell. It is not difficult to envision how such an abnormal balance could be instituted in an initially "normal" cell by any of a wide range of physical, chemical, and biological carinogenic agents. If such an electroosmotic mechanism is active in malignancy, agents or treatments that would permit or induce restoration of a high negative potential level selectively in the cancer cell would obviously be of great therapeutical value.

SUMMARY

Observations of synchronized populations of L-strain fibroblasts, both in monolayer and suspension culture, have revealed that pronounced variations in cell volume and electrical potential accompany the initiation of mitosis *in vitro*. These experimental variations can be satisfactorily correlated and interpreted in terms of electroosmotic theory on the assumption that the Na^+ concentration in the cell remains constant during the polarization, but increases significantly during the depolarization period. The two most important implications of the proposed theory and experimental-variation interpretation are that prophase initiation is brought about by an osmotically induced volume increase which results in an increase in the Na^+ concentration of the cell when a "threshold" value of the surface/volume ratio is reached, and that the

membrane potential level may have a significant regulatory influence on metabolic preparations for mitosis. Experimental results show that hypotonic pulsation with Na^+ has a pronounced stimulatory effect on mitosis initiation in the L-strain cell. The results of experiments intended to simulate the maintenance of various negative intracellular potential levels imply that the percentage of cells of a given population entering division will be significantly reduced by increasingly negative potential values and that a sufficiently negative potential level will completely block mitosis. This implication appears to be borne out by existing experimental data for somatic cells *in vivo*. Two characteristic aberrancies of cancer cells, an exceptionally small negative potential and a deficiency of Ca^{++}, when examined in light of the foregoing theoretical and experimental results, strongly suggest the potential involvement of an abnormal electroosmotic balance in the maintenance of malignant proliferation.

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