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Regulation of the Cell Cycle of 3T3 Cells in Culture by a Surface Membrane-Enriched Cell Fraction

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Addition of a suspension of a surface membrane enriched fraction prepared from confluent 3T3 cells to sparse 3T3 cells in culture results in a concentration dependent and saturable decrease in the rate of DNA synthesis. The inhibition of cell growth by membranes resembles the inhibition of cell growth observed at confluent cell densities by a number of criteria: 1) In both cases the cells are arrested in the G_1 portion of the cell cycle; 2) the inhibition by membranes or by high local cell density can to a large extent be compensated for by raising the serum concentration or by addition of fibroblast growth factor plus dexamethasone. Membranes prepared from sparse cultures inhibit less well than membranes from confluent cultures in a manner which suggests that binding of membranes to cells is not by itself sufficient to cause inhibition of cell growth. The inhibitory activity has a subcellular distribution similar to phosphodiesterase (a plasma membrane marker) and appears to reside in one or more intrinsic membrane components. Maximally, membranes can arrest about 40% of the cell population in each cell cycle. Plasma membranes obtained from sparse 3T3 cells are less inhibitory than membranes obtained from confluent cells. This suggests either that the inhibitory component(s) in the plasma membrane responsible for growth inhibition may be in part induced by high cell density, or that this component(s) may be lost from these membranes during purification.

Key words: fibroblasts, plasma membranes, contact inhibition, growth control

The growth of fibroblasts in culture is sensitive to local cell density and the concentration of soluble factors in the bulk medium [1, 2]. This control of cell growth is clearly defined by the results of wounding a quiescent confluent monolayer. Under these conditions cells migrate into the wound and initiate DNA synthesis while cells in the same medium in

Abbreviations: DME, Dubelcco's modified Eagle's medium, PDE, phosphodiesterase, FGF, fibroblast growth factor, EGF, epidermal growth factor; Hepes, N-2-hydroxyethylpiperazine-n′-2′-ethane sulfonic acid, dT, thymidine.

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the confluent areas remain quiescent [3]. Thus local cell density regulates initiation of DNA synthesis. This phenomenon is termed topoinhibition of growth. Its mechanism of action may involve inhibition by direct cell–cell contact [2] or local depletion of soluble factors required for growth [4]. If the concentration of serum is lowered below a critical level there is a proportionate decrase in the fraction of cells in the wound which initiate DNA synthesis [3]. Thus the concentration of serum factors in the bulk medium also regulates initiation of DNA synthesis. When quiescent confluent cells receive fresh medium containing a higher serum concentration a certain fraction of cells initiate DNA synthesis [5] and the final density at which quiescence is again achieved is proportional to the serum concentration [6]. Thus serum contains factors which are able to compensate for topoinhibition. These results suggest that the ability of a cell to initiate DNA synthesis is a function of positive regulatory signals provided by serum (and other soluble factors) [1] and negative signals provided by high local cell density. Cells arrested in low serum at a sparse density, or at confluency, appear to be blocked in the G_1 region of the cell cycle [1, 7].

We have previously shown that addition of a surface membrane-enriched fraction of 3T3 cells to sparse growing 3T3 cells mimics the effect of confluent density on initiation of DNA synthesis [8]. In this previous work we showed that inhibition by a surface membrane-enriched fraction was both time- and concentration-dependent, and was reversible. Inhibition was shown to be specific since addition of 3T3 membranes to 3T3 cells resulted in inhibition, while addition of 3T3 membranes to simian virus 40-transformed 3T3 cells (SV3T3) produced no inhibition. This specificity was consistent with the loss of growth control by SV3T3 cells. Inhibition by membranes was shown not to result from depletion of factors in the bulk medium required for DNA synthesis. In this communication we compare by several criteria the growth inhibitory activity of membranes compared to the inhibition of growth observed at confluency in order to provide evidence that membranes exert this effect by providing an inhibitory factor or factors normally provided by adjacent cell surfaces at confluency.

MATERIALS AND METHODS

3T3 Cell Culture

Swiss 3T3 cells were obtained from Dr. Howard Green and were grown and maintained as previously described [8]. Calf serum was from K.C. Biological, Lenexa, Kansas. Fibroblast growth factor was from Collaborative Research. Dexamethasone was from Sigma. In some experiments cells were grown with a mixture of calf plasma and calf serum.Fibrin was removed from the plasma as follows: calf plasma containing sodium citrate was obtained from Grand Island Biologicals; it was dialyzed against phosphate buffered saline, then against 0.15 M NaCl and 1.3 mM $CaCl_2$ buffered with 0.02 M Hepes, pH 7.4. Insoluble proteins were removed after heating at 56° for 30 min by centrifugation at 27,000 × g for 30 min. The plasma was filter sterilized (0.2 μm pore size, Millipore) after removing particulate matter. Calf serum and calf plasma concentration are expressed as a percent by volume.

Surface Membrane Preparation

A surface membrane-enriched fraction from confluent 3T3 cells was prepared as previously described [8].

Surface membranes were prepared from sparse cultures by a modification of the same method. Cells, plated at 6×10^4 cells/150 mm diameter dish (Falcon) and grown for 4 days with one medium change, were harvested at a density of $1.2–2.6 \times 10^3$ cells/cm^2.

The cells were removed by scraping in Ca^{++} and Mg^{++}-free Hanks' salt solution containing 1 mM EDTA, buffered with 0.02 M Hepes at pH 7.4 and collected by centrifugation. The cells were suspended at 4° in 10 ml of Hanks' solution buffered at pH 7.4 with 0.02 M Hepes and containing bovine serum albumin (5 mg/ml). The cells were counted and after centrifugation typically $1-2 \times 10^7$ cells were suspended in 1 ml of homogenization buffer (0.25 M sucrose/10 mM Tris, pH 7.4/0.2 mM $MgCl_2$, bovine serum albumim 5 mg/ml) with 125 μg DNase I. The cells were disrupted in a small Dounce homogenizer. The homogenate was sedimented at 27,000 × g for 15 min. The pellet was then resuspended and layered on a discontinuous Ficoll gradient consisting of 2.1 ml 9% (w/v) Ficoll in homogenization buffer and 2.1 ml 25% (w/v) Ficoll in homogenization buffer. The gradient was spun at 104,000 × g for 8–10 h in an SW50.1 rotor. For determination of marker enzymes, fractions were collected from the gradient and assayed directly except where indicated. Control experiments showed that Ficoll had no measurable effect on the level of enzyme activities. For use in experiments where fraction B-1 was added to cells in cultures it was suspended in 4 ml of 10 mM Tris, pH 7.4/140 mM NaCl/bovine serum albumin, 5 mg/ml and pelleted at 39,000 × g for 20 min. The membrane pellet was then suspended in 1 ml of the above solution for determination of the level of phosphodiesterase (EC 3.1.4.1) activity [8]. Six ml of DME/5% calf serum was then added to the membranes and after mixing they were sedimented at 39,000 × g for 20 min. The membranes were then resuspended with a vortex mixer in DME/5% calf serum/L-glutamine, 0.1 mg/ml to the desired membrane concentration to be added to the cells in culture. Determination of total phosphate was as previously described [8]. NADH diaphorase (EC 1.6.99.X) was assayed as described [8] except that the oxidation of NADH was measured fluorometrically using a Farrand Optical fluorometer (model A). The initial NADH concentration was 10 μM in the reaction mixture. Phosphodiesterase (EC 3.1.4.1) was assayed by a modification of the method of Touster et al [9]. The reaction was carried out at 37° in 25 mM Tris (pH 9.0) containing 0.54 mM thymidine-5′-monophospho-α-naphthol ester and enzyme in a final volume of 0.4 ml. The reaction was slowed by adding 0.8 ml 65 mM glycine 0.2 M NaCl, pH 10.9 at 0°. The fluorescence of α-naphthol liberated was determined immediately using a Farrand Optical fluorometer (model A) and compared to an α-naphthol standard. Acid phosphatase (EC 3.1.3.2) was as described [8] except the reaction mixture contained 1 mM α-naphthol phosphate and release of α-naphthol was determined as described above.

The relative protein in various cell fractions was determined by uniformly labeling the cells for 2 days in culture with 1μCi/ml [^{3}H] L-leucine in leucine free medium with 10% calf serum. The specific activity before addition to the media was 57.4 Ci/mmol. Trichloroacetic acid precipitable counts were determined as described [8].

Measurement of DNA synthesis was performed in Linbro dishes (FB-16-24-TC, ~ 2 cm^2 area) as described [8]. [^{3}H] dT incorporation into TCA insoluble material after a two-hour pulse was normalized to cell number determined with a Coulter counter in duplicate dishes. Unless otherwise stated each value is the average from two dishes. It was shown previously [8] that changes in the [^{3}H]dT incorporation rate agree well with changes in the fraction of autoradiographically labeled cell nuclei following a pulse with [^{3}H]dT. This indicates that [^{3}H]dT incorporation rate is a measure of DNA synthesis. In most of these experiments, membranes were sterilized with UV light before addition to cells. Experiments have been carried out with membranes that have not been exposed to UV sterilization with the same results. The distribution of cells in different regions of the cell cycle (G_1, S and G_2 + M) were kindly determined by flow microfluorometry by K. Balmer and M.M. Burger, Biozentrum Basel. For this purpose DNA was stained with

mithramycin after fixation of trypsinized cells in 70% (v/v) ethanol/150 mM NaCl. Data were analyzed essentially as described [10].

In some experiments the number of cells remaining on a dish incubated with plasma membranes was less than would be expected from the cell content of control dishes and from the observed inhibition of DNA synthesis by membranes. This effect was not observed consistently and was observed less frequently at high serum concentrations. We presume that this may reflect some effect of membranes on the ability of the cells to attach to the dish. We have recorded the cell recoveries in the legends to the figures. It should be clear, however, that this observed loss of cells from the dish should not be equated to inhibition of cell growth observed with membranes. If, as we will show below, cells are blocked by the addition of membranes early in the G_1 portion of the cell cycle, then one generation after addition of membranes to cells, where we maximally observe a 50% inhibition of dT incorporation into DNA (or of cells in S phase), we would not expect the dishes to which membranes have been added to contain a significantly smaller number of cells than control dishes with no membranes. After 2 generations at saturating membrane concentration when the rate of dT incorporation into DNA is inhibited by 75% we would only expect a 30% difference in cell number between dishes with membranes and control dishes.

RESULTS

It is the purpose of this paper to further define the inhibition of growth of 3T3 cells by a plasma membrane-enriched fraction, and to compare this inhibition with that observed at high cell density. The experiments described below try to define the position in the cell cycle at which membranes block cell division and compare the kinds of growth inhibition brought about by membranes and by confluency. As has been shown previously a plasma membrane-enriched fraction from confluent 3T3 cells will inhibit cell division of sparse 3T3 cells. Other possibly fractions obtained during the preparation of membranes by isopyonic density centrifugation [8] will also inhibit cell division in proportion to this content of phosphodiesterase, a surface membrane marker (Fig. 1). It has not been possible to measure the activity of whole homogenates of 3T3 cells, possibly because other soluble components interfere with the assay. The data in Table I show that the inhibitory activity cannot be extracted from the membranes with 0.13 M Na pyrophosphate. This high concentration of a divalent metal chelator will extract a number of extrinsic proteins from the membranes. These data taken together support the view that the inhibitory activity is localized in an intrinsic membrane component present predominantly at the cell surface.

Heat Lability of Membrane Inhibitory Activity

When surface membranes prepared from confluent 3T3 cells were heated, at 60°, 70°, 80°, and 90°C for 10 min, there was an increasing loss of the ability of the membranes to inhibit DNA synthesis as the temperature was raised (Fig. 2). When membranes were heated at 60° there was a progressive loss of inhibitory activity with time (results not shown). These results are consistent with a role for a membrane protein in the process of inhibition, although raising the temperature could also produce a general alteration of membrane structure. One expects that molecules such as lipids or carbohydrates would not be affected by heating at 70–80° for 10 min, since the biological activities of these species are not usually influenced by brief heating.

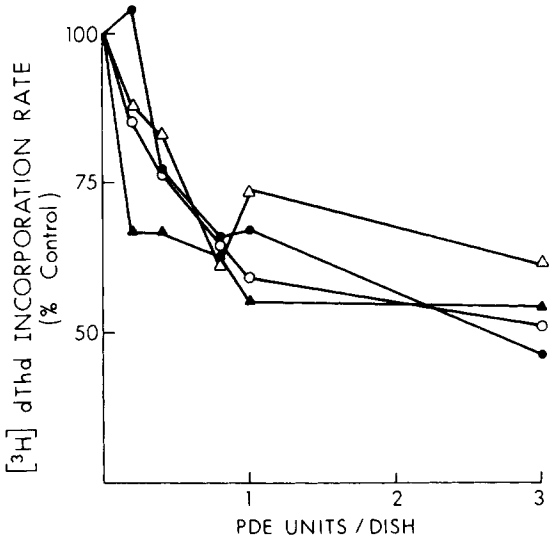

Fig. 1. Inhibition of cell division by membrane fractions. Membrane fractions were prepared by isopycnic centrifugation, as described previously, from confluent 3T3 cells. ○, Material at top of 9% Ficoll (B-1), ●, Material at 9 to 25% Ficoll interphase (B-2), △, Material at the 25 to 35% interphase (B-3) and ▲, the pellet at the bottom of the centrifuge tube (P). The enrichment of these fractions for phosphodiesterase relative to homogenate were: B-1, 14-fold; B-2, 12-fold; B-3, 3-fold; P, 1-fold. Cells were plated in 10% serum; after 1 day the medium was changed to 5% serum, and after 2 days the medium was changed to fresh medium containing 5% serum and the membrane fraction indicated.

TABLE I. The Inhibitory Components are Tightly Bound to the Plasma Membrane Fraction

	Concentration in assay μg/dish	Heat treatment	[^{3}H]dT incorporation % of control
Pyrophosphate extract	24	–	86
		+	84
Extracted membrane	16	–	45
		+	81
	47	–	38
		+	115

The plasma membrane enriched fraction was prepared from confluent 3T3 cells by standard methods but without the addition of serum albumin to any of the buffers. The membranes were suspended at a concentration of 0.5 mg/ml in 0.13 M Na pyrophosphate, pH 7.4, 0.1 mM dithiothreitol 0.1 mM phenylmethylsulfonylfluoride. After incubation at 4° for 30 minutes with occasional agitation with a Vortex mixer, the suspension was centrifuged at 106,000 × g for 30 min. The pellet was gently rinsed with 10 mM K phosphate, pH 7.5, 0.1 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride and finally suspended in the same buffer at a concentration of 0.4 mg/ml. The supernatant fluid and the suspension of the pellet were dialyzed for 16 hours afainst several changes of the same buffer. Before dialysis, ovalbumin was added to the solutions to a concentration of 1 mg/ml. The dialyzed solutions were tested for their ability to block the incorporation of [^{3}H]dT into DNA after 48 h incubation with 3T3 cells. As a control, the extract and membranes were heated at 80° for 10 min before addition to the cells. 30% of the membrane protein was extracted with Na pyrophosphate. In similar experiments, non-extracted membranes show maximal inhibition of DNA synthesis at 5 to 10 μg protein/dish, but the membranes used for extraction were not assayed for activity before extraction. Saturating levels of protein were used in all the experiments reported in this table. The results are expressed relative to control dishes which did not receive membranes or extract.

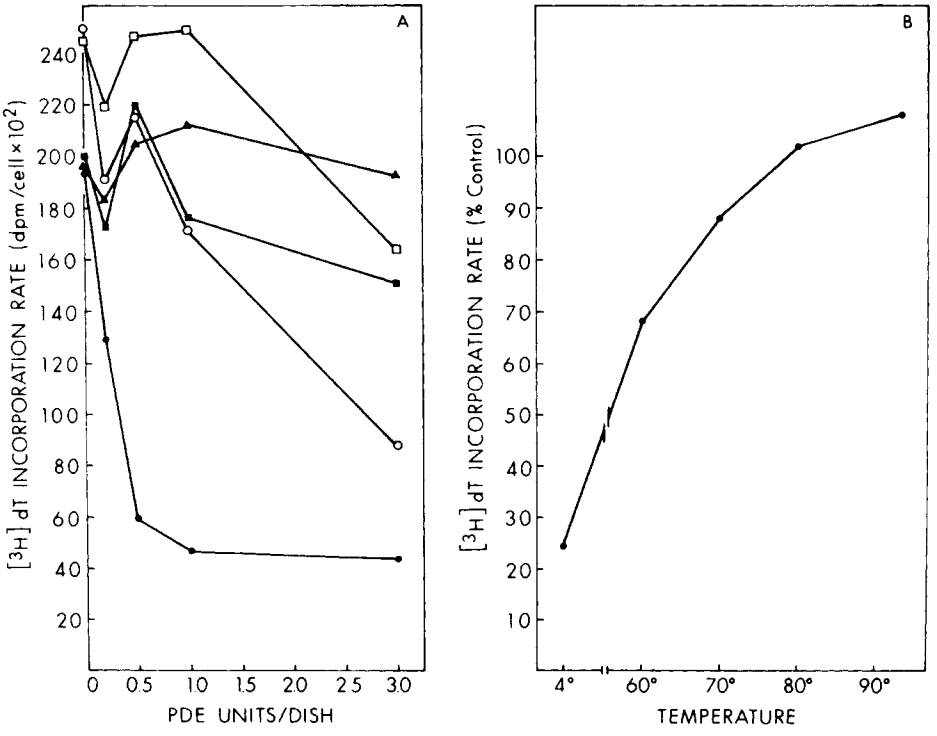

Fig. 2. The heat lability of membrane inhibitory activity. 3T3 cells were plated in Linbro dishes at 1.6×10^3 cells/dish in DME with 10% calf serum, and the medium was changed after 24 hours. 48 hours after plating, the medium was replaced with medium containing surface membranes as follows: Surface membranes prepared from confluent 3T3 cells were resuspended in DME at a concentration of 20 PDE units/ml. Aliquots were heated at the indicated temperatures for 10 min or kept at 4°C. The solutions were cooled and an equal volue of DME with 20% calf serum was added. The controls received medium prepared by heating DME to the appropriate temperature which was then diluted with an equal volume of DME with 20% calf serum. Dishes received 0.3 ml of medium. 48 h after adding membranes cells were pulsed with 1 μCi/ml [^{3}H]dT (2.2 Ci/mmol) as described in Materials and Methods. (a.) [^{3}H]dT incorporation rate as a function of membrane concentration for membranes treated at (●), 4°; (○), 60°; (■), 70°; (□), 80°; (▲), 90°. (b.) [^{3}H]dT incorporation from panel A carried out in the presence of 1 PDE unit/dish as a function of heating temperature. Incorporation rate is expressed as a percentage of the control not receiving membranes for each set.

We previously reported that the inhibition activity of membranes was sensitive to trypsin [8]. In recent experiments this effect has only been observed in about 50% of the experiments, while trypsin had no effect in the other experiments. We do not at present understand the source of this variability.

Position of the Membrane Inhibited Cells in the Cell Cycle

Accumulating evidence indicates that when normal fibroblasts in culture cease growth because of high density, serum deprivation, or nutrient deprivation, nearly all the cells in the population arrest in the G_1 phase of the cell cycle [1, 7, 11]. Not all agents which arrest the growth of cells do so by arresting cells in G_1. For example, Shodell has shown that 3T6 cells can be reversibly arrested in G_2 by lowering the culture temperature to 15°C. This effect of temperature could be overcome by decreasing the proportion of saturated fatty acids in the cell membranes [12]. Pardee has shown that nonphysiological

blocking agents such as hydroxyurea and colchicine block BHK cells in early S phase and mitosis, respectively [11]. If inhibition of growth produced by incubating cells with surface membranes occurs by the same mechanism as density dependent inhibition of growth, then membrane inhibited cells should arrest in G_1. To ask whether this is the case we first determined whether inhibition by membranes causes 3T3 cells to accumulate in a limited region of the cell cycle by testing whether membranes could be used to synchronize entry of cells into S phase. We then determined the position of the restriction point for membrane-inhibited cells in relation to the restriction point for serum which is known to lie in G_1 [1]. Finally we determined the DNA content of membrane-inhibited cells and compared this to sparse growing and confluent cells to determine whether membranes increase the fraction of the cell population in G_1.

It has been shown that confluent 3T3 cells can be released from a quiescent growth state and induced to enter S phase when trypsinized and replated [7]. The cells enter S phase after a characteristic lag giving rise to a synchronous rise in the rate of DNA synthesis. As shown in Fig. 3, trypsinized and replated confluent cells began to enter S phase as measured by a rise in [^{3}H]dT incorporation rate between 12 and 18 h after replating. The [^{3}H]dT incorporation rate reached a maximum at about 24 h and then decreased at 30 h. Confluent cells which received fresh medium remained essentially quiescent. When sparse growing 3T3 cells were trypsinized and replated the rate of DNA synthesis fell and then returned to normal after 18 h. When membrane-inhibited (66% inhibition) sparse 3T3 cells were trypsinized and replated the cells behaved essentially as predicted from the extent of inhibition of DNA synthesis. This is illustrated in Fig. 3b which compares the observed rate of DNA synthesis from replated membrane-inhibited cells, and the calculated value from a mixture of confluent cells (66%) and sparse cells (34%). The same pattern is observed if the number of cells in S phase is determined by autoradiography of thymidine labeled cells rather than by incorporation into trichloroacetic acid insoluble counts (data not shown). Thus the results are not due to inhibition of thymidine uptake. The membrane-induced block was reversed between 12 and 18 h and there was a rise in the apparent rate of DNA synthesis which reached a maximum about 24 h and then declined. The lag followed by a peak of DNA synthesis was not the result of replating itself since when sparse control cells were replated the cells consistently returned to a normal level of DNA synthesis between 12 and 18 h while replated membrane-inhibited cells and confluent cells consistently showed a larger lag with a peak of DNA synthesis at 24 h (Fig. 3). These results show that density-inhibited and membrane-inhibited cells accumulated in a limited region of the cell cycle before S phase. The accumulation of the cell population in a limited region resulted in a synchronous entry of cells into S phase following release of the block. The region of the cell cycle where cells accumulated could not be narrowly defined since the peak of DNA synthesis was broad and there was a lag before resumption of cell growth. These results, however, rule out a random block throughout the cell cycle produced by membranes since in this case there would have been a failure to synchronize cell entry into S phase.

The number of cells in the dishes incubated for 48 h with membranes was 71% of the number of cells in control dishes. This decrease in cell number is expected from inhibition of mitosis by membranes during this time period.* The efficiency of plating was close to 100% for control cells and membrane-inhibited cells. Thus we have not selected for a

*The decrease in cell number observed in the presence of membranes is only presented as an indication that in the presence of membranes there does not occur an excessive loss of cells leading to a selection of a subpopulation of cells. The decrease in cell number is too small and variable to be used as a measure of inhibition of growth by plasma membranes.

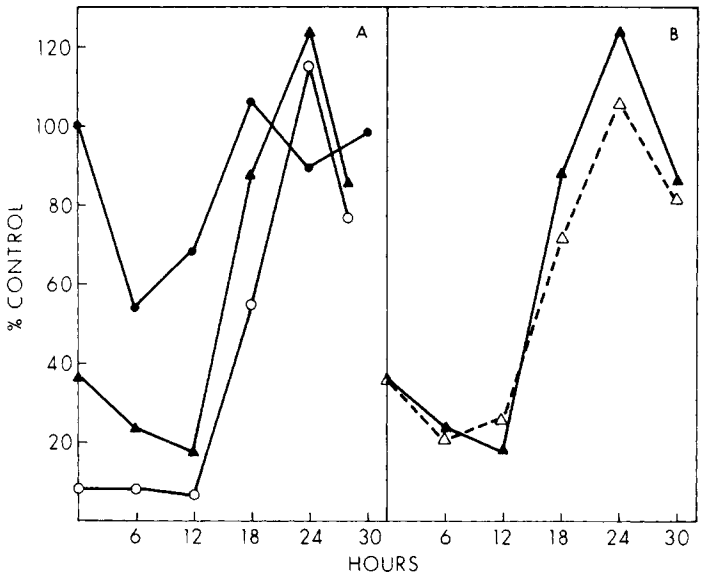

Fig. 3. Time course for entry of trypsinized and replated confluent cells and membrane inhibited cells into S phase. 3T3 cells were plated and grown in DME with 10% calf serum. Cells plated at 0.16×10^4/Linbro dish received surface membranes prepared from confluent 3T3 cells 48 h after plating, while cells plated at 0.08×10^4/dish received no membranes. Cells plated at 3×10^4/dish grew to confluency. Dishes received 0.3 ml of medium. 48 h after adding membranes cells were rinsed with CMF (Ca^{++} and Mg^{++}-free Hanks' salt solution buffered with 0.02 M Hepes at pH 7.4), and incubated with 0.02% crystalline trypsin (Sigma) in CMF for 10 min at 37°. Cells were gently suspended in an equal volume of DME/10% calf serum containing 0.02% soybean trypsin inhibitor (Sigma). Cells, pelleted at 300 × g for 5 min and suspended in DME/10% calf serum, were plated at 0.5×10^4 cells/Linbro dish in 0.5 ml DME/10% calf serum. At the indicated times after plating, cells were pulse labeled with 3 μCi/ml [^{3}H]dT (6.6 Ci/mmol) as described in Materials and Methods. Some cells at sparse density with and without membranes or at confluency were not trypsinized. The data have been normalized to the rate of incorporation of thymidine into cells of control sparse cultures; the values are taken as 100% (6 dpm/cell). ●, Replated sparse cells; ○, Replated confluent cells; ▲, Replated membrane inhibited cells. Panel A shows the observed data, and panel B shows the data obtained for the cells incubated in the presence of membranes as well as the predicted data (△) for a mixture of 34% sparse cells and 66% confluent cells. All data are the average of three experiments. Not shown are the data for membrane-inhibited cells which were not replated. These cells remained inhibited and at 24 hours showed a rate of deoxythymidine incorporation 20% of control sparse cells. Cells which were not replated were originally plated at the following densities: Sparse control cells – 0.08×10^4/dish (0 h), 0.06×10^4/dish (6–18 h), 0.04×10^4/dish (24–30 h); sparse cells plus membranes – 0.16×10^4/dish (0 h), 0.12×10^4/dish (6–30 h); confluent cells – 3×10^4/dish. This plating schedule is followed so that the cultures remain sparse at the time of assay, and are not partially inhibited by high cell density.

subpopulation of the membrane inhibited cells during the replating procedure, and it is reasonable to conclude that the block induced by membranes or confluency is characteristic of the majority of the inhibited cell population.

When membrane inhibited cells similar to those in Fig. 3 were washed with five changes of one ml of medium in an attempt to remove membranes and reverse the inhibition of DNA synthesis the peak rate of [^{3}H]dT incorporation was approximatley 50% of that of a sparse control culture of 3T3 cells without membranes. This suggests that the

membranes bind tightly to the cells and that at best only a fraction of the membranes can be removed without trypsinization.† We previously showed that both membrane-inhibited and control cells have the same plating efficiency and grow into cell colonies following trypsinization and replating [8]. Since confluent 3T3 cells normally undergo a limited number of rounds of cell division following trypsinization [13] and since many rounds of division are required to form a colony, it seems unlikely that trypsin provides a mitogenic stimulus which indirectly overcomes the inhibition by membranes.

When the growth of 3T3 cells is limited by serum the cells arrest in the G_1 portion of the cell cycle [1]. As shown in Fig. 4 when sparse 3T3 cells arrested in 0.4% serum

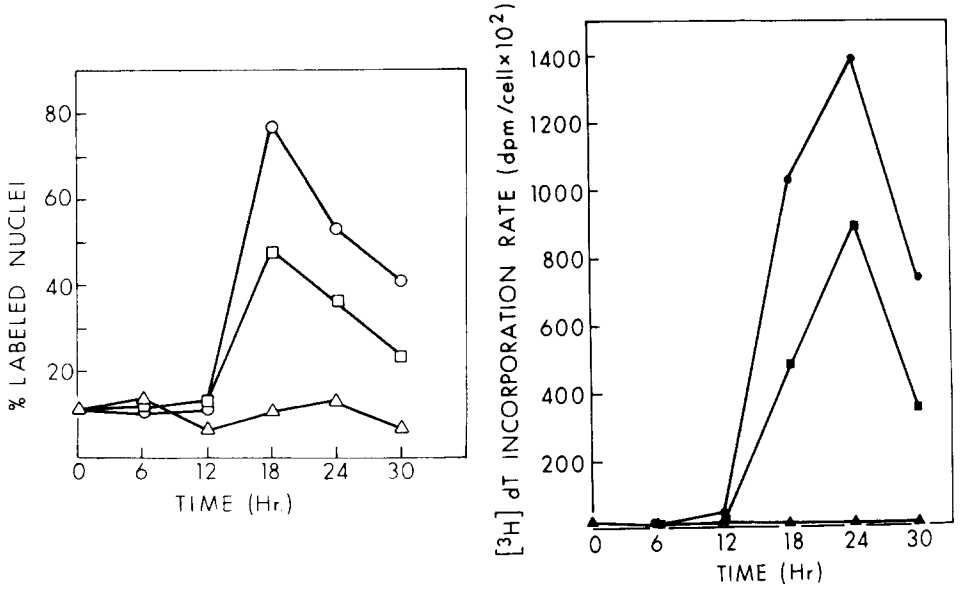

Fig. 4. Ability of membranes to block serum stimulated entry into S phase. Left panel: Fraction labeled nuclei versus time. 3T3 cells were plated at 0.6×10^4/dish in Linbro trays in DME with 10% calf serum. After 24 h cells were rinsed with DME and changed to DME with 0.4% calf serum. After 48 h (0 h on the fig.) the medium was changed on some dishes to 0.3 ml DME with 10% calf serum with or without 6 PDE units of surface membranes prepared from confluent 3T3 cells. At time 0 other dishes received 0.3 ml fresh DME with 0.4% calf serum. At the times indicated, dishes were pulsed with [^{3}H]dT and processed for autoradiography as previously described [8]. Each point is the average of duplicate dishres. (△), 0.4% calf serum; (○), 10% calf serum; (□), 10% serum plus membranes. The area under the curve with added membranes (□) is approximately 43% the area under the curve with no membranes (○). Right panel: [^{3}H]dT incorporation rate versus time. 3T3 cells were plated at 0.8×10^4/dish in Linbro trays in DME with 10% calf serum. After 24 h cells were rinsed with DME and changed to DME with 0.2% calf serum. After 72 h in DME with 0.2% calf serum with one medium change cells were treated as in the left panel with some cells receiving 8.7 PDE units/dish. At the times indicated after raising the serum concentration [^{3}H]dT incorporation rate was determined as described in Materials and Methods. (▲), 0.2% calf serum; (●), 10% calf serum; (■), 10% calf serum plus membranes. The area under the curve with added membranes (■) is approximately 48% the area under the control curve (○). Cell numbers at 24 h were 0.46×10^4/dish for cells in 0.2% serum; 0.66×10^4/dish for cells in 10% serum; 0.72×10^4/dish for cells in 10% serum plus membranes.

†Direct measurements of binding of radioactively labeled membranes to sparse cells have been carried out and will be reported separately [M. Lieberman, T. Woolsey, D. Raben, B. Whittenberger and L. Glaser, unpublished observations].

received fresh medium with 10% serum the fraction of cell nuclei labeled during a pulse with [^{3}H]dT increased after a lag, peaking at about 18 h. This result shows that serum arrested cells in G_1 enter S phase synchronously after the serum concentration is raised. Pardee has described a method for ordering restriction points in G_1 [11]. We have used this method to determine whether the steps for cell cycle progression which are regulated by membranes precede, overlap, or follow the steps regulated by serum. Since cells inhibited by membranes accumulate in a limited region of the cell cycle it seems reasonable to assume that a limited number of steps in cell cycle progression are regulated by membranes. We asked whether addition of membranes could block the entry of serum stimulated cells into S phase. As shown in Fig. 4 addition of membranes prevented about 50% of the stimulated cells from entering S phase. When cells randomly distributed in the cell cycle received membranes, the rate of DNA synthesis decreased by a maximum of about 40% in 19 h. Since the doubling time for 3T3 is about 19 h, this suggests that about 60% of the cells escape inhibition by membranes during each cell cycle. The fact that essentially the same degree of inhibition per generation is observed with a random cell population suggests that in each cell cycle a fraction of the population (approximately 50%) is not inhibitable by membranes and that the factors which determine the inhibition event are expressed early in the cell cycle. If the steps regulated by membranes preceded the steps regulated by serum, addition of membranes would have no effect on entry of serum stimulated cells into S phase. The steps involved in cell cycle progression regulated by membranes must therefore occur at the same place in the cell cycle or be subsequent to the steps regulated by serum. Since the serum regulated steps lie in G_1 it follows that the membrane regulated steps also lie in G_1. The same result as in Fig. 4 was obtained if membranes were added to the cells 8 h before the addition of serum, showing that the 50% inhibition is truly a saturating level rather than a fortuitous result of the relative time required for added membranes to inhibit the cells compared to the time required for cells to become committed to enter S phase. The same results are obtained by measuring incorporation of [^{3}H]dT into trichloroacetic acid insoluble counts or by the determination of labeled nuclei by autoradiography (Fig. 4) thus either method represents a true measure of the number of cells in S phase.

Further evidence that addition of surface membranes to sparse cells increases the fraction of the cell population arrested in G_1 comes from the flow microfluorometric analysis shown in Fig. 5. The percentage of the cell population with G_1, S, or G_2 + M amount of DNA is shown for confluent quiescent cells, sparse growing cells, and membrane inhibited cells. It can be seen that upon arrest at confluence when the [^{3}H]dT incorporation rate was about 5% of that of a sparse culture, the fraction of the cell population with S and G_2 + M amount of DNA decreased and the fraction with G_1 amount of DNA increased when compared to sparse cells. This shows that confluent cells accumulate in G_1. When membranes were added to sparse cells the average [^{3}H]dT incorporation rate in three separate experiments was reduced to 54.4% of that of sparse control cultures. The fraction of the membrane-inhibited cell population with S amount of DNA decreased and with G_1 amount of DNA increased when compared to sparse control cells. Using the paired difference test [14] the difference in the percentage of sparse cells and membrane-inhibited cells in G_1 was significant at the $> 99\%$ confidence level and for cells in S phase the difference was significant at the $> 95\%$ confidence level. The changes in the percentage of membrane-inhibited cells with G_2 + M amount of DNA were too small to be significant.

Based upon the reduction in [^{3}H]dT incorporation rate in membrane-inhibited cell cultures it was possible to predict the percentage of the cell population within G_1 and S.

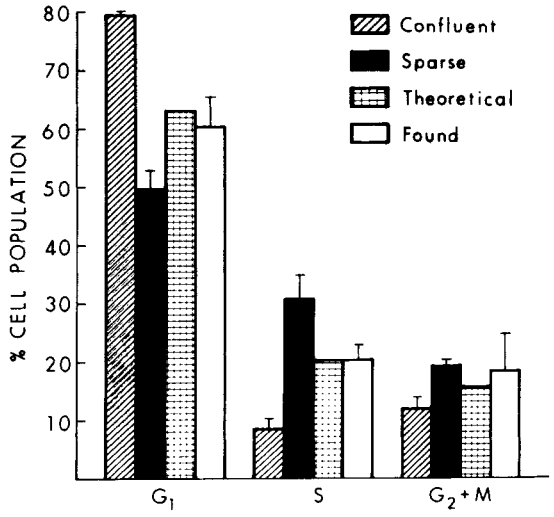

Fig. 5. Flow microfluorometric analysis of membrane inhibited 3T3 cells. 3T3 cells were plated in 60 mm dishes (Falcon) at 0.4×10^4/dish for the sparse control and 0.5×10^4/dish for the membrane inhibited cells in 3 ml DME/10% calf serum. Twenty-four hours later medium was replaced with fresh medium. Cells were also plated in Linbro dishes as in Fig. 2. Forty-eight hours after plating cells 60 mm dishes received 3 ml DME/10% calf serum with or without 42 PDE units of surface membranes prepared from confluent 3T3 cells. Cells in Linbro dishes received 0.3 ml DME/10% calf serum with or without 3 PDE units/dish. Cells were also grown to confluency in 60 mm dishes. Forth-eight hours after adding membranes [^{3}H]dT incorporation rate was measured in the Linbro dishes as described in Materials and Methods. Flow microfluorometric analysis was performed as described in Materials and Methods using about 3×10^6 cells from sparse 60 mm dishes and 1×10^7 cells from confluent 60 mm dishes. The data presented are th eaverage of three experiments. The mean [^{3}H]dT incorporation rate in the presence of membranes was 54.4% of a sparse control not receiving membranes. To compute the theoretical fraction of cells in G_1, S, or G_2 + M for membrane inhibited cells the following formula was used:

$$\text{Theoretical} = (0.456)\ \text{confluent value} + (0.544)\ \text{sparse value}.$$

The cell number in 60 mm dishes receiving membranes averaged 86% of the cell number in dishes not received membranes (corrected for the size of inoculum).

The theoretical and measured percentages for membrane-inhibited cells agreed rather well, as shown by comparing the bars in Fig. 5. It is clear that the reduction in [^{3}H]dT incorporation rate in membrane-inhibited cell cultures reflects primarily a decrease of the fraction of the cell population in S phase and an increase of the fraction of the population in G_1. The flow microfluorometry experiments, therefore, are in agreement with the expectation that both density inhibited 3T3 cells and membrane-inhibited cells arrest in the G_1 portion of the cell cycle. That membrane-inhibited cells arrest in G_1 provides additional evidence for specificity in the inhibition of growth by membranes.

The Effect of Serum Concentration of Inhibition of DNA Synthesis by High Cell Density and by Surface Membranes

Since it has been shown previously that increasing the serum concentration will increase the saturation density of 3T3 cells (the final stable density achieved when cells receive regular medium changes) [6] we decided to examine the effect of serum concentration on inhibition of DNA synthesis by surface membranes. If inhibition of growth by

membranes and inhibition of growth by high cell density occur by similar mechanisms then raising the serum concentration should decrease the inhibition of growth by membranes. We first determined the rate of [^{3}H] dT incorporation as a function of cell density in DME with 5% calf serum or 20% calf serum as shown in Fig. 6. [^{3}H] dT incorporation rate provided a measure of the fraction of cells which entered S phase since results similar to those in Fig. 6 were obtained when the fraction of cell nuclei that was autoradiographically labeled following a pulse with [^{3}H] dT was determined (not shown). The fraction of cells in S phase at the lowest density was similar in DME with 5% and 20% calf serum. As the cell density was increased the fraction of cells in S phase decreased, but it decreased more steeply in 5% serum comapred to 20% serum. Thus at any cell density the fraction of the cell population which was growing was greater in DME with 20% calf serum than with 5% calf serum. These results are consistent with the fact that 3T3 cells in DME with 20% calf serum grow to a saturation density which is 2–3 times higher than that of cells grown in DME with 5% calf serum. The results would be in agreement with the assumption that serum can compensate for high density and that it is the combination of these two signals that determines whether a cell will initiate DNA synthesis. Cells grown in DME with 5% calf serum were distributed on the dish in a manner which appear under the phase microscope to be identical to cells grown in DME with 20% calf serum. Thus the effect of lower serum concentration was not to increase the number of cells growing in islands and therefore the local cell density.

When sparse 3T3 cells were incubated with various concentrations of surface membranes, there was a concentration dependent decrease in the rate of DNA synthesis. The concentration of membranes required to maximally inhibit DNA synthesis was dependent on the serum concentration, but the maximal inhibition appeared to be independent of the serum concentration. Two typical experiments are shown in Fig. 7. The data in Fig. 7a were obtained by incubating sparse 3T3 cells either in the presence of 5% serum or 20% serum. In this particular experiment membranes equivalent to 3 to 4 phosphodiesterase units were required for maximal inhibition in 5% serum, while 15 units of phosphodiesterase were required to achieve the same inhibition of DNA synthesis in 20% serum.

In some experiments, addition of membranes to cells in the presence of less than 10% serum resulted in a loss of cells from the dish relative to control greater than that which could be accounted for by the inhibition of DNA synthesis. On the assumption that this loss was due to failure of the cells to adhere to substratum at low serum concentration, we have supplemented the growth medium with 5% plasma when the serum concentration is low. Plasma (data not shown) was only weakly mitogenic for 3T3 cells but appeared to prevent cell loss in these experiments. The data in Fig. 7b were similar to those in Fig. 7a but the medium was also supplemented with 5% plasma. While the membrane preparation used in this experiment were considerably more active than the one used in the experiment in Fig. 7a, both experiments show that higher concentrations of membrane is required to achieve the same degree of inhibition of DNA synthesis in the presence of 20% serum than in the presence of 5% serum. The observation that by volume calf plasma is less potent in compensating for inhibition of DNA synthesis by membranes than calf serum (data not shown) is in agreement with observations by others that a factor released by platelets is responsible for this compensation [15–17].

Gospodarowicz et al have shown that fibroblast growth factor (FGF) can substitute for platelet factors [16, 17] in promoting the growth of Balb/c 3T3 cells [15]. It has also been shown that addition of FGF can cause initiation of DNA synthesis in confluent quiescent Swiss 3T3 cells [5]. We decided to determine if addition of FGF reduced the

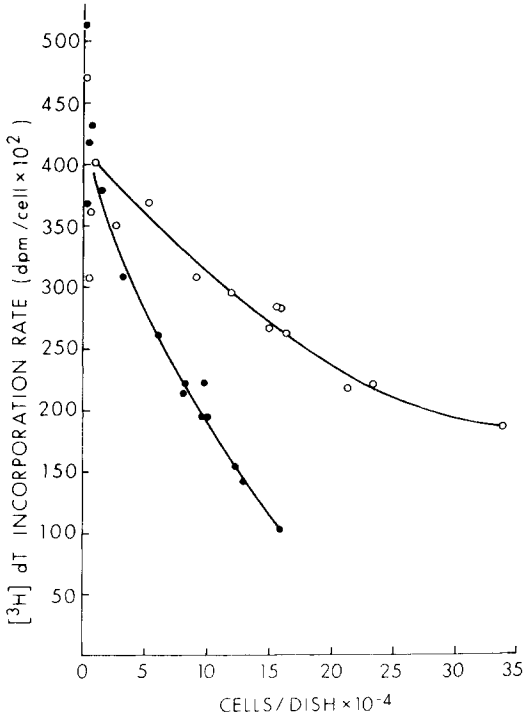

Fig. 6. [^{3}H]dT incorporation rate as a function of cell density in 5% calf serum and 20% calf serum. 3T3 cells were plated between 0.01 and 3 × 10^4 cells/Linbro dish in 1 ml DME with 10% calf serum. Twenty-four hours later medium was replaced with 1 ml DME with 5% or 20% calf serum. Medium was changed every 24 h. Seventy-two hours after the first medium change cells were pulsed with 1 μCi/ml [^{3}H]dT (2.2 Ci/mmol) as described in Materials and Methods. (•), 5% calf serum; (○), 20% calf serum. The cell counts shown in the abscissa are those observed at the time when thymidine incorporation was determined.

inhibitory effect of membranes on DNA synthesis. As shown in Fig. 8, at any given membrane concentration the apparent rate of DNA synthesis was higher in dishes supplemented with either FGF or FGF plus dexamethasone, than in control dishes without these additions. In this experiment the rate of DNA synthesis appeared to level off at a higher rate in cultures receiving FGF plus dexamethasone. In another experiment not shown, the rate continued to decrease with addition of more membranes. These results provide additional evidence that addition of membranes to sparse cells mimics topoinhibition since addition of mitogenic factors such as FGF and dexamethasone, or those found in serum, compensates for inhibition by membranes much in the same way it compensates for topoinhibition at high local cell density.

Since completion of these experiments, a very ingenious approach to the same problem has been reported by Peterson et al [18]. These investigators used 3T3 cells arrested in G_1 by serum deprivation. These cells can be induced to divide by the addition of epidermal growth factor. When surface membranes from a 3T3 mutant which cannot bind epidermal growth factor [19] are added to such cells, they prevent the initiation of DNA synthesis induced by epidermal growth factor. These experiments are in agreement with our observations but, in addition, by the use of membranes prepared from cells which

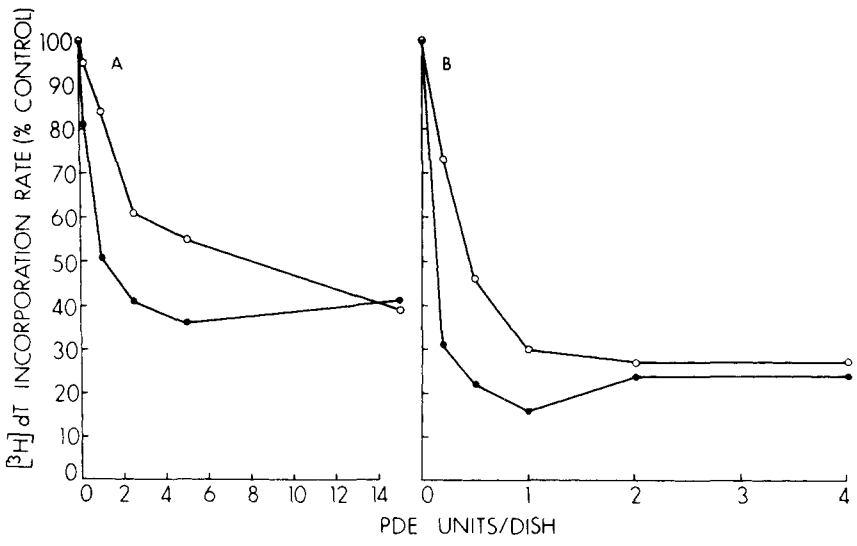

Fig. 7. [^{3}H]dT incorporation rate as a function of membrane concentration in 5% serum and 20% serum or 5% plasma with 5% serum or 20% serum. Cells were plated at 0.08 and 0.16 × 10^4/Linbro dish (A) or 0.12 × 10^4/Linbro dish (B) in DME with 10% calf serum. Twenty-four hours after plating the medium was replaced with DME with 5% calf serum (dishes plated at 0.16 × 10^4) (•) or with 20% calf serum (dishes plated at 0.08 × 10^4) (○) (Panel A) or with DME with 5% calf plasma plus 5% calf serum (•) or with 5% calf plasma plus 20% calf serum (○) (Panel B). Forty-eight hours after plating, medium was replaced with 0.3 ml fresh medium plus or minus surface membranes prepared from confluent cells which were added at the indicated concentrations. Forty-eight hours after adding membranes cells were pulse labeled with 3 μCi/ml [^{3}H]dT (6.6 Ci/mmol) in DME with 5% or 20% dialyzed calf serum (Panel A) or in DME with 10% dialyzed calf serum (Panel B) as described in Materials and Methods. [^{3}H]dT incorporation rate in control dishes not receiving membranes was: Panel A, 5% serum 12.54 dpm/cell; 20% serum 11.87 dpm/cell. Panel B, 5% plasma/5% serum, 11.83 dpm/cell; 5% plasma/20% serum, 14.89 dpm/cell. In Panel A the cell numbers in control dishes were 0.66 × 10^4 in 5% serum and 0.70 × 10^4 in 20% serum; the cell numbers in dishes receiving 15 PDE units were 0.35 × 10^4 in 5% plasma/5% serum and 1.01 × 10^4 in 5% plasma/20% serum; the cell numbers in dishes receiving 4 PDE units were 0.41 × 10^4 in 5% plasma/5% serum and 0.58 × 10^4 in 5% plasma/20% serum.

cannot bind epidermal growth factor, these investigators have succeeded in ruling out the possibility that membranes act by removing epidermal growth factor from the medium, or decrease its concentration near the cell surface.

The Time Course for Inhibition of DNA Synthesis by Membranes

We have shown previously that inhibition of DNA synthesis increases with time of incubation of cells with surface membranes [8]. In the experiment shown in Fig. 9, 3T3 cells were incubated with 0.4 or 5 PDE units/dish of surface membranes in DME containing 5% plasma with either 5% serum or 20% serum. The apparent rate of DNA synthesis decreased logarithmically with time suggesting that a constant fraction of the cell population was arrested in G_1 per unit time. The fraction arrested was increased by adding more membranes (compare panels A and B), or at a given membrane concentration by lowering the serum concentration (compare 5% plasma plus 5% plasma plus 20% serum).

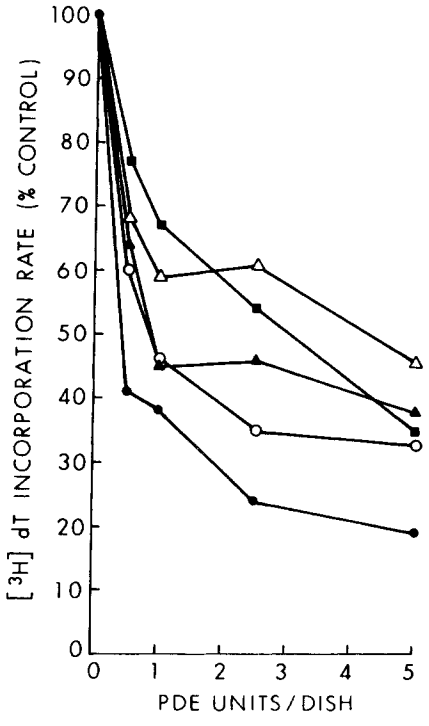

Fig. 8. [^{3}H]dT incorporation rate as a function of membrane concentration in medium supplemented with FGF, dexamethasone, or FGF and dexamethasone. Cells were plated at 0.10 × 10^4 cells/Linbro dish as in Fig. 7. After 24 h the medium was changed to DME with 5% plasma and 5% serum with or without 25 ng/ml FGF, 1 μg/ml dexamethasone, or 25 ng/ml FGF plus 1 μg/ml dexamethasone, or to DME with 20% calf serum. Forty-eight hours after plating medium was replaced with the above media (0.3 ml/dish) with various amounts of surface membranes prepared from confluent 3T3 cells. Forty-eight hours after adding membranes [^{3}H]dT incorporation rate was determined as described in Materials and Methods using 1 μCi/ml (2.2Ci/mmol) in DME with 10% dialyzed calf serum. DME with 5% plasma/5% serum (●); 5% plasma/5% serum plus 25 ng/ml FGF, (○); 5% plasma/5% serum plus 1 μg/ml dexamethasone, (▲); 5% plasma/5% serum plus 25 ng/ml FGF plus 1 μg/ml dexamethasone, (△); 20% serum, (■). [^{3}H]dT incorporation rate in dishes not receiving membranes: 5% plasma/5% serum, 2.48 dpm/cell; plus FGF, 3.35 dpm/cell; plus dexamethasone, 2.72 dpm/cell; plus FGF plus dexamethasone, 3.03 dpm/cell; 20% serum, 2.72 dpm/cell. Cell number (× 10^{-4}) in control dishes and dishes receiving 5 PDE units/dish, respectively: 5% plasma/5% serum, 1.17 and 0.69; plus FGF, 0.58 and 0.33; plus dexamethasone, 1.24 and 0.86; plus FGF plus dexamethasone, 1.02 and 0.64; 20% serum, 2.18 and 1.30.

Inhibition of DNA Synthesis by a Surface Membrane-Induced Fraction From Sparse 3T3 Cells

All the work reported on the inhibition of 3T3 cells by plasma membranes has used membranes prepared from confluent 3T3 cells or membranes prepared from SV30 transformed 3T3 Cells [8]. The latter showed lower activity than membranes from 3T3 although they were clearly inhibitory to 3T3 cells.‡ It is therefore of interest to determine whether the plasma membrane from subconfluent (ie, actively growing) 3T3 cells show inhibitory activity. We have therefore prepared a surface membrane fraction from sparse

‡Neither membranes from 3T3 cells or from SV40 transformed 3T3 cells inhibited the growth of SV40 transformed 3T3 cells.

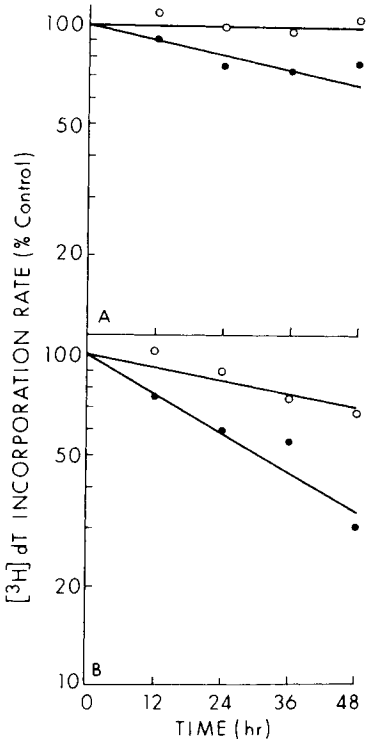

Fig. 9. [^{3}H]dT incorporation rate vs time after addition of surface membranes in 5% plasma with either 5% serum or 20% serum. Cells were plated and treated as in panel B of Fig. 7. Cells were pulsed with [^{3}H]dT (1 μCi/ml, 2.2 Ci/mmol) at the indicated times after adding membranes as described in Materials and Methods. Incorporation rate is expressed as a percentage of control not receiving membranes for each time point. Each point is the average of the determinations from four dishes. Panel A, 0.4 PDE units/dish; Panel B, 5.0 PDE units/dish. (○), 5% plasma plus 20% serum; (●), 5% plasma plus 5% serum. Cells were plated at the following densities for the indicated points: 5% plasma/5% serum, 12 h and 24 h, 0.48 × 10^4/dish; 36 h and 48 h, 0.24 × 10^4/dish; 5% plasma/ 20% serum, 12 h, 0.48 × 10^4/dish; 24 h and 36 h, 0.24 × 10^4/dish; 48 h, 0.12 × 10^4/dish. It is necessary to plate cells initially at various densities so that they are still at a sparse density when the rate of DNA synthesis is measured, and at a high enough cell density that an accurate cell count can be obtained.

3T3 cells essentially by the same procedure used in preparing membranes from confluent cells. The distribution and activities of protein and marker enzymes for fraction B-1 obtained from sparse 3T3 cells in an individual experiment are shown in Table II. The specific activity of the surface membrane marker phosphodiesterase was increased in B-1, the surface membrane fraction used in our experiments, about 9-fold over the activity in the homogenate. Based upon enrichment of phosphodiesterase, fraction B-1 prepared from sparse 3T3 cultures was similar to fraction B-1 prepared from confluent 3T3 cultures [8], but is clearly contaminated with other organelles. In general, the enrichment of marker enzymes in fraction B-1 prepared from sparse 3T3 cultures has been more variable compared to fraction B-1 prepared from confluent 3T3 cultures.

The apparent rate of DNA synthesis measured by [^{3}H]dT incorporation rate per cell decreased in a concentration dependent and saturable manner when a suspension of surface membranes (Fraction B-1 of Table I) prepared from sparse or confluent 3T3 cells was

TABLE II. Distribution and Activities of Protein and Subcellular Markers in Cell Fractions From Sparse Culture 3T3 Cells

Fraction	Protein	Phosphodiesterase	Acid phosphatase	NADH Diaphorase
Homogenate	(100)	1.14	1.14	58.6
Supernatant (27,000 × g)	(69.2)	0.22 (13.4)	0.62 (37.5)	40.8 (48)
B-1	(2.8)	4.45 (10.8)	1.41 (3.4)	58.8 (3)
B-2	(3.2)	3.88 (10.8)	2.28 (6.3)	82.4 (5)
Pellet	(5.9)	1.91 (9.9)	1.72 (8.9)	71.4 (7)
Washed B-1	(0.9)	9.9 (8.2)	2.26 (1.9)	393.3 (6)

Sparse cultures of 3T3 cells were fractionated as described in Materials and Methods. Fraction B-1 was collected from the 0%/9% Ficoll interface. Fraction B-2 was collected from the 9%/25% Ficoll interface. Specific activities are expressed as 10 nmoles/hr per 10^6dpm [^{3}H] leucine for phosphodiesterase and acid phosphatase, and as nmole/min per 10^6dpm for NADH diaphorase. The numbers in parentheses are the percent recovery of protein ([^{3}H] leucine dpm) or total enzyme activity in the homogenate taken as 100%. There were 37 × 10^6dpm [^{3}H] leucine from 2.2 × 10^7 cells in the homogenate. Fraction B-1 was washed (washed B-1) by resuspending in 4 ml 10 mM Tris, pH 7.4/140 mM NaCl/ bovine serum albumin, 5 mg/ml, and pelleting at 39,000 × g for 20 min. The pellet was then resuspended in 1 ml of the above buffer for assay. The washed B-1 fraction corresponds to the material added to cells in the experiments in Figure 10. In a number of experiments, recovery of protein in the washed B-1 fraction has ranged between 0.9 and 1.6% and 1.6% of the protein in the homogeante. Enrichment of specific activities in the washed B-1 fraction with respect to the homogenate has ranged between 8.7 and 24.0-fold for phosphodiesterase, between 2.0 and 3.5-fold for acid phosphatase, and between 0 and 6.7-fold for NADH diaphorase.

added to sparse 3T3 cells in culture as shown in Fig. 10. After 48 h in the presence of a saturating level of membranes from confluent cells DNA synthesis was 30% of the control value observed in the absence of membranes. Cells grown in the presence of a saturating level of membranes prepared from sparse 3T3 cells showed a rate of DNA synthesis about 60% of control. This difference between membranes prepared from sparse and confluent cells is reproducible and was seen in a number of different experiments. The fact that membranes from sparse cells, even at saturation, are less inhibitory than membranes from confluent cells is consistent with a model in which binding of membranes to cells is necessary for biological activity, but the degree of inhibition depends on subsequent interactions between the bound membranes and the cell surface, and that these interactions are less effective with membranes obtained from sparse cells as compared to confluent cells. These data may also suggest that the inhibitory components present in the plasma membrane may be "induced" at high cell density. However the alternative view that the inhibitory component is more labile in membranes from sparse cells, or lost during membrane preparation, cannot be ruled out.

DISCUSSION

We have approached the study of the role that cell-cell interaction plays in regulating growth by examining the effect of addition of suspensions of a surface membrane enriched fraction from 3T3 cells on the growth of 3T3 cells in culture. We have made the assumption that the cell surface components involved in density dependent inhibition of growth are able to exert their inhibitory effect on growth when added to cells in culture in the form

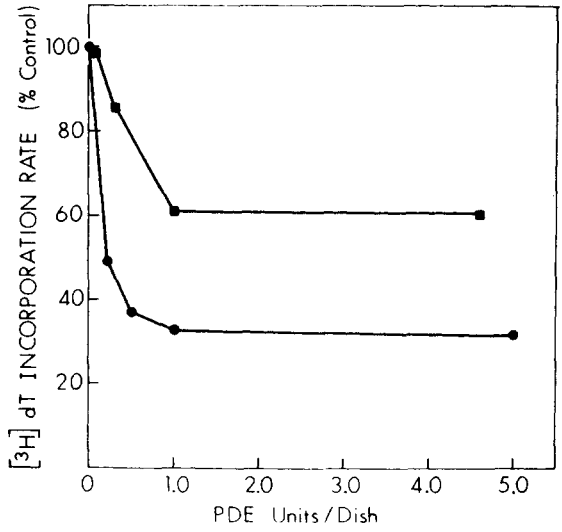

Fig. 10. [^{3}H]dT incorporation rate by 3T3 cells as a function of concentration of confluent or sparse 3T3 cells as a function of concentration of confluent or sparse 3T3 culture membranes. 3T3 cells were plated in Linbro dishes at 1.6 X 10^3 cells/dish in DME (Dulbecco's modified Eagle's medium) with 10% calf serum. Twenty-four hours later the medium was changed to DME with 5% calf serum. About 48 h later a suspension of surface membranes prepared from confluent (•) or sparse (▪) 3T3 cultures was added in 0.3 ml DME with 5% calf serum. Membrane concentration is expressed in units of phosphodiesterase (PDE) activity added per dish (one unit of activity hydrolyzes ten nmoles of substrate per h). Controls received no membranes. [^{3}H]dT incorporation rate into trichloroacetic acid insoluble counts was determined 48 h after adding membranes. The control incorporation rate was 2.38 dpm/cell. The cell number in control dishes was 9.3 X 10^3 cells/dish. The cell number in dishes receiving 5 PDE units of membranes from confluent cultures was 5.1 X 10^3 cells/dish and in dishes receiving 4.6 PDE units of membranes from sparse cultures it was 6.5 X 10^3 cells/dish.

of isolated membranes. The use of membranes is the first step in the purification of cell surface components involved in toporegulation of growth. It is important, therefore to characterize the inhibition of growth by membranes to determine whether it behaves in a manner characteristic of density-dependent inhibition of growth.

At the current stage of this work, it is not possible to state with absolute certainty that the inhibition activity is localized exclusively in the surface membrane. Since the purity of plasma membrane fractions is never absolute, the only approach to this problem until the components are purified is the use of nonpenetrating reagents that might destroy the activity in whole cells prior to membrane preparation. Proteases would fulfill the role, but in this case fail to inactivate the inhibitory component in whole cells and in most membrane preparations. This approach has been successful in delineating the location of a mitogenic signal for Schwann cells on the surface of neurites [20], a situation in which cell to cell contact results in stimulation of cell division. As shown previously, the inhibitory activity is found in a plasma membrane-enriched fraction [8]. Figure 1 shows that, based on phosphodiesterase activity (a plasma membrane marker), the activities of various particulate fractions are essentially similar. Thus the inhibitory activity in the membrane fraction is enriched to the same extent as phosphodiesterase. Extrinsic membrane proteins, or protein which simply adsorb to the membrane, can often be removed by extraction with high salt or chelating agents. The data in Table I show that high concentrations of Na

pyrophosphate, which also acts as an effective divalent metal chelator, fail to extract the inhibitory activity from the membrane. Thus it appears likely that the inhibitory activity resides in one or more intrinsic membrane components.

Density-Inhibited and Membrane-Inhibited Cells Accumulate in G_1

Our results and those of others show that density arrested 3T3 cells accumulate in G_1. Moreover, our results show that 3T3 cells incubated in culture with a surface membrane enriched fraction accumulate in G_1. This conclusion is supported by two lines of evidence. First, membrane inhibited cells accumulate in a limited region of the cell cycle. This was shown by the result that trypsinization and replating of membrane inhibited cells resulted in a synchronization of the entry of cells into S phase (Fig. 3). The steps in the cell cycle progression regulated by membranes are the same or occur later in the cell cycle than the serum restriction point. This was shown by the ability of membranes to prevent about 50% of the cell population from leaving G_1 after serum stimulation (Fig. 4). Since the maximal fraction of the cell population arrested by membranes in one cell cycle was no more than about 40% in a randomly growing cell population it seems reasonable to conclude that the arrested cells in the serum stimulation experiment represented that fraction of the cells which could be inhibited by membranes during any given cell cycle.

The second line of evidence that membrane-inhibited cells arrest in G_1 comes from flow microfluorometric determination of the DNA content of cells. The fraction of the cell population with a G_1 amount of DNA increased when sparse cells were incubated with membranes, while the fraction of the cell population with an S phase amount of DNA decreased. Taken together, these experiments show that membrane-inhibited cells, like density-inhibited cells, arrest in the G_1 portion of the cell cycle, thus satisfying a critical test of specificity of inhibition of growth by membranes.

Serum or FGF With Dexamethasone Compensate for Inhibition by Membranes and High Cell Density

When the rate of DNA synthesis was determined for 3T3 cells at various cell densities in DME with low or high serum, the rate of DNA synthesis was higher in the higher serum for any inhibitory cell density. Similarly, when sparse cells received various amounts of membranes in DME with low or high serum the rate of DNA synthesis was higher in high serum at any nonsaturating membrane concentration. FGF and dexamethasone together substituted for serum in ability to reduce inhibition by membranes. These results show that membranes inhibit cells in a manner which mimics topoinhibition since in both cases serum or defined mitogens such as FGF compensate for this inhibition [5, 15].

Serum appears to make the regulation of entry of cells into S phase less sensitive to changes in cell density or to changes in membrane concentration. The possible mechanisms by which serum or defined mitogens could compensate for inhibition by high density or by membranes depend upon the mechanism of topoinhibition. High cell density or addition of membranes could result in a local depletion of serum factors required for initiation of DNA synthesis if delivery of these factors was locally diffusion limited and if these factors were removed from the medium by cells or membranes [4]. Raising the serum concentration would compensate for this by increasing the rate of delivery of these limiting factors to the cell surface.

Recently Peterson et al [18] have measured the ability of surface membranes to inhibit the mitogenic effect of epidermal growth factor on 3T3 cells. By the use of the membranes which cannot bind EGF [18] but which blocked the mitogenic effect of EGF,

they could essentially rule out the possibility that in that particular case the membranes act by binding EGF. We have recently presented evidence that topoinhibition with these cells (in 10% serum) is not caused by a limitation of diffusion of medium components to the cell surface [21]. Alternatively, if one assumes that cell–cell contact or membrane–cell contact directly provides a negative signal for growth then the effect of serum could occur by independently providing a positive signal for growth, or in addition, by regulating the extent of cell–cell or membrane–cell interaction.

A Constant Fraction of Cell Population is Arrested by Membranes in Each Cell Cycle

The extent of inhibition of DNA synthesis increases with time of incubation with membranes. The data in Fig. 8 show that at saturating amounts of membrane about 40% of the cells are arrested in G_1 in 19 h. Since the doubling time for 3T3 cells is about 19 h, and since the inhibition of DNA synthesis continues to increase for up to 60 h [8], the membranes appear to arrest a maximum of about 40% of the growing cells during each cell cycle. When the membrane concentration is lowered the fraction of growing cells arrested during each cell cycle is reduced. Also, at a given membrane concentration raising the serum concentration lowers the fraction of growing cells arrested during each cell cycle.

Even at membrane levels which are saturating for the inhibition of DNA synthesis the degree of inhibition increases with time, therefore the time dependence of inhibiton does not result from an increase in the extent of binding of membranes to cells. If certain cells in the population were more sensitive to inhibition by membranes than other cells then it would be possible for only a fraction of the cell population to be arrested during each cell cycle. Our results are consistent with a model which states that the distribution of sensitivities remain the same for growing cells during each cell cycle since the fraction of cell cells arrested during each cell cycle remains constant. This would require that the sensitivity of each growing cell which escaped inhibition by membranes to be randomly reset after each cell cycle. If this were not the case then after one cell cycle there would be no further arrest of growing cells, since all growing cells would be insensitive to inhibition by membranes.

Martz and Steinberg have published data which supports the notion that single 3T3 cells in a population differ in their sensitivity to regulation by local cell density [22]. Using time-lapse cinematography these authors observed that all-around cell–cell contact or high local cell density present throughout G_1 did not produce immediate inhibition of mitosis in about 50% of the observed cells, while the remainder of the population was arrested.

The data shown in Figs. 6 and 7 provide additional support for the notion that among a 3T3 cell population some cells will be trapped in G_1 while others will continue into S phase under identical conditions of serum concentration. Raising the serum concentration decreases the fraction of the cell population which is arrested in G_1 per unit increase in cell density or membrane concentration. This change can only occur if after raising the serum concentration some cells arrest at a higher cell density or membrane concentration than other cells in the same population. Others have shown that when a population of confluent quiescent 3T3 cells receives fresh medium a small fraction of the population undergoes cell division [23], again suggesting that not all cells are equivalent in their ability to be arrested at a particular cell density and serum concentration.

The following model is useful for illustrative purposes to attempt to explain these observations. Suppose that at a critical point in the cell cycle the level of a factor(s) must reach a threshold value for cells to escape G_1 and enter S. The rate of accumulation of this factor(s) could be under positive control by serum and negative control by cell density or membranes. The difference in ability to leave G_1 among different cells in a population

would be explained if each cell had a different threshold for escape. In order to explain why a constant fraction of the population is arrested by membranes during each cell cycle it is necessary that the threshold for escape for each growing cell be randomly reset each cell cycle. Evidence for randomness in the threshold for initiation of cell replication among sparse growing cells [24] or among cells undergoing serum stimulation has been presented [25].

We have shown that the regulation of cell growth by addition of a surface membrane-enriched fraction to 3T3 cells in culture reproduces two critical characteristics of density-dependent inhibition of growth. Membrane inhibited cells are arrested in G_1 and membrane inhibition can be compensated for by raising the serum concentration or by adding FGF plus dexamethasone. These features of inhibition of growth by membranes, plus the others previously demonstrated [8], support the notion that membranes can be used to study the role that cell–cell interaction plays in regulation of growth. At the present level of purification the nature of the inhibitory activity is obscure, although its heat lability suggests a role for a protein component. Membranes should provide a reasonable step from which further purification can be achieved.

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