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Control of Somatic Cell Mitosis by Simulated Changes in the Transmembrane Potential Level

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Abstract. Experiments designed to test the premise that variations in intracellular ionic concentrations accompanying different levels of the electrical transmembrane potential difference might serve as a mechanism for control of mitosis in somatic cells have yielded results supporting the validity of the premise. Employing naturally synchronized CHO cells *in vitro* and test media designed to produce intracellular concentrations of Na^+, K^+, and Cl^- approximating those which would exist at various naturally-generated E_m levels *in vivo*, it was found that mitosis was completely, but reversibly, blocked in both short and long-term tests by simulated E_m levels of –70 mV and greater. This blockage was furthermore found to result from complete, but reversible, prevention of DNA synthesis. These findings that simulated high E_m levels introduce a reversible G_1 mitotic block in somatic cells *in vitro* lend credence to the supposition that natural modulation of cellular E_m levels *in vivo* may constitute a basic mechanism for mitosis control *in vivo*.

Key Words
Mitotic regulation
DNA synthesis control
Cancer theory
Cell surface polymers
Intracellular ionic hierarchy
CHO cell line
Natural synchronization

Introduction

In a recent paper, CONE [1969] advanced the premise that variations in intracellular ionic concentrations corresponding to different levels of the electrical transmembrane potential difference (E_m), might play a key role in the control of cell division, possibly through alteration of specific metabolic events of the G_1-period with consequent prevention of DNA synthesis. In a subsequent paper [CONE, 1970], this thesis was substantially expanded, and utilized to explain a number of observed aspects of oncogenesis. It was

pointed out that cells which continuously maintain a highly negative E_m level (e.g. nerve and muscle, –70 to –90 mV) seldom if ever enter mitosis under normal conditions, while cells having very small negative E_m levels (e.g. established cell lines in tissue culture, –10 mV) normally undergo vigorous and continuous proliferation, thus suggesting a relationship between E_m level and mitogenic activity. The fact that E_m level was greatly reduced in a range of malignant cell types *in vivo*, relative to their normal homologs, was also noted. In view of evidence that apparently all somatic cells possess negative E_m levels of various magnitudes, determination of whether these potential differences and their associated electroosmotic balances are in fact capable of exerting a controlling influence on mitosis initiation is a matter of considerable interest and importance from both the general standpoint and that of malignancy.

Unfortunately, determination of the effects of E_m level and its associated ion balance on the mitotic activity of somatic cells by directly imposed variations in the actual membrane potential *per se* is not feasible experimentally. From a practical standpoint, however, such difficulties can be overcome and meaningful simulations of desired E_m levels obtained by exposing test cells to external media of such compositions that, upon equilibrium, the intracellular concentrations of the various ions approximate those which would exist if the cells were actually able to generate and maintain the E_m level being simulated. Design of the ionic compositions of such 'potential simulation' media can be based either upon existing experimental concentration data for cells of known E_m level (e.g. nerve, muscle), or upon estimated values obtained from conventional membrane transport theory using appropriate assumptions regarding permeabilities and whether active or passive transport is occurring for the various ion species.

Utilizing this potential-simulation technique, the present experimental investigation was conducted to establish the effect of E_m level variation on the mitotic activity of a representative mammalian cell line *in vitro*. Of primary interest was the question of whether sufficiently negative E_m levels could *reversibly* block mitosis in cells which, at their normal low E_m level *in vitro*, exhibit vigorous and continuous proliferation. The results demonstrate conclusively for the cell line examined that ionic conditions corresponding to various E_m levels do in fact exert a pronounced control over mitotic activity and, furthermore, that the total but reversible arrest of proliferation found to accompany very negative E_m levels is brought about by prevention of DNA synthesis, in accord with the basic premise advanced by CONE [1969].

Materials and Methods

Plan of Experiments

Using monolayer populations of naturally synchronized cells[1] so as to preclude introduction of metabolic and mitotic abnormalities by the synchronization process itself, the minimum E_m level[2] (i.e. the corresponding simulation medium composition) which blocked all mitosis was determined in preliminary tests using a graded series of E_m simulation media covering the maximum E_m range normally found *in vivo* (–10 to –90 mV). The quantitative effects of this minimum-E_m medium on mitotic activity and DNA synthesis were then determined by monitoring the time increase of both total and ^{3}H-thymidine pulse-labeled cells during continuous incubation of newly synchronized cell populations for a period of approximately 2T (T = normal cell cycle period *in vitro*), followed by a mitotic recovery period of one T during which the test medium was replaced with normal culture medium. The data of the first period established the mitosis and DNA synthesis blocking effectiveness of the (simulated) 'high E_m' condition. Those of the second period established the time and degree of mitosis and DNA synthesis recovery upon return to normal E_m conditions, thus demonstrating the relative reversibility of the E_m-imposed mitotic block. Additionally, a 'long-term' test in which newly synchronized populations were monitored for cell number increase during continuous maintenance in the high E_m medium for a period of approximately 6T was run to ascertain that spontaneous recovery of mitotic activity did not occur as long as the cells were maintained at the high E_m condition, and also to determine the rate of cell death during prolonged mitotic blockage.

Cells

All experiments were performed with naturally synchronized Chinese hamster cells [CHO cell line, TJIO and PUCK, 1958] in monolayer culture. The CHO line was chosen because of its excellent suitability for natural synchronization. Synchronization was accomplished by the metaphase shake-off technique [PETERSON *et al.*, 1969], which generally yielded test cell populations consisting initially of 85 to 90% metaphase cells.

Culture Media

Stock cultures and experimental controls were maintained in F-10 medium [HAM, 1963] without added Ca^{++} and supplemented with 10% calf serum and 5% fetal calf serum (Balt. Biol. Co.). The generation cycle period of CHO cells may vary considerably with media prepared from different lots of serum [TOBEY *et al.*, 1967]; all experiments of the present study were performed using the same serum lot.

The primary test medium, designated HEM_{100} ('high E_m medium'), was designed to produce intracellular osmotic conditions and concentrations of Na^+, K^+, and Cl^- in the

[1] Obtained by shaking rounded metaphase cells free from the surface of large monolayer cultures.

[2] Since the potential of somatic cells is always negative relative to the bathing medium, reference only to the numerical value of the E_m level is made herein. Thus 'high E_m level' denotes a large negative value (e.g. –60 mV) while 'low E_m level' denotes small negative values (e.g. –10 mV).

CHO cells approximating those of mammalian muscle at E_m = –90 mV (table I). This HEM_{100} formulation was based on the assumption that the equilibrium intracellular concentrations of Na^+, K^+, and Cl^- in CHO cells maintained in this medium would be essentially the same as in the medium itself, since the basic E_m level of the cells *in vitro* is so small[3]. HEM_{100} was made by substituting for the usual NaCl, KCl, Na_2HPO_4, and $NaHCO_3$ components of the normal F-10 medium [HAM, 1963], the proper amounts of KCl, K_2SO_4, K_2HPO_4, $KHCO_3$, and sucrose to yield the required osmolarity and Na^+, K^+, and Cl^- concentrations, with due allowance for the Na^+ and K^+ content of the serum used (see table II). In determining the osmotic balance, the cell was assumed to be impermeable to SO_4^{--}.

To obtain test media with ionic compositions approximating lower E_m levels, the primary HEM_{100} was diluted with increasing amounts of normal F-10 medium. This medium series was designated HEM_{xx}, where the subscript expresses the percentage of HEM_{100} in the particular test medium (e.g. HEM_{55} consists of 55% HEM_{100} and 45% normal F-10 medium). Thus, as the test medium varies from 100 to 0% HEM_{100}, the corresponding (simulated) E_m level varies from –90 mV to –10 mV (the E_m level of interphase CHO cells in F-10 medium). Although the relationship between the percentage HEM_{100} (i.e. between the resulting $[K^+]_i/[Na^+]_i$) and the corresponding simulated E_m level is probably not precisely linear (due to the logarithmic terms in the Nernst equation), assumption of a linear relation should give a reasonably accurate first order approximation of the simulation E_m level corresponding to each concentration.

In reality, it was not possible to make up HEM_{100} medium *per se* since the $[Na^+]$ contribution of the serum component used was itself in excess of the required 12 $\frac{\mu mole}{ml}$ for –90 mV simulation. The highest E_m simulation medium actually attainable was HEM_{89}; all concentrations of the E_m simulation series herein are, however, expressed in terms of equivalent dilution of HEM_{100} with normal F-10, although actually prepared by dilution of HEM_{89}. Final Na^+ and K^+ concentrations in the test media were verified by direct atomic absorption spectrographic analysis.

Table I. Ion Distribution in Mammalian Muscle Cells[1]

Ion[2]	Intracellular	Extracellular
Na^+	12	145
K^+	155	4
Cl^-	4	120
$(Others)^-$	8	34
$A+A^-$	155	57

[1] Concentrations in μmole/ml; E_m = –90 mV.

[2] (A) denotes inorganic molecules; (A^-) denotes inorganic anions.

[3] The validity of this assumption has been implied by the results of atomic absorption spectrographic analysis of total intracellular Na and K of interphase CHO cells equilibrated in F-10 and in HEM_{85}.

Table II. Modification of F-10 Medium Salts to Prepare HEM_{89}[1]

Medium[2, 3]	NaCl	Na_2HPO_4	$NaHCO_3$	KCl	K_2SO_4	K_2HPO_4	$KHCO_3$	Sucrose	H_2O
F-10	7.400	0.154	1.200	0.285	—	—	—	—	to 1 liter
HEM_{89}	—	—	—	0.984	9.951	0.160	1.215	5.169	to 1 liter

[1] All weights given in grams.

[2] Only salt composition was varied; all other medium components identical to normal F-10 medium [HAM, 1963].

[3] Na and K contents of combined sera used were 156.0 and 4.6 μmole/ml, respectively.

Test Procedures

A preliminary test was conducted to determine the minimum HEM concentration (i.e., E_m level) which completely blocked division in the CHO cells. Pooled, synchronized cells obtained by shake-off were chill-accumulated [PETERSON *et al.*, 1969] in F-10 for a given test, and inoculated into Falcon plastic culture flasks with F-10 at 37°C. 50 min (a period determined in separate tests) were allowed for the cells to complete initial division and attach; the end of this period was designated time zero (t = 0) and corresponded to very late telophase. During this settling period, the open flasks were exposed to a 5% CO_2 atmosphere [PETERSON *et al.*, 1969]. The test medium concentrations studied ranged from HEM_0 (i.e. the normal F-10 control) to HEM_{89}; duplicate test flasks were used for each medium concentration. All cells for a given test were taken from the same shake-off population. Cell surface densities (~150 cells/mm²) in test flasks were sufficiently sparse and uniform to preclude any mitotic inhibition effects due to confluency up to intervals of several T. At confluency, CHO cell surface density is approximately 1800 cells/mm². At t = 0, the F-10 of the test flasks was replaced with test medium, following two washings with test medium, and the cells incubated at 37°C. Viable-cell counts were made visually within specific ruled areas of the flasks at t = 0 and at 24-h intervals for 96 h, or until confluence occurred. The ruled areas of each flask contained a total of approximately 500 initial cells (t = 0). The minimum HEM concentration required for complete mitotic blockage was HEM_{85} (~–75 mV); this medium was consequently used for all subsequent tests. The pH was monitored throughout the experimental period by observation of the medium indicator color; pH was maintained between 6.8 and 7.0.

The effects of HEM_{85} on mitotic activity were determined for naturally synchronized cells using exactly the same procedures as outlined above for the preliminary test, only cell counts were made at 2-h intervals from t = 0 to t = 33 h. At t = 33 h, the test medium was replaced with normal F-10 and the cell counts continued for an additional 20-h period to determine if mitosis recovery occurred. A long-term test was conducted similarly, only the cells were maintained continuously in the HEM_{85} medium for 120 h (6 T) with periodic counts of the viable cell number, following which the HEM_{85} was replaced with F-10 and the culture observed for resumption of mitotic activity. A comparison long-term test was also run using F-10 containing 0.40 mg/ml of 5-aminouracil (the minimum concentration found to block mitosis in CHO cells) as the test medium.

The effects of HEM_{85} on DNA synthesis activity were determined by pulse labeling with ^{3}H-thymidine. The tests were conducted with cell samples prepared in exactly the

same manner as those above for both the short- and long-term mitotic blockage and recovery tests; the test cells were incubated, however, on plastic slides (made from plastic flask bottoms) immersed in HEM_{85}. Samples slides were removed periodically from the HEM_{85} and incubated for 15 min in HEM_{85} containing 0.5 μM/ml of ^{3}H-thymidine (activity = 15 c/mM), following which they were washed, fixed (3:1 ethanol: acetic acid), dried, coated with NTB-3 liquid emulsion (Kodak), and exposed for 12.2 days. Time of fixation was taken as the test sample time (relative to time zero). Following development in Dektol (Kodak), nuclear staining was performed with 0.25% toluidine blue. DNA synthesis activity was determined by scoring the fraction of cells with labeled nuclei corresponding to each sample time using 1000 randomly selected cells per sample slide.

Results

Determination of Minimum E_m Level for Complete Blockage of Mitosis

The effects of simulated E_m level on the mitotic activity of naturally synchronized CHO cells over a 96-h period are shown in figure 1. Immediate mitotic suppression (i.e. within 24 h) begins at about HEM_{45} (~ −46 mV) reaches the condition of complete arrest at HEM_{85} (~ −75 mV). Interestingly, this latter level is essentially the same as that maintained *in vivo* by nondividing neurons. Above HEM_{84} cell division is completely ar-

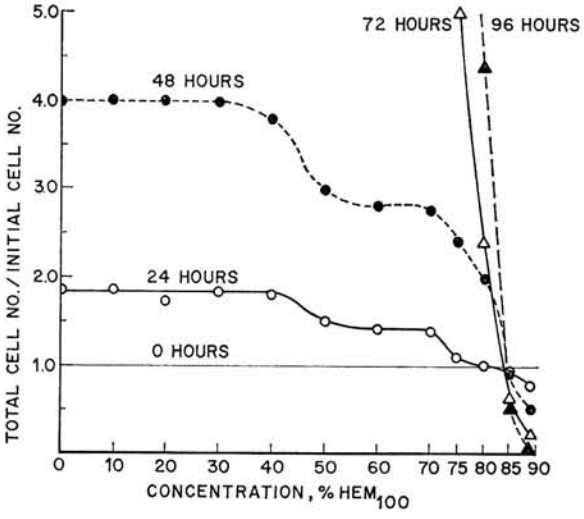

Fig. 1. Effect of simulated E_m level (HEM_{100} concentration) on mitotic activity in naturally synchronized CHO cells at 24-h intervals.

rested and cell death slowly reduces the population at HEM_{85} and more rapidly at HEM_{89}. As ascertained by direct observation, no cell death above the normal control (i.e. HEM_0 or F-10) value of 4% of a given population per 24-h period, occurred for HEM_{80} and below during the 96-h period. The crossover point between net proliferation and net population decrease occurs for all time periods in the vicinity of HEM_{84}; at this E_m level, therefore, the small fraction of cells which die in each time period is exactly replaced by the small fraction of cells which divide, and population constancy is maintained. Below HEM_{84}, total cell division exceeds cell death while above HEM_{84} all cell division is blocked and population decrease follows gradually from cell death. The relatively rapid decrease in viable cells in HEM_{89} indicates that the conditions of this E_m level, or at least the abrupt imposition of this level, on CHO cells are relatively toxic. To insure complete mitotic blockage in the ensuing tests, HEM_{85} was taken as the minimum E_m for mitotic arrest.

Effect of High E_m Level on Mitotic Activity

The effect of HEM_{85} medium on CHO cell mitotic activity is shown in figure 2. As expected from the preliminary tests, HEM_{85} entirely blocks cell division during the 33-h treatment, whereas the control cells in normal F-10

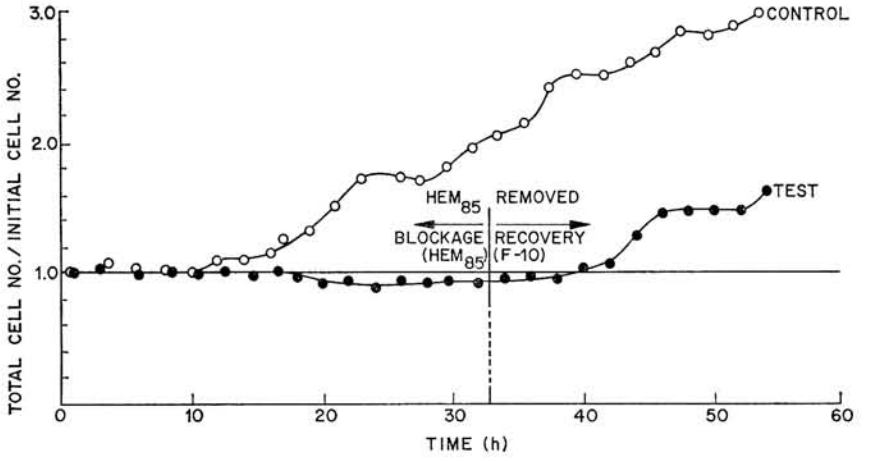

Fig. 2. Effect of simulated E_m level of –75 mV (HEM_{85} test medium) on mitotic activity in naturally synchronized CHO cells. Control cells maintained in normal F-10 medium. HEM_{85} test medium was replaced with F-10 medium at 32.8 h.

proceed to divide in a synchronous wave with a cycle time of 20 h. Direct visual observation again demonstrated no cell death beyond control values during the 33-h blockage period. The data of this figure also demonstrate that the E_m-induced mitotic blockage is fully reversible; the midpoint of the mitotic rise occurs some 11 h after removal of the HEM_{85}. The percentage of the previously blocked cells which divided was essentially the same as for the first mitotic wave of the unblocked controls.

The results of tests to ascertain the long-term blocking effect of HEM_{85} are shown in figure 3. It is seen that mitosis is in fact completely blocked so long as the cells are maintained at the high E_m level, as determined by direct visual observation over the 120-h period. The percentage of dead cells increased very slowly up to 60 h (3 T), following which the death rate began to increase. At t = 120 h (6 T) some 26% of the initial population were still viable and morphologically normal in appearance except for slight rounding. Following replacement of the test medium, mitotic activity resumed in the remaining cells such that 40% had divided within 26 h. Also shown in figure 3 are the results of a comparison long-term mitotic blockage test in which 5-aminouracil (5-AU) was used as the blocking agent. The 5-AU concentration used was the minimum required for blocking all mitosis in CHO cells in F-10, as determined by separate tests. As is evident,

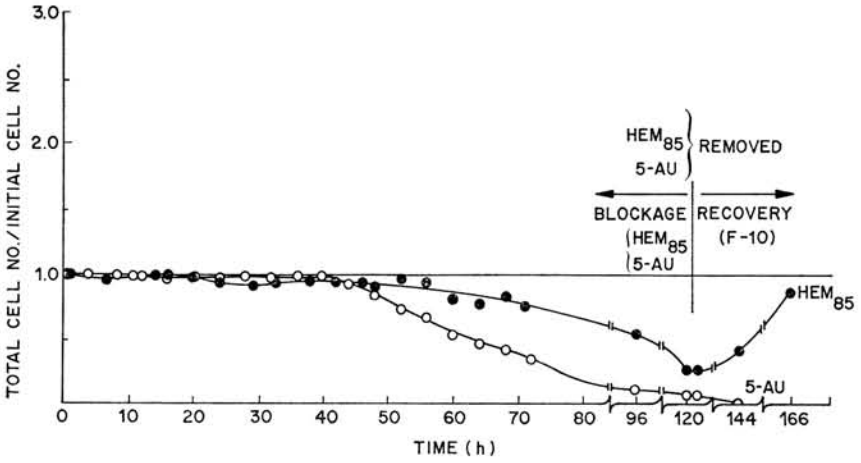

Fig. 3. Long-term effect of continuous simulated E_m level of –75 mV (HEM_{85} test medium), and treatment with 5-AU (0.4 mg/ml) on mitotic activity and cell death in naturally synchronized CHO cells. Test media replaced with F-10 medium at 120.6 h. F-10 control same as in figure 2.

long-term cell death with 5-AU appreciably exceeds that with HEM_{85}, beyond 50 h. In addition, the 5-AU blocked cells underwent grotesque morphological changes, forming into multinucleate giant cells; these cells subsequently died without division following replacement of the 5-AU medium with F-10. It thus appears that while the cell death rates during long-term mitotic blockage with HEM_{85} and 5-AU are roughly comparable, such mitotic blockage is reversible only in the case of the HEM_{85}. This reversibility of HEM-blocked cells was further demonstrated by an additional experiment in which unsynchronized CHO cells were maintained in HEM_{89} for approximately 168 h (~8.5 T). Although only about 5% of the cells were viable at the end of this period, these cells appeared quite normal and resumed mitosis upon return to F-10, soon proliferating to the original population level.

Effect of High E_m Level on DNA Synthesis

The effect of HEM_{85} on DNA synthesis in CHO cells is shown in figure 4; these data correspond to the mitotic blockage and recovery test results presented in figure 2, the test cells coming from the same original population. The data clearly demonstrate that the simulated high E_m level corresponding to HEM_{85} fully (but reversibly) blocks DNA synthesis, and thus implies that the mitotic blockage shown in figure 2 is a consequence of the prior blockage of DNA synthesis. From figure 4, the time required for attainment of maximum DNA synthesis activity in F-10 (control cultures) is ~15 h. This is essentially the combined lengths of the G_1 (7.5 h) and S (7.0 h) periods for CHO cells with a 20-h cycle period. If we assume that the

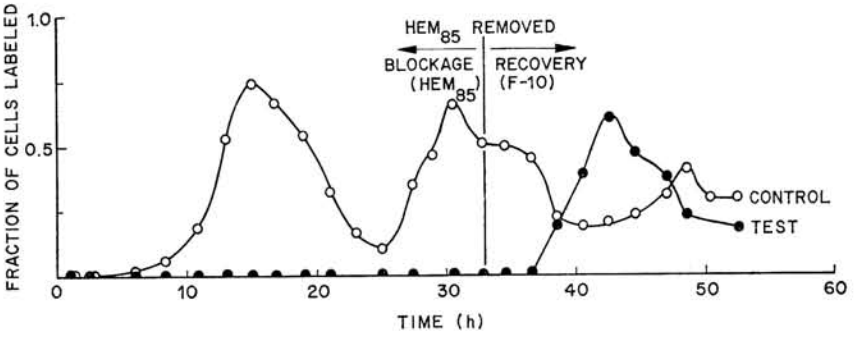

Fig. 4. Effect of simulated E_m level of –75 mV (HEM_{85} test medium) on DNA synthesis activity in naturally synchronized CHO cells as determined by ^{3}H-thymidine pulse-labeling. Control cells maintained in normal F-10 medium.

DNA synthesis period of the cells previously blocked in HEM_{85} is also 7 h (as seems reasonable from the data), then it appears that G_1 mitotic blockage occurs at a point roughly 3 h before the end of the G_1 period, since maximum DNA synthesis activity occurs some 10 h after removal of the HEM_{85}. Thus, if no appreciable time is required for recovery and resumption of mitotically directed metabolism after removal of the HEM_{85}, mitotic blockage by HEM_{85} apparently occurs late in the G_1 period. The autoradiographic studies of DNA synthesis conducted as part of the long-term tests (fig. 3) revealed, as might be expected, the complete absence of DNA synthesis during the treatment period.

One series of (short-term, 33-h) blockage tests was run using CHO cells in suspension (spinner) culture with electronic counting. These tests yielded essentially the same DNA and mitosis blockage and recovery as observed with the monolayer cells, only the cycle period was slightly longer.

Discussion

The present experimental study was designed to provide an essential qualifying test, using an arbitrary but representative cell system, of the basic premise of CONE [1969] that variations in the intracellular ion hierarchy accompanying different E_m levels in somatic cells might constitute a mechanism for natural *in vivo* control of mitosis, possibly through G_1 period blockage of metabolic events leading to DNA synthesis. In order to provide a preliminary validation of this premise for the *in vivo* case, it is first necessary to demonstrate conclusively for at least one representative somatic cell type that an effective increase of the E_m level to values representative of nondividing cells *in vivo* imposes a reversible G_1 blockage of DNA synthesis, and hence of mitosis. In regard to the *in vivo* applicability of the premise, demonstration that the mitotic block occurs in the G_1 period and is reversible is a mandatory feature of the validation, since it is well established that practically all mature *in vivo* somatic cells reside in G_1 and that mitotic homeostasis is maintained in most somatic tissues by controlled mitotic activation from within this pool of G_1-blocked cells. The underlying rationale of the original premise and of the present experimental design is based upon the precepts of conventional membrane potential theory and their proposed implications [CONE, 1969] for electroosmootic feedback control of the intracellular ionic and osmotic environment, and hence of the cell's metabolic activities.

The selection of the CHO cell line for these experiments was based on the possibility of obtaining large numbers of naturally synchronized cells. Additionally, the diploid uniformity of these cells would be expected to produce a relatively high uniformity in population response to imposed experimental conditions. Although complete electrochemical potential data for Na^+ and K^+ for the CHO cycle are not yet available, preliminary analyses on interphase CHO cells have clearly demonstrated that K^+ is in fact concentrated and Na^+ extruded, implying operation in these cells of the basic Na^+–K^+ regime of conventional theory and thus validating a key criterion in the design of the present experiments. Very late telophase was intentionally selected as the time of test medium application on the assumption that at this time the cells would just be reestablishing their cytogenetic-metabolic activity and would thus activate the integrated pattern of genome expression most compatible with their new intracellular ionic regime (equivalent to the new E_m level). Presumably, this time of application would also impose minimum shock on the already-established aspects of cell metabolism.

In regard to the effects of the HEM_{100} concentration range (fig. 1), it is interesting that concentrations in the range of HEM_0 to HEM_{40} (E_m between –10 and –40 mV) exert no early influence on mitotic activity, while despite the diploid uniformity of the CHO test cells, the relative number of cell divisions which occur within a given initial population within a given time period decreases continuously in the range from HEM_{40} to HEM_{84} (E_m between –40 and –75 mV). It is within the upper half of this latter range that the E_m levels of most *in vivo* somatic tissue cells appear to reside. The higher concentrations of HEM_{100} (i.e. HEM_{89}, $|E_m|>75$ mV) appear to be somewhat toxic in that appreciable cell death occurs within a relatively short period (2 to 3 T). This effect might be attributed to the possible shock these cells experience when suddenly exposed to such substantial alterations in their $[K^+]_i/[Na^+]_i$, after years of adaptation to growth at an E_m level of –10 mV. However, the fact that some cells (~5%) of any population survive indefinitely in the G_1-blocked condition in HEM_{89}, with subsequent resumption of normal mitosis upon return to F-10 demonstrates that long-term CHO existence in HEM_{89} is possible and suggests that perhaps a more gradual adaptation to the HEM_{89} conditions might result in much less early cell death at the higher concentrations. Other factors probably influencing cell death in HEM_{89} are discussed subsequently. Experiments are presently in progress to establish whether clones of 'HEM_{89}-resistant' CHO cells can be developed.

The basic results of figures 2 and 3 demonstrate that intracellular ionic conditions approximating those which would theoretically accompany an E_m level in the vicinity of –70 mV effectively block all mitotic activity in CHO cells so long as the simulated E_m level is maintained, and that reversion to lower E_m levels results in active resumption of mitosis even after long-term blockage. A gross spectrographic analysis of naturally synchronized interphase CHO cells demonstrated that a decrease in total (free and bound) intracellular Na and an increase in K commensurate with an E_m level of –70 mV ($[K^+]_i/[Na^+]_i$ from approximately 4 to 8) occurs when the cells are transferred from F-10 to HEM_{85} medium, the actual physical E_m level changing only slightly (–10 mV to –6 mV), thus implying that the intended changes in intracellular ion concentration levels necessary for simulating high E_m levels were in fact achieved in the present experiments.

Cells permanently blocked in G_1 will, of course, ultimately die since all cells have finite lifetimes, terminated by either death or division. It is thus only to be expected that all cells of a given CHO population continuously blocked in HEM_{85} will ultimately die out since division is impossible; presumably this factor is one basic cause of the gradual population depletion observed in the long-term experiment of figure 3. It might be argued, however, in the case of the present experiments that in reality the observed cell death is due to an immediate and substantial upset of cell metabolism induced by the HEM_{85} conditions, and that the mitotic blockage is but a consequence associated with this ultimately lethal metabolic upset. On the other hand an appreciable percentage of a given CHO population will live for a period equivalent to several cell cycles under the HEM_{85} conditions, thus indicating that the essential aspects of cell metabolism cannot be too severely affected by the high E_m conditions. Furthermore, the mitotic blockage which occurs is entirely reversible upon return to normal *in vitro* (i.e. low E_m) conditions, as shown not only by the results of figure 2 but also by the fact that cells held in mitotic arrest for 120 h (6 T) and longer resume active division upon return to low E_m conditions. Thus the existence of a drastic alteration of metabolism in these cells would not be expected.

The general similarity of the long-term cell death histories in both HEM_{85} and 5-AU blocked CHO cells (fig. 3), despite the obviously different overall effects on metabolism of these two agents, also suggests that the cell death seen with HEM_{85} may be more a product of long-term mitotic blockage *per se* rather than a specific toxic effect of the intracellular Na^+–K^+ change. It must also be realized in this regard, however, that the present experiments, while giving a useful first-order simulation of E_m level changes in

relation to intracellular ionic conditions, are by no means a complete simulation of the *overall* conditions which would exist in the case of *naturally* modulated changes by variations in the actual E_m level of the membrane itself. In particular, the $[K^+]$ and $[Na^+]$ seen by the exterior surface of the cell in HEM_{85} are quite different from those experienced in F-10 (or in normal interstitial fluid *in vivo*), and these differences could conceivably lead in long-term treatment to changes in membrane and/or surface structural and functional integrity. These alterations in turn could lead to changes in absolute and selective membrane permeabilities with subsequent effects on metabolism or even cell viability. Moderate blebbing of the cell surface has in fact been observed in some CHO cells after long-term treatment with HEM_{89}. It is thus possible that at least part of the cell death observed in the longterm experiments with HEM_{85} (fig. 3) could be associated with changes in the membrane, ultimately induced by long-term exposure to the abnormal external ionic environment. The same factor could be associated with the relatively early toxic effects of HEM_{89} (fig. 1). The significance of the present results, however, is that cell division can be effectively and reversibly blocked in CHO cells for extended periods by conditions simulating high E_m levels. Under *in vivo* conditions, where E_m changes would be accomplished by the natural functioning of the membrane itself, the rate of cell death in long-term mitotic blockage by high E_m levels might be grossly reduced; (i.e. the average lifetime of 'E_m-blocked' cells *in vivo* might be much longer than that obtained in the present simulation experiments).

Unlike mitotic blockage by high E_m conditions (i.e. HEM_{85}), long-term blockage by 5-AU is irreversible and leads to highly aberrant morphological changes and cell death. Apparently, the blockage imposed by 5-AU (although involved with only one aspect of DNA precursor metabolism) substantially alters the overall cell metabolism in long-term treatments. The absence of morphological abnormalities and the resumption of normal mitosis upon return of HEM_{85} blocked cells to low E_m conditions (i.e. F-10), on the other hand, suggests that such mitotic control by E_m-level regulation must occur by relatively natural mechanisms, and hence could serve as an *in vivo* mechanism for mitotic regulation.

The results of figure 4 indicate that mitotic blockage is imposed in the latter half of the G_1 period and is associated, as suggested [CONE, 1969] with an arrest of DNA synthesis. Since completion of DNA synthesis is generally a prerequisite for mitosis initiation [MAZIA, 1961] especially for cells such as the present CHO line having low ploidy and teny, it appears reasonable to assume that the observed mitotic blockage by simulated high E_m level is a

direct result of DNA synthesis blockage. The apparent location of the mitotic block in the latter half of G_1 suggests that prevention of DNA synthesis might be due to metabolic or enzymic alterations involving blockage of DNA precursor or polymerase synthesis or activation. Experiments to elucidate the biochemical sources of this DNA synthesis blockage by changes in $[K^+]_i$ and $[Na^+]_i$ are currently in progress.

In view of the present overall results, it appears reasonable to conclude that the premise [CONE, 1969, 1970] that mitotic activity and associated DNA synthesis might be blocked by intracellular ionic conditions accompanying high E_m levels is in fact valid for the CHO cell line. Although such validity has been demonstrated experimentally only for these particular cells, the CHO line is considered to be generally representative mitotically of a wide range of somatic cell types in culture and hence it seems reasonable to expect that such cell lines will also exhibit the same mitotic blockage in response to imposition of simulated high E_m conditions. The positive results of the present study with the CHO line in culture therefore give credence to the basic concept regarding E_m level variations as a fundamental mechanism of mitosis control, and provide a basis for designing extended investigations of *in vivo* systems. In view of the present results for cells *in vitro*, it seems quite feasible that natural variation of the E_m level of somatic cells could serve as an effective mechanism of mitosis control *in vivo*.

If such a mitotic regulatory mechanism as proposed were found to be generally active *in vivo*, mitosis control would be intimately involved with the particular aspects of cellular environment and metabolism which govern E_m generation and E_m level determination. Of particular interest are the cell surface and its contact associations, including specific membrane permeabilities and their changes, and active ionic transport variations, all of which may be intimately coupled by feedback mechanisms with the cellular metabolism [CONE, 1969, 1970]. In regard to this implied importance of the cell surface, it seems significant that adaptation of normal (*in vivo*) tissue cells of many types to growth in culture is characterized by a pronounced decrease in the E_m level of the cells (upon disaggregation) and that this depolarization is accompanied by a gross increase in mitotic activity.

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