Contact Inhibition of Division: Involvement of the Electrical Transmembrane Potential [1]

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ABSTRACT Measurements of simultaneous mitotic activity, electrical transmembrane potential (E_m), and cell density levels in both 3T3 and Chinese hamster ovary (CHO) cell cultures reveal that a 5- to 6-fold increase in the E_m level is associated with development of mitotic arrest at saturation densities. This rise occurs both in confluent monolayers and in interior areas of isolated colonies, and is independent of the rate at which confluence is attained. The E_m rise is accompanied by a substantial decrease in intracellular Na. Electron microscopy of saturated CHO monolayer sections shows from 46 to 63% of the cell surfaces to be in close apposition (< 300 Å spacing). These results for contact-inhibited cultures support the hypothesis that mitotic activity may be functionally coupled with the E_m level and associated ionic concentration levels. It is suggested that contact inhibition of mitosis may result from a reduction in synthesis of mitogenically essential RNA following a decrease in intracellular Na produced by contact-induced alteration of surface ion-transport activity.

Many cell systems exhibiting contact (density-dependent) inhibition of mitosis at confluency can be maintained for long periods in a viable and nonproliferating state arrested in the G_1 stage (Todaro and Green, '63; Todaro et al., '64; Nilausen and Green, '65; Defendi, '66; Vasiliev et al., '66, '69; Todaro et al., '67), yet can be stimulated to resume mitosis by a variety of chemical and physical treatments (Todaro et al., '65, '67; Holley and Kiernan, '68; Wiebel and Baserga, '68; Kolodny and Gross, '69; Dulbecco and Stoker, '70; Vasiliev et al., '70, '71). Such systems provide useful models for investigation of mitogenic control mechanisms in normal cells and the alterations induced therein by malignant transformation (Temin and Rubin, '58; Vogt and Dulbecco, '63; Todaro et al., '63, '64; Defendi, '66; Eagle et al., '70). Evidence from such studies has strongly implied that the cell surface plays a key role in mitotic control. The actual mechanism(s) by which the cell surface presumably acts to exert control over intracellular mitogenic metabolism however, is presently undefined.

One mechanism recently proposed is based upon observations suggesting that a functional correlation exists between the degree of mitotic activity of a cell and the ionic concentrations associated with the level of its electrical transmembrane potential (E_m). Changes in the intracellular ionic concentration levels resulting from changes induced in active and passive ion transport through the plasma membrane by various surface conditions, are hypothesized to constitute a basic controlling influence by modulating, either directly or indirectly, one or more key metabolic events required for the initiation of mitogenesis (Cone, '69, '71). Support for this hypothesis has been provided by demonstrations that imposition of intracellular ionic concentrations corresponding to a high E_m level fully, but reversibly, block log phase cultured cells in the G_1 stage (Cone and Tongier, '71; Orr et al., '72). In a continuing examination of this hypothesis, the study reported here investigated the changes in E_m level and intracellular ionic concentrations associated with the development of mitotic arrest in various culture forms at saturation densities. The results are found to be in accord with the precepts of the general hypothesis and suggest, in association with other recent electrophysiological and bio-

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chemical findings, a sequence of basic interactions involved in contact inhibition of mitosis.

MATERIALS AND METHODS

Cell lines and media

The principal cell line studied was the Chinese hamster ovary (CHO) line (Tijo and Puck, '58), although several parallel experiments were made using the 3T3 line (Todaro and Green, '63). The CHO cells were originally obtained from Dr. R. A. Tobey and are the same line used in previous studies of E_m-ion regulation of mitogenesis (Cone and Tongier, '71). CHO stock cultures were maintained in F-10 culture medium (Ham, '63) containing 10% calf and 5% fetal calf serum (Bio-Quest) equilibrated with 5% CO_2 (pH 7.2), using the same culturing procedures described previously (Cone and Tongier, '71). The 3T3 cell line used was obtained from Dr. H. Green. 3T3 stock cultures were cultivated in 30 ml plastic flasks with Dulbecco and Vogt's modification of Eagle's medium (Eagle et al., '57) containing 10% calf serum (BQ) and equilibrated with 10% CO_2 (pH 7.2). The cells were maintained using culturing procedures previously described (Todaro and Green, '63). In view of the effects of different serum lots on mitotic properties (Tobey et al., '67; Cone and Tongier, '71), all tests were performed with media using the same serum lot. Test cultures were maintained in the respective media types used for stock cultivation. Cultures were free of PPLO.

Culture systems

Monolayer cultures. (a) CHO cells. Time-lapse cinematographic recordings were made of monolayer cultures to provide detailed information on the simultaneous local mitotic index and cell density during development of confluency and at saturation; E_m measurements were made at several density levels in chambers which were replicates of those used for time-lapse recordings. For checks, E_m measurements were made directly on some chambers which had been recorded in time-lapse up to the time of measurement. Monolayer test cultures were maintained at 37° C in 26 mm diameter perfusion chambers (Cone and Tongier, '69) using coverslips cut from plastic Petri dish bottoms. Chambers were seeded with 1.1×10^5 cells/cm^2 and observed in time-lapse for up to 130 hours. During filming, chambers were perfused continuously with fresh medium (pH 7.2) at the rate of 1 ml/day. For E_m measurements, the coverslips were removed from the chambers and placed in Petri dishes. In tests to establish that the E_m level at saturation is independent of the rate at which confluency develops, some chambers were seeded with 2.0×10^5 cells/cm^2. For ion content (Na, K) determinations, cultures were grown to desired test densities at pH 7.2 in 250 ml plastic flasks. Chamber cultures used for electron microscopy of cell contact at saturation were replicates of those used for filming and E_m measurements. *(b) 3T3 cells.* E_m, mitotic index, cell density, and ion content determinations for 3T3 cells were made for the log through saturation phases of monolayer cultures. The cells were grown in 60 mm plastic Petri dishes (250 ml flasks for ion determinations) at 37° C from a seeding density of 0.3×10^4 cells/cm^2, using "conditioned" medium (i.e., originally fresh medium which had been in contact with nearly saturated 3T3 monolayer cultures for 48 hours; 20 ml medium per 250 ml flask). Medium was changed every other day.

Colony cultures. E_m, mitotic index, and cell density were also determined for isolated, clonal colonies of CHO and 3T3 cells, as functions of radial position within the colony. The cells were seeded in 60 mm plastic Petri dishes at a density of two to three singly-dispersed cells/cm^2, and colonies allowed to develop until mitotic activity ceased in the interior of the clones selected for observation. Sequential photographs of selected colonies were made during growth to determine colony developmental rates and characteristics.

Measurement procedures

Cell density. The cell density for CHO monolayer cells was determined from time-lapse films. Preselected areas of cultures were filmed in a Sage (model 100) time-lapse unit at 1.0 frame/minute using Zeiss phase-contrast optics at a magnification of 10 × (0.460 mm^2 field). Cell density was determined by counts of cells per frame, with independent data being ob-

tained from photographs of additional areas in the filmed cultures and by electronic counts (Coulter counter, model B) of trypsinized populations of replicate chambers. Density determinations for 3T3 cultures were obtained by electronic counts of trypsinized dish cultures. Within colony cultures, the local cell density was determined by visual counts from photographs of cells in local (annular) areas (same as used for mitotic counts) just prior to E_m measurements, and verified by nuclear counts in fixed and stained colonies of similar size.

Mitotic activity. The local mitotic index (fraction of cells dividing per unit time) was determined for CHO monolayer cells from time-lapse films using an analysis procedure described previously (Cone and Tongier, '69). Mitotic activity in 3T3 cultures was inferred from the rate of cell density increase. Mitotic activity in colony cultures was determined by counts of rounded cells in local (annular) areas in hourly photographs taken over the ten hour period immediately preceding E_m measurements. The distribution of activity was independently determined in colonies of similar size by local metaphase counts following an eight hour colchicine treatment (0.1 mg/ml medium).

Transmembrane potential. E_m was measured by cell impalement with glass ultramicroelectrodes filled with 3 M KCl (tip diameter $<0.25\ \mu$; resistance >30 megohms; tip potential <5 mV), using a Leitz micromanipulator system (model 52-21a) and fixed-stage microscope. Contact with the pipet fillant and culture medium was made through Ag-AgCl wire electrodes connected to an ELSA-2 (Electronics for Life Sciences) negative capacitance amplifier, and output voltages were fed through a Bell and Howell 1-172-12 galvanometer amplifier and recorded on an S-134 Bell and Howell Datograph lightbeam recorder. Resistance and voltage calibrations were made using a Tektronix dual beam oscilloscope (model 555). The micromanipulator-microscope unit was entirely enclosed in a Lucite glovebox where a temperature of $37 \pm 0.3°$ C and proper humidified CO_2 atmosphere for each cell type were maintained.

E_m measurements for CHO monolayer cultures were made at several stages of development up through saturation (one replicate culture at each density level, ~ 50 cell measurements in each of three separate fields per culture). For these measurements, selected areas were first identified by a mark on the outside of the coverslip and photographed for density determinations; the perfusion chambers were then opened within the glove-box and the cell-containing coverslip placed in a 60 mm Petri dish containing perfusion medium at pH 7.2. E_m measurements were made in the same areas photographed, for correlation of the E_m level with the coexisting density. E_m measurements for 3T3 monolayers were made for the log through saturation phases using cultures which were replicates of those used for density determinations (1 replicate culture per density condition, ~ 50 cells measured in each of 3 separate fields per culture). E_m was determined in CHO and 3T3 colonies as a function of local radial position in colonies whose mitotic index and cell density distributions had been monitored photographically over the preceding ten hours. Measurements were obtained within small local (annular) areas along the radius used for density and mitotic activity determinations (3 colonies of each line examined, ~ 200 cells measured per colony).

Only cell voltage recordings which were constant for five seconds or longer following electrode penetration were utilized in determining the E_m level. Special care was taken to perform measurements under conditions of minimum vibration, since electrode vibration was found to severely inhibit the ability to accurately record the higher E_m levels, presumably because of impairment of sealing of the membrane to the electrode, with subsequent leakage-current discharge of the cell. 3T3 cells were found to yield a higher percentage of successful penetrations (steady voltage recordings) than CHO cells, especially for isolated cells, apparently due to better sealing of the 3T3 cell membrane to the glass electrode.

Ion concentrations. Total intracellular Na and K were determined for CHO and 3T3 log and saturation phase monolayers. Special care was taken to minimize errors arising from washing procedures. Cells were grown to desired density in replicate

250 ml plastic Falcon flasks. The cells from one flask were removed by trypsin and cell number, mean cell volume, and the volume frequency distribution determined electronically (model B Coulter counter coupled with a 4,096 channel Kicksort model 711A pulse-height analyzer). Trypsinization was accomplished using a five minute treatment with 1.0 ml of 0.1% trypsin in Tris-buffered choline chloride solution (pH 7.2) containing sufficient choline chloride to produce exact isotonicity relative to the culture medium (292 milliosmols/liter). This procedure, developed in a separate study using volume-time extrapolation techniques, required minimum correction for the effects of trypsinization on cell volume ($<3\%$ volume change). Test flasks were emptied of medium, washed six times with 20 ml aliquots of Tris-buffered isotonic choline chloride at 4° C, the monolayer removed by treatment *in situ* with 10 ml of 12 N HCl for 15 minutes, and then washed three times with distilled H_2O. This washing procedure, developed in separate studies using Na and K tracers, was found to minimize intracellular Na and K losses during washing while providing adequate removal of extracellular ions. The resulting suspension was centrifuged, the cell pellet digested for 24 hours in 12 N HCl, and recombined with the supernate. Na and K were determined by atomic absorption spectroscopy and reported as μmol per milliliter of total cell volume (3 replicate flasks measured per density condition, two independent experiments performed). Checks on the relative levels of Na in log and saturated CHO cells were performed by electron probe microanalysis (Cameca microprobe, model MS46).

Surface proximity and contact. Chamber monolayer cultures of CHO cells in the saturation phase were fixed, stained, and embedded (Brinkley et al., '67), and thin sections (800 Å) examined (1500× and 30,000×) for contact extent and morphology. Serial sections at spaced intervals through several contiguous cells were used to construct surface contours for estimating the percentage of surface in cell-cell contact in saturated cultures. Sections taken from two locations in each of two replicate chambers were analyzed.

RESULTS

Characteristics of confluency development

Monolayers. A brief lag in mitotic activity (~ 5 hours) follows plating of CHO cells, during which some cell motility is observed. Cellular movement decreases due to contact as mitoses begin, and small patches of loosely contacting cells develop. With continuing mitosis, these merge to form a confluent low-density monolayer which briefly maintains an appreciable level of mitotic activity, but as the daughter cells from these divisions press back into the monolayer the cell density rises to the saturation level (4.2×10^5 cells/cm^2) and mitotic activity ceases. 3T3 monolayers follow essentially the same development pattern as the CHO line, but mitosis declines while the monolayer density is still quite low, and ceases at a much lower saturation density (5.0×10^4 cells/cm^2). At saturation, the 3T3 cells appear to be flatter, with more discernable overlapping of cell peripheries than the CHO cells.

Colonies. In the development of CHO colonies from single cells, the daughter cells of the first few generations tend to migrate outward, away from one another (through contact inhibition of inward movement), but the rate of this dispersive motility is sufficiently low that continuing mitoses soon fill the open areas and a roughly circular confluent colony is established (~ 300 cell stage). Mitosis continues within the colony for a time after confluence is reached, resulting in an increasing cell density, but ultimately ceases in the central area as saturation density is attained there (~ 1000 cell stage). The density at which mitotic activity ceases locally (4.3×10^5 cells/cm^2) is practically the same as the saturation density of monolayer cultures. Mitotic activity proceeds undiminished in the peripheral area where continuing outward migration of cells maintains open and low-density areas. The 1.8 to 2.2 mm diameter colonies which were used for the E_m measurements presented herein developed from single cells in nine to ten days, and contained some 7000 to 8000 cells. 3T3 colonies develop in a similar manner but there is more active cell migration. Although even at

the 60–80 cell stage many 3T3 colonies are still not fully confluent, there is much overlapping of peripheries in adjacent cells and cell groups and a definite decrease in overall proliferation rate is observed. Similar results were obtained in a previous study of 3T3 colony development (Fisher and Yeh, '67). In both cell lines, considerable variation in colony growth rate is observed among the separate clones.

Correlation of E_m level, mitotic activity, and cell density

Monolayers. The simultaneously occurring changes in E_m level, mitotic index, and cell density for CHO cells during development of monolayer cultures are shown in figure 1. The measurements were made on cells within preselected areas of the developing monolayer and not confined to cells of a particular isolated colony in the monolayer. After a short lag phase ($\sim$5 hours), mitotic activity rises rapidly to the growth phase level. The average mitotic index during the period of active growth is 0.05 (fig. 1b) which, together with the 87% plating efficiency observed for these cells, indicates a cycle period of 17.5 hours; this value agrees well with the average period (17.8 hours) determined by timing the cycle of individual cells in time-lapse films. Although numerous patches of closely associated cells develop during the active growth period (10 to 27 hours) and the (monolayer average) cell density rises rapidly (fig. 1a), the agreement of the observed cycle period and mitotic index of these contacting cells with those of essentially free log-phase CHO cells indicates such contact has relatively little effect on mitotic activity at this stage. The mitotic index begins to decrease after 26 hours, coincident with development of increasingly larger areas of confluence. By 40 hours the monolayer has become fully confluent (4.0×10^5 cells/cm^2), but a low level of mitotic activity continues

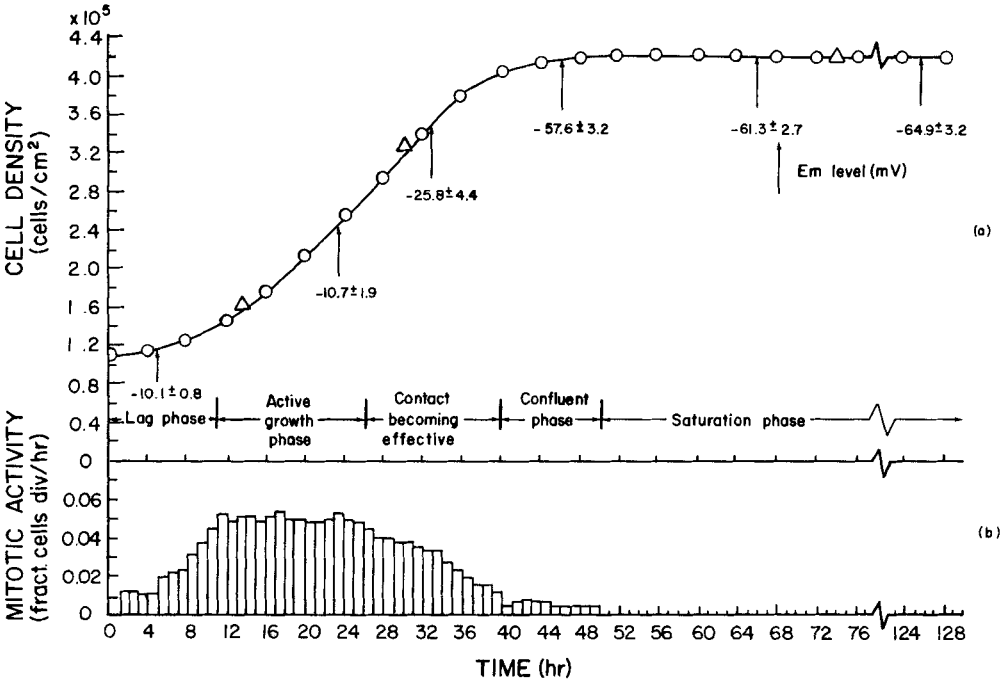

Fig. 1 Variation of cell density, E_m, and mitotic index levels with time in developing CHO monolayer cultures. Density and mitotic results are pooled data from three time-lapse films of selected areas of monolayer cultures; groups of 1600 to 5800 cells analyzed per data point. ○, counts from time-lapse films; △, electronic counts of whole chambers. The E_m value at each density condition is the mean ($\pm$ S.D.) of ∞ 150 cell measurements.

until full saturation density (4.2×10^5 cells/cm^2) is reached at 50 hours. The density values obtained from time-lapse films, although based on counts of localized areas, agree well with averages obtained using entire perfusion chamber cultures.

The E_m changes for CHO cells (fig. 1a) correlate directly with the density changes, the level rising steadily with increasing density, and also directly with the mitotic activity level (fig. 1b), which decreases steadily as the E_m level rises. The E_m level under log phase conditions is quite low (-10.7 mV), as appears typical of actively proliferating cells in vitro (Cone, '69), but the average level increases significantly (-25.8 mV) as appreciable areas of confluence begin to develop (26 to 40 hours). During this period, E_m measurements of cells well within large confluent regions show values considerably greater than this average (up to -50 mV) while cells in the peripheral areas of such regions are the same as those of log phase cells (-11 mV), thus closely mirroring the distribution of mitotic activity observed in these same regions. The E_m level increases further (-57.6 mV) in the fully confluent but unsaturated condition and reaches a value of -61.3 mV at full saturation density. E_m continues to increase slightly with time (126 hours) to a stable level of -64.9 mV. This E_m increase at constant density appears to be related to the consolidation of the cells in an observable matrix of extracellular material. High E_m levels have previously been observed in monolayer cells under conditions which promote extracellular collagen synthesis (Swift and Todaro, '68). The final saturation E_m level observed in the present CHO monolayers is almost as high as that recorded *in situ* for a number of tissues (Giebisch, '58; Johnstone, '59; Balitsky and Shuba, '64; Kuffler et al., '66; Jamakaosmanović and Loewenstein, '68; Sperelakis and Shigenobu, '72).

Although the saturation density value for a given cell line appears to be characteristic for that line, tests to confirm that the E_m level of saturated CHO monolayers is independent of the rate and manner in which saturation is reached were conducted (table 1). Despite an almost twofold increase in the time required by the sparsely inoculated cultures to reach saturation, the final densities and E_m levels attained were practically the same as those of the more heavily inoculated cultures, indicating that the E_m level is determined principally by the final saturation condition.

The same positive correlation of E_m level, mitotic index, and cell density as observed for CHO monolayers is found for 3T3 monolayers (fig. 2), which reach saturation at only 5.0×10^4 cells/cm^2. The onset of contact effectiveness is taken when the cell doubling period begins to increase beyond the log phase value. Although the E_m levels during active growth are essentially the same for both lines (-11 to -12 mV), and also at saturation (-61 to -63 mV), the 3T3 line begins to show contact-effectiveness in regard to E_m and mitotic activity level changes at much lower cell densities ($\sim 3.3 \times 10^4$ cells/cm^2). The fact that mitotic activity ceases in 3T3 monolayers at nearly the same E_m level as in CHO cells, but at only one-eighth the cell density, would seem to indicate a closer relationship between the E_m level and degree of mitotic activity than between cell density per se and mitotic activity in these lines (see APPENDIX). Previous studies have also indicated the existence of such a mitotic activity-E_m relationship by showing that changes in cellular Na and K simulating

TABLE 1

E_m and density levels at saturation for CHO cultures reaching saturation in different times

Inoculation density cells/cm^2	Saturation [1] density cells/cm^2	Time to saturation hours	Saturation [2] E_m mV ± S.D.
2.0×10^5	4.3×10^5	28	-63.7 ± 2.9
1.1×10^5	4.2×10^5	50	-64.9 ± 3.2

[1] Data obtained from time-lapse films.
[2] Average of 85 impalements at each condition.

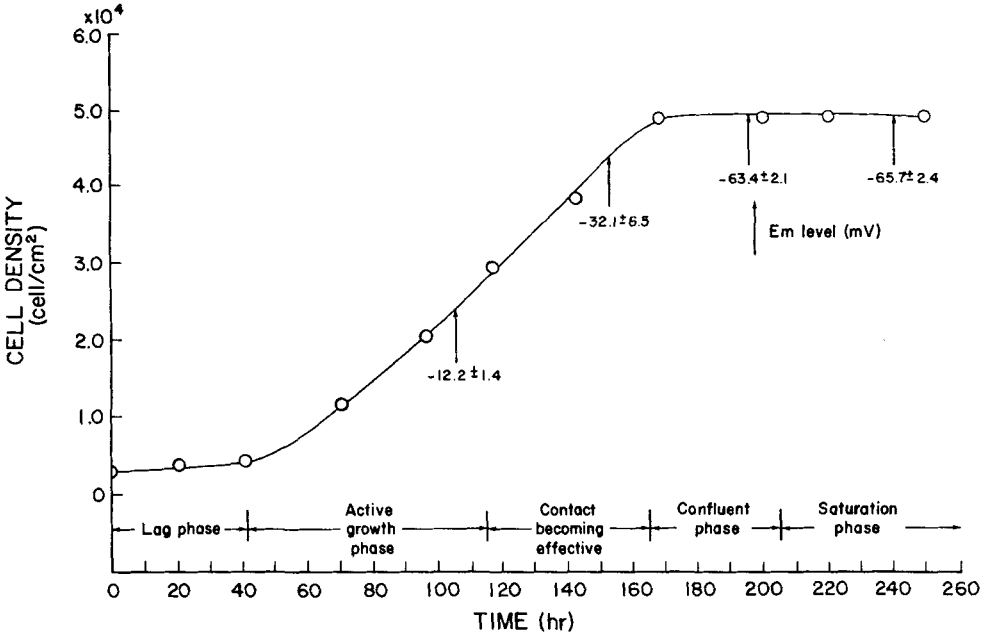

Fig. 2 Variation of cell density and E_m levels with time in developing 3T3 monolayers. Each density point is the mean of counts of three replicate dishes; each E_m value is the mean ($\pm$ S.D.) of $\sim$ 150 cell measurements.

those which would presumably accompany rises in E_m level of the magnitude found in the present study effectively block mitogenesis (Cone and Tongier, '71).

Colonies. The radial variations of local E_m, mitotic activity, and cell density for representative CHO and 3T3 colonies are shown in figure 3. The sizes selected for illustration are such that mitotic activity has ceased in the central region of the colony, where densities close to the respective monolayer saturation values exist. All colonies examined exhibited these same general correlations. The correlations of the local E_m and mitotic index with local cell density are essentially the same as those obtained with monolayers, despite the difference in the manner in which confluence develops and the fact that the local cell age varies from the center to the periphery of the colony. These findings again imply that the E_m and associated mitotic activity level are a direct function of the cell density or, more probably, the relative degree of cell contact as expressed by the magnitude of the local cell density relative to the saturation density (see APPENDIX). Mitotic activity in CHO colonies larger than the 1000-cell stage, and in 3T3 colonies larger than the 500-cell stage, is confined primarily to the peripheral areas, with the highest degree of activity in the open and low density confluent areas at the periphery. The distribution of mitotic activity for the 3T3 colony, determined from recordings of actual mitoses, agrees well with the distribution of DNA synthesis activity observed in previous studies of 3T3 colonies (Fisher and Yeh, '67). In large size colonies, conditions are very similar to those shown in figure 3; mitotic activity extends inward from the colony edge about the same distance ($\sim$ 0.6 mm) while the large central area is mitotically quiescent. The results of mitotic index distributions determined by colcemid treatment of colonies generally agreed quite well with those determined by photographic recordings (fig. 3) of mitoses. However, it is necessary to restrict the colcemid treatment to relatively short periods (6 to 8 hours) since the accumulation of increasing numbers of cells in the rounded metaphase form soon begins to affect the extent of local cellular contact in mitotically active areas.

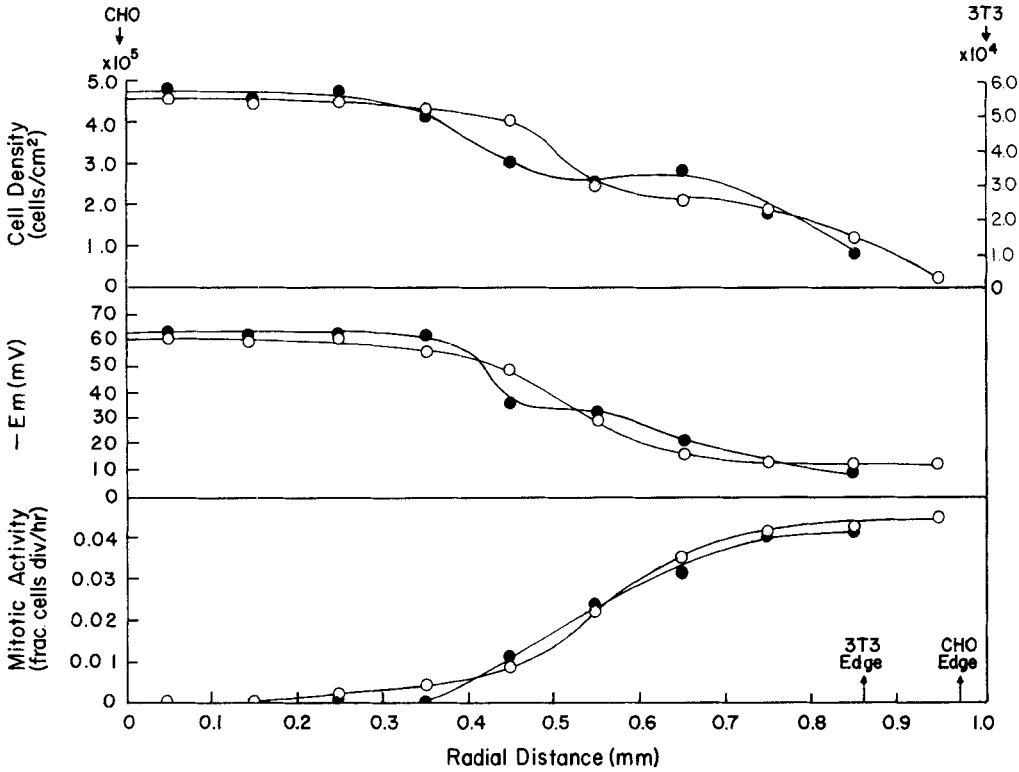

Fig. 3 Radial variation of cell density, E_m, and mitotic index within representative CHO (○) and 3T3 (●) colonies after attainment of saturation density in colony interiors. Density and mitotic index points are based on a group of 150–200 (CHO) and 90–110 (3T3) cells at each radial location. E_m points are the mean of ~20 cell measurements at each radial location. The colony sizes shown contained 7×10^3 (CHO) and 8.5×10^2 (BT3) cells.

Changes in intracellular Na and K levels

The Hodgkin-Huxley theory of potential generation in excitable cells proposes that the E_m level is established primarily by active membrane extrusion of Na^+ (Hodgkin, '64) resulting in a relatively low steady-state $[Na^+]_i$. Several studies have indicated that a similar mechanism is also involved in E_m generation in nonexcitable cells (Aull, '67; Hempling, '58, '62). To establish if significant changes in intracellular ion content accompany the E_m increases observed in development of saturation densities, cellular Na and K were determined for log and saturation phase CHO and 3T3 monolayer cultures. The results (table 2) show that for each cell line the increase in E_m level is associated with a substantial decrease in cellular Na, with relatively small changes in cellular K. The "concentration" values cited were obtained by dividing the total Na and K per cell by the mean volume and hence are not actual cytoplasmic concentrations. Since the free and bound (or internally compartmented) fractions of total Na and K were not determined individually, the relationship of the intracellular Na and K ion levels with the measured E_m levels in terms of the electrochemical potential gradient cannot be evaluated quantitatively, but the pronounced decrease in cellular Na which accompanies the E_m increase at saturation densities is qualitatively in agreement with the Hodgkin-Huxley theory. The Na results shown in table 2 for CHO cells were qualitatively substantiated by electron probe microanalysis of single cells in freeze-dried log and saturated monolayers grown on silicon disks and washed in the same manner as the flask monolayers. The microprobe results indi-

TABLE 2

Na and K levels in log phase and saturated CHO and 3T3 monolayers [1]

Ion	CHO		3T3	
	Log	Saturated	Log	Saturated
Na	15.3 ± 1.8	7.9 ± 2.1	17.6 ± 1.5	8.6 ± 0.8
K	186.1 ± 5.3	185.9 ± 6.2	204.5 ± 3.6	197.0 ± 4.8

[1] Levels are expressed as μmol of element per milliliter of mean total cell volume, ± S.D. Each value is the mean of six flask determinations; separate mean cell volume determinations were made for each flask set corresponding to each value.

cated a log phase-to-saturation phase Na ratio of $\sim$ 1.8, as determined by relative Na x-ray emission intensities.

Surface contact in saturated monolayers

Previous studies have indicated that substantial areas of cell-cell contact occur in confluent monolayers (Todaro et al., '64; Schutz and Mora, '68; Brunk et al., '71). Since appreciable areas of cell surface remain freely exposed under saturation conditions, however, those areas which do make cell-cell contact are presumably responsible for effecting the pronounced E_m changes observed in the present study. To establish the nature and extent of cell-cell contact in CHO cultures under conditions where high E_m levels exist, transverse sections of saturation monolayers were examined in detail by electron microscopy. These studies showed that although the CHO cells appear to constitute a monolayer of relatively discrete cells when viewed from above with light microscopy, there is in reality extensive overlapping of the cell peripheries, the basic pattern being essentially that shown by the right cell pair of figure 4. Because of this overlapping, large areas of intimate cell-cell contact occur, with outer membrane surface separation distances of only 100 to 200 Å (fig. 4). Three-dimensional surface contours constructed from serial sections through saturated CHO monolayers indicate that some 46 to 63% of a cell's surface is in contact with or in close proximity to other cells (intermembrane distances $< \sim 300$ Å). Previous electron microscopic studies of saturated 3T3 cell monolayers have revealed similar areas of close surface contact at saturation (Todaro et al., '64). As viewed by high magnification phase microscopy, the 3T3 cells used in this study tend to assume a roughly stellate form in saturated monolayers with a high degree of overlap of the thin cellular mantels, while the CHO cells appear thicker and more elongated with a high density of cell packing in the saturated monolayer. The possible significance of cellular volume and shape with regard to cell density and contact effectiveness in saturated monolayers is discussed in the APPENDIX.

DISCUSSION

The results of this study have revealed that a pronounced rise in E_m level is associated with onset of mitotic arrest at saturation density in contact-inhibited CHO and 3T3 cultures. This E_m rise correlates directly with the increase in cell density, which in turn is attended by the development of a significant amount of

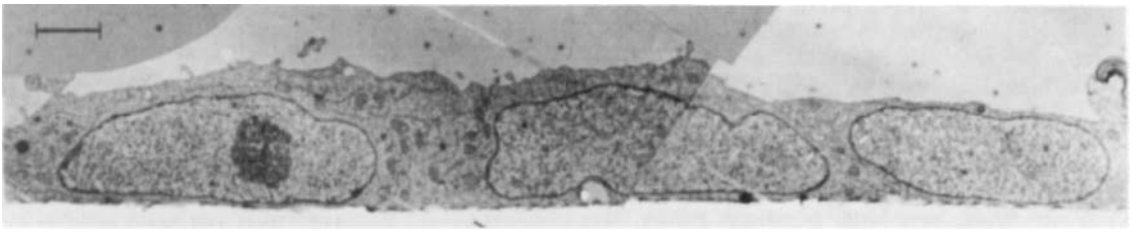

Fig. 4 Electron micrograph of typical transverse section of a saturated CHO monolayer, showing peripheral overlap and close contact of cell surfaces; calibration line represents 1 μ.

intimate cell-cell contact as the saturation level is approached. The results further reveal that a substantial decrease in cellular Na accompanies the E_m rise. This latter result is in accord with previous findings that low Na, high K concentrations (simulating the higher E_m levels) effectively but reversibly block log phase cells in the G_1 stage of the cell cycle (Cone and Tongier, '71). Similar blockage effects were observed with a BHK cell line using a high K, low Na medium (Orr et al., '72). The fact that the present correlations were observed in two independent cell lines, and in both the full-monolayer and colony forms of confluence suggests the possible generality of their involvement in contact inhibition of mitosis.

These findings for contact-inhibited systems are in agreement with the predictions of the hypothesis previously advanced on E_m-related ionic modulation of mitogenesis activity (Cone, '69, '71) and suggest, in terms of this hypothesis, a possible model for the mechanism of contact inhibition of mitosis. In view of the present results, contact of appreciable areas of adjacent cell surfaces at (local) saturation apparently alters the ion transport activity of the membrane in such a manner that a net extrusion of cellular Na results, with an accompanying increase in the steady state E_m level. This contact-induced alteration could be in the form of an increase in active ionic transport or a change in membrane permeability to specific ions, or both.[2,3] The resulting alteration of cellular ionic levels is then presumed to lead, directly or indirectly, to blockage of G_1 metabolic events essential for the initiation of mitogenesis. Other aspects of metabolism might be concomitantly affected; significant changes in surface glycoprotein composition and synthesis of cellular exudates have been observed to accompany development of saturation in confluent monolayers (Meezan et al., '70; Sanders and Zalik, '72; Swift and Todaro, '68).

Although a range of enzyme-activating ions or ionic conditions could conceivably be involved in such mitogenesis control (Cone, '69), the present results in association with previous findings make the possible role of the intracellular Na level worthy of consideration. Previous studies of the sequence of metabolic events in serum-stimulated mitogenesis in contact-inhibited cultures (Todaro et al., '65) have implied that regulation of RNA synthesis constitutes the basic means by which mitogenesis is controlled in such systems. Although the apparent *early* increase in RNA synthesis rate observed by these authors following fresh serum addition has subsequently been shown to reflect only an increased uptake and incorporation of tagged uridine rather than an actual increase in the RNA synthesis rate (Cunningham and Pardee, '69; Ceccarini and Eagle, '71), the overall results of these and other studies, both in vitro (Lieberman et al., '63; Bollum and Potter, '59) and in vivo (Tsukada and Lieberman, '61) have demonstrated that an increase in RNA synthesis does in fact precede the increase in specific enzyme activities associated with DNA biosynthesis. An early rise in RNA polymerase activity, preceding the increase in RNA synthesis rate, has been observed in regenerating liver (Tsukada and Lieberman, '61), and may be pertinent in view of the finding that exposure of cultures to fresh serum for even short periods results in a fraction of the cells becoming committed to proceed through DNA synthesis and division (Todaro et al., '65). More recently, studies in which continuous recordings were made of the E_m level in single cells in contact-inhibited cultures before and following serum application (Hülser and Frank, '71) have shown that the first observable result of treatment with serum is a rapid

[2] Activation of an increased Na-pumping rate and/or a decrease in Na permeability of the membrane upon surface contact would, for example, eventually result in an increase in E_m level at steady state conditions, if the membrane permeability for K^+ were greater than that for Na^+ at saturation and Cl^- were passively distributed (e.g., Cone, '69). Studies of BHK cells (McDonald et al., '72), which behave similarly to the present CHO cells in mitotic response to different Na^+/K^+ media, show that changes in E_m are almost immediate upon change of external K, and indicate that the membrane permeability for K is in fact greater than for Na in these cells under nonconfluent growth conditions. Similar results have been found for ascites cells (Hempling, '58; Aull, '67).

[3] The overall K levels indicated in table 2 are total values and since a considerable fraction of cellular K may be effectively concentrated or compartmented in mitochondria, these values cannot be meaningfully correlated with the pericellular E_m level. The fraction of total cellular K immobilized in this way might also vary between the log and saturation phases, depending upon the relative number and K-concentrating activity of the mitochondria in each phase.

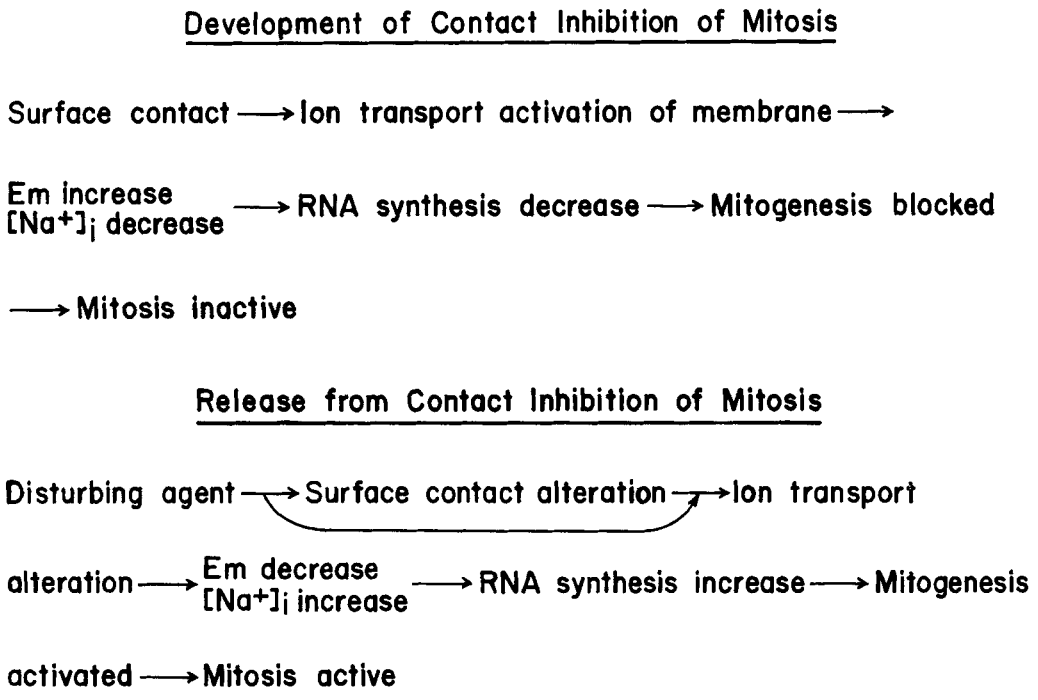

Fig. 5 Model of hypothetical system of interactions underlying development of and release from contact-inhibition of mitosis.

and pronounced depolarization of the cell within two minutes after serum application. Since the present results indicate that a significant decrease in intracellular Na accompanies the increase in E_m level at saturation, it might be expected that a corresponding rise in cellular Na would accompany the E_m decrease induced by serum treatment. To evaluate this premise, the E_m levels and relative cytoplasmic Na contents (electron microprobe analysis) of cells in control and serum-stimulated saturated 3T3 cultures were determined four hours after fresh serum addition. The results revealed that 9.1% of the serum-treated population had E_m levels below -20 mV and 8.7% had Na concentrations more than 1.8 that of the control cultures at four hours, thus demonstrating that a significant increase in cellular Na is produced in a fraction of the cells by serum treatment.[4] Although it was not possible to determine whether the cells of the low E_m and of the high Na fractions were those which eventually divided, the similarities of the percentages of these cells with the total percentage of cells dividing after serum treatment (8.5%) are suggestive of such a correlation.

The fact that a rise in RNA synthesis follows serum-induced depolarization (with presumed increase in cellular Na) thus suggests that E_m-related Na level changes may possibly be involved in the regulation of RNA synthesis. Significantly, perhaps, the concentration level of extranuclear Na has in fact been found to effectively control RNA polymerase ac-

[4] At 0.5 hour following fresh serum addition, only 8% of the cells had E_m levels larger than -20 mV, and only 1% had Na levels greater than 1.25 times that of the control cells. The increased percentage of cells (90.9%) having high E_m levels ($E_m > -20$ mV) at four hours indicates a repolarization of most of the cells following the initial serum-induced depolarization; this ability of most of the cells to repolarize to the saturation E_m level within a few hours after serum addition may possibly explain why most of the population does not subsequently divide. The fact that the Na level had not increased significantly, even though pronounced depolarization had occurred after 0.5 hour of treatment, suggests that the depolarization induced by serum may be somewhat similar in nature to the action potential of excitable membranes, where membrane depolarization occurs quite rapidly but without immediate alteration of the bulk intracellular concentrations of Na^+ and K^+, and thus implies that rapid, but transient, membrane permeability changes may be induced by serum.

tivity in cell nuclei (Congote et al., '69); variation of the K level was without effect. The ability of increased intracellular Na levels to decrease the cell cycle period and to further increase the degree of mitotic synchrony in chemically synchronized cultures has also been demonstrated (Cone, '69). More recently, increased proliferation rates in CHO cells obtained by use of trypsin in the culture medium were found to be associated with a three-fold increase in intracellular Na in the treated cells (Cone, Sidney, Cone, unpublished). Although Na might be directly involved in various aspects of RNA synthesis (at least of the mitogenically essential RNA species), it could also conceivably be active in other phases of mitogenesis, e.g., in effecting ion-exchange release or potentiation of DNA polymerase-activating ions such as Zn^{++} (Mikac-Devic, '70; Scrutton et al., '71). The fact that many agents capable of inducing mitotic activity in saturated cultures are surface active and behave much like serum in their basic effects (Vasiliev et al., '70) suggests that a common mechanism of mitotic activation may be involved in which cellular depolarization plays a significant role. The essential features of such a model of contact inhibition and release are summarized in figure 5.

Although the mechanism of contact inhibition outlined above is based upon results obtained with chemical releasing agents, particularly serum, the same pronounced depolarization has been found to accompany mitotic release induced by physical "monolayer wounding" of saturated cultures, a condition where direct physical disruption of surface contact is the only disturbing influence (Cone and Tongier, '73). These findings again suggest a basic involvement of surface contact per se and E_m-associated ionic changes in contact inhibition of mitosis.

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APPENDIX

It has often been stated that the 3T3 cell line is "particularly sensitive" to contact inhibition of mitosis because of its exceptionally low saturation density level. However, the results of the present study, as interpreted in the following analysis, raise some question as to the general validity of this conclusion and suggest that the mitotic sensitivity of the 3T3 line to contact may in fact be very similar to that of the CHO line studied, despite an eightfold difference in the saturation densities. From the data of table 3, the average volume V of 3T3 cells at saturation is three times that of CHO cells at saturation. If, for purposes of analysis, 3T3 and CHO cells are considered to have geometrically similar shapes in their respective monolayers, assumed as a crude approximation to be very thin circular disks, the radius r of such disks may be roughly estimated from the measured cell density σ, $r = (\pi\sigma)^{-1/2}$, and the disk thickness t from r and the measured cell volume V, $t = \frac{V}{\pi r^2}$. (In reality the value of r as determined here would be considerably less than the true value existing in the monolayer since there is a great deal of cellular overlapping at saturation (fig. 4). This is particularly true of the flat 3T3 cells; the CHO cells are considerably more rounded.) The total surface area A_s of the disk can then be calculated using r and t, or more directly from the relation $A_s = 2[\sigma^{-1} + \pi^{1/2}\sigma^{1/2} V]$.

TABLE 3

Density and mean volume of CHO and 3T3 cells in saturated monolayers

Cell line	Saturation density cells/cm²	Saturation [1] volume μ^3
CHO	4.2×10^5	872
3T3	5.0×10^4	2549

[1] Mean values for 9×10^5 cells per line.

Such calculations, using the present data for σ and V at saturation (table 3) show that the surface area of the 3T3 cell is 6.3 times that of the CHO cell, and in reality is probably considerably larger because of the extreme thinness and extensive overlapping of the 3T3 peripheral areas. On the other hand, the saturation density of the CHO monolayer is 8.4 times that of the 3T3. These estimations, therefore, reveal the interesting result that despite the great difference in saturation density for the two lines, they actually possess roughly the same amount of total cell surface area per unit of culturing surface area at saturation. If, as seems reasonable, approximately the same percentage of this total surface is in cell-cell "contact" in each line at saturation, then roughly equal contact areas exist at saturation, and hence the 3T3 line cannot be considered more highly sensitive to contact than the CHO line. The fact that essentially identical E_m levels exist at saturation for both lines, despite the pronounced difference in cell density, also correlates well with the present indication that roughly equal areas of effective contact may exist. The extreme geometrical complexity of the three-dimensional shape and surface contours of the actual monolayer cells (fig. 4) precludes a more precise quantitative development of the factors outlined here, but the present results are of interest in their suggestion that the total amount of cell-cell contacting surface per unit of substrate area may be a parameter of primary importance in contact inhibition of mitosis in these lines, rather than cell density per se.