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STUDIES ON CONTACT INHIBITION OF GROWTH IN THE MOUSE FIBROBLAST, 3T3

I. CHANGES IN CELL SIZE AND COMPOSITION DURING 'UNRESTRICTED' GROWTH

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SUMMARY

Growth of the contact-inhibited 3T3 and its SV_{40}-transformed derivative, 3T3T is unbalanced. Despite obvious exponential increase in cell number, cell volume, RNA and protein show a marked decrease with increasing population density in both cell types, greater in 3T3 than 3T3T. Daily medium change does not correct this growth pattern but merely seems to equalize initial growth rates of the 2 cell types and prolong the period during which the cell number continues to increase. The evidence suggests that the lag period that ensues following plating of the cells is a period of intense metabolic activity during which a large proportion of cellular protein and RNA is synthesized in preparation for exponential growth.

INTRODUCTION

The mouse embryo fibroblast line 3T3, developed by Todaro & Green (1963), is admirably suited for studies on the control of cell growth in culture because of its high degree of contact inhibition. Since it appears clear that long-term cultivation *in vitro* may in itself alter growth requirements, in part by a process of inadvertent selection (Eagle, 1965), an appropriate control cell which is not contact inhibited must be used for comparison. The 3T3 transformed by the oncogenic virus SV_{40} (3T3T) (Todaro & Green, 1966) was used in these studies. Henrici (1928) reported that bacterial cells increase in size during the lag which precedes cell division in a newly inoculated culture, and become smaller again during the period of declining growth. That a similar sequence of events ensues during the growth of eukaryotic cells in culture is not clearly documented. In the experiments to be described, we will demonstrate analogous changes in the 3T3 and 3T3T. These changes along with those in cell composition will be used to provide a rationale for the hypothesis that contact inhibition of growth is the result of series of events that follow from the progressive intracellular depletion of vital small-molecular-weight building blocks such as amino acids. These deficiencies occur secondarily to changes in the plasma membrane which are apparently population density dependent. In this paper we merely wish to document the cell size and some changes in composition with growth, as well as the evidence that growth of the 3T3 and 3T3T is unbalanced in the sense defined below.

We use the phrase 'unrestricted growth' with the following reservation. In so far as the medium does not limit the growth by the concentration of any nutrilite contained therein, it is unrestricted. For example, growing the 3T3 in medium containing 5 times the concentration of cofactors and nutrilites does not increase the rate of growth or the growth yield (unpublished data). On the other hand, as has been shown by others (Todaro, Lazar & Green, 1965; Holley & Kiernan, 1968; Becker & Levitt, 1968), increasing the concentration of serum alone will stimulate the extent of growth.

The term 'balanced growth' is used in the sense stated by Campbell (1957), that growth is said to be balanced over a time interval if, during that interval, every extensive property of the system increases by the same factor. Our data, we feel, indicate that at almost no time during the growth of the 3T3 is balanced growth achieved. A qualitatively similar but much less marked deviation is noted in 3T3T.

MATERIALS AND METHODS

Cell cultures

The 3T3 and its transformed derivative were generously supplied by Dr Howard Green. In accordance with the original directions, the growing cell stocks of 3T3 were plated at 1:100 dilutions in order to preserve the requisite degree of contact inhibition. Cultures were maintained in Dulbecco's modified Eagle's medium (Grand Island Biological Co.) with 10% calf serum (Colorado Serum Co.). About every 2 months all cultures were discarded and fresh cells were grown from frozen stocks. Cultures were periodically checked for Mycoplasma contamination by culturing cells in both liquid Tryptose broth containing 1% glucose and 1% serum, and solid Tryptose media containing 1% agar (Maniloff, 1969). Cells were grown at 37 °C in a humidified atmosphere containing 10% CO_2 in plastic dishes or flasks (Falcon Plastics). The medium was changed at 3–4 days intervals for stock cultures and 24–48 h prior to each experiment. Thereafter it was changed daily for the duration of the experiment.

Cell volume

Cells were grown on 60-mm Petri dishes to the desired surface density. The medium was removed and the cells washed twice in an excess of buffer. Trypsinization was performed using 0·1% trypsin (Difco) treatment in Tris A for 12 min (Tris A: NaCl, 138 mM; KCl, 5·0 mM; Na_2HPO_4, 0·7 mM; tris(hydroxymethyl)aminomethane, 25 mM pH 7·4). The trypsin was neutralized by an equal volume of medium and brought up to adequate volume for counting and sizing of the suspended cells. These dilutions were made using Ca^{2+}- and Mg^{2+}-free Earle's Balanced Salt Solution.

The counting and sizing operation was performed using a Celloscope particle counter (Particle Data Inc., Elmhurst, Ill.) equipped with a 76-μm orifice. The output was tabulated and recorded by a Nuclear Data Pulse Height Analyser, Series 2200 (Palapine, Ill.). A minimum of 8000 events was recorded in 128 channels. Polystyrene particles (Particle Information Service, Los Altos, Cal.), 14–48 μm diameter, which had been previously sized under the microscope using a micrometer eyepiece, were used for calibrating the analyser. All counting and sizing operations were performed in triplicate. Average diameters were calculated by multiplying the number of cells counted in a given channel by the diameter which the particular channel represented, summing the results from each channel, and dividing by the total number of cells counted. Average cell diameters computed by counting 10^3 cells with the micrometer eyepiece agreed within 15% with those obtained by electronic sizing. In general, the electronic sizing method tended to give lower values than those obtained under the microscope.

Cell water

Cells were plated on 15-mm circular glass coverslips, no. 2 thickness (Corning no. 583120) contained in 100-mm diameter Petri dishes. When the desired surface density was achieved, the medium was aspirated, and the coverslips washed in Tris A. A few coverslips were set aside for determination of cell density. To the remainder, Tris A containing 0·1 % glucose, 2 μCi/ml of [^{14}C]thiourea (S.A. 39·8 mCi/mmol) and 4 μCi/ml of [^{3}H]mannitol (S.A. 3·14 mCi/μmol) was added to each plate of coverslips. Quantities of radioactivities were chosen such that the ^{14}C cpm differed from the ^{3}H cpm by at least 100 per cent.

The plates containing the coverslips were incubated for 30 min at 37 °C. The coverslips were removed, dipped twice into buffer, quickly drained and added to scintillation vials containing 0·5 ml of NCS solubilizer (Nuclear Chicago). Scintillation fluid was added and the radioactivity determined in a liquid scintillation counter. A minimum of 6 determinations was performed for each density. In any given experiment at least 4 different population densities were examined for both the 3T3 and 3T3T. An aliquot of incubation medium was counted to relate radioactivity and fluid volume. The intracellular fluid was taken as the difference between the apparent thiourea space and the apparent mannitol space (see Discussion).

Protein and RNA content

The medium was aspirated from the plates and the cell layers gently washed twice with Tris A. Cell protein was determined in duplicate by a modified Lowry procedure (Oyama & Eagle, 1956). RNA was determined on sister plates by dissolving the cell layer in 1·0 % sodium deoxycholate (Mann Research Labs.). The lysate was precipitated with 0·6 N perchloric acid. The precipitate was washed, sedimented by centrifugation and dissolved in 0·3 N KOH at 37 °C. Hydrolysis was allowed to proceed for 1 h with occasional stirring to ensure that the precipitate had completely dissolved (Munro & Fleck, 1966). The mixture was then neutralized with perchloric acid and the resulting precipitate removed by centrifugation. The supernatant was then acidified with additional perchloric acid resulting in the precipitation of protein and DNA. The supernatant was collected and the pellet washed again in perchloric acid. After centrifugation, the supernatants were combined and neutralized. They were evaporated to dryness and resuspended in an amount of Tris buffer sufficient to give adequate optical density at 260 nm. Contamination of the RNA hydrolysate by protein could not be detected.

Measurement of radioactivity

Radioactivity was measured in an MI Liquid Scintillation Spectrometer (Nuclear Chicago). [^{14}C]Thiourea was obtained from Schwarz/Mann and [^{3}H]mannitol from New England Nuclear Corp. The double-label counting was performed by a Channels Ratio method using an external standard. The toluene-based scintillation fluid consisted of 15·1 g of diphenyloxazole and 50 mg of 1,4-bis-2(phenyloxazolyl)-benzene (Amersham Searle) dissolved in 4 l. of toluene. The efficiency for ^{14}C was 60 % and for ^{3}H, 15 %. All other reagents were obtained from Mallinckrodt.

RESULTS

Growth characteristics of 3T3 and 3T3T

Cells were plated on 60-mm Petri dishes and 24 h later the medium was changed. This was considered zero time. Thereafter, the medium was changed daily. Under these conditions, the 3T3 reached a density of approximately 13×10^4 cells/cm^2, which is more than twice the density achieved when medium is changed semiweekly. In our experience, cells which achieve population densities greater than 6×10^4 cells/cm^2 by semiweekly medium change can achieve this very high density by daily medium

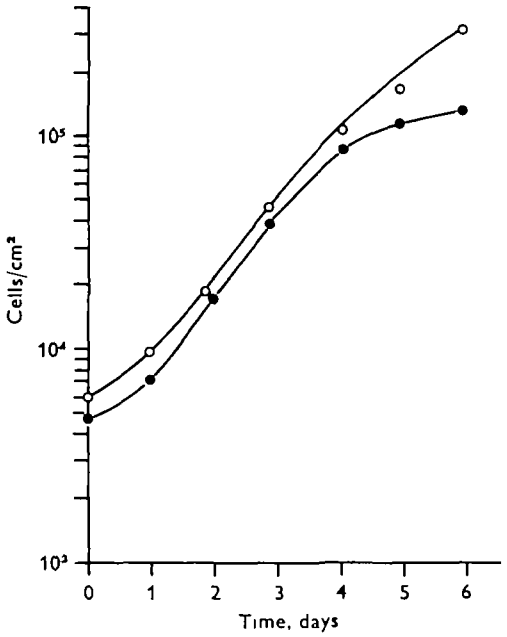

Fig. 1. Growth of 3T3 (●) and 3T3T (○). Medium was changed daily. Time 0 is 24 h after plating. Cell suspensions from duplicate plates were counted in triplicate. Each point is the average from 2 plates.

change. On the other hand, cells which cease dividing at densities less than 5×10^4 respond less well to daily medium change.

By extrapolating the linear portion of the growth curves in Fig. 1 to zero, a lag of approximately 12 h and 1 h (36 and 25 h if we consider the actual time of plating) is noted for the 3T3 and 3T3T, respectively. In experiments not shown, we have determined that the higher the population density of the cells in the stock flasks prior to the experiment, the more prolonged will be the lag phase after plating. This relationship applies if the cells are plated at densities greater than $0{\cdot}5 \times 10^4$ cells/cm^2.

The initial growth rates during exponential increase tend to be greater in the 3T3T (Meisler, 1973). Changing the medium daily tends to abolish this difference. Invariably, whether the medium is changed in the usual manner or daily, the 3T3T continue to grow for a more extended period than the 3T3, though not necessarily in an exponential manner. Arithmetic increase in 3T3T at high population densities is especially prominent when the medium is changed only twice each week. In the experiment shown, it is evident that the 3T3 are in exponential growth between 24 and 80 h. Thereafter growth gradually ceases. In the case of the 3T3T, exponential growth continues further, until nearly the termination of the experiment.

Cell volume

The experiment shown in Fig. 2 is representative of others performed in an entirely comparable manner and demonstrates what appears to hold true generally with these

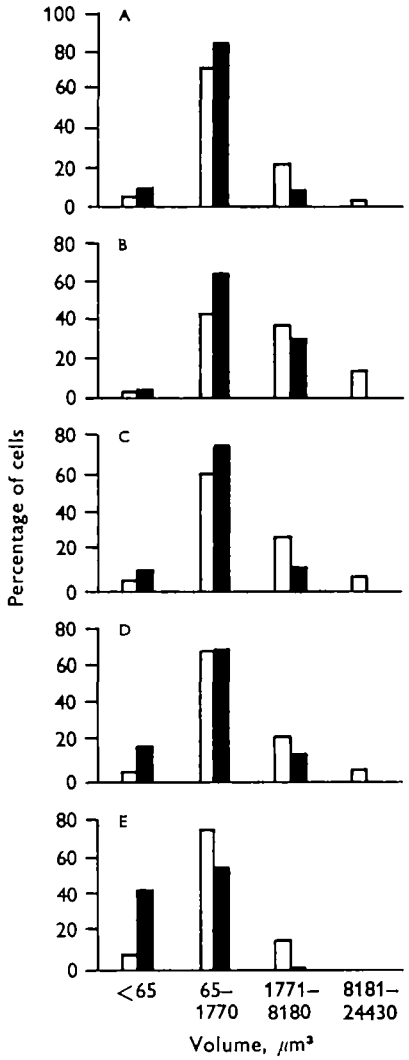

Fig. 2. Variation in cell volume with increasing population density. These population densities were obtained from a single experiment in which cells were inoculated on to 60-mm Petri dishes. Plates were processed as described under Methods. Medium was changed daily. The open bars represent 3T3 and the darkened bars, 3T3T. Times, in h, after inoculation and population densities $\times 10^{-4}$ for 3T3 and 3T3T, respectively were: A, 0, 1·40, 1·86; B, 22, 1·67, 4·27; C, 51, 3·40, 12·09; D, 74, 6·44, 24·92; E, 146, 11·98, 48·50.

cells. It shows the change in cell size distribution as a function of time from an arbitrary zero (24 h after plating). In this experiment, the medium was changed daily and the growth curve is shown in the inset of Fig. 3. The volume intervals on the abscissa were chosen such that if the tops of the bars were connected by a continuous line, the curve obtained would mirror the frequency distribution of sizes seen in the oscilloscope screen of the cell sizer. The distribution tends to be similar

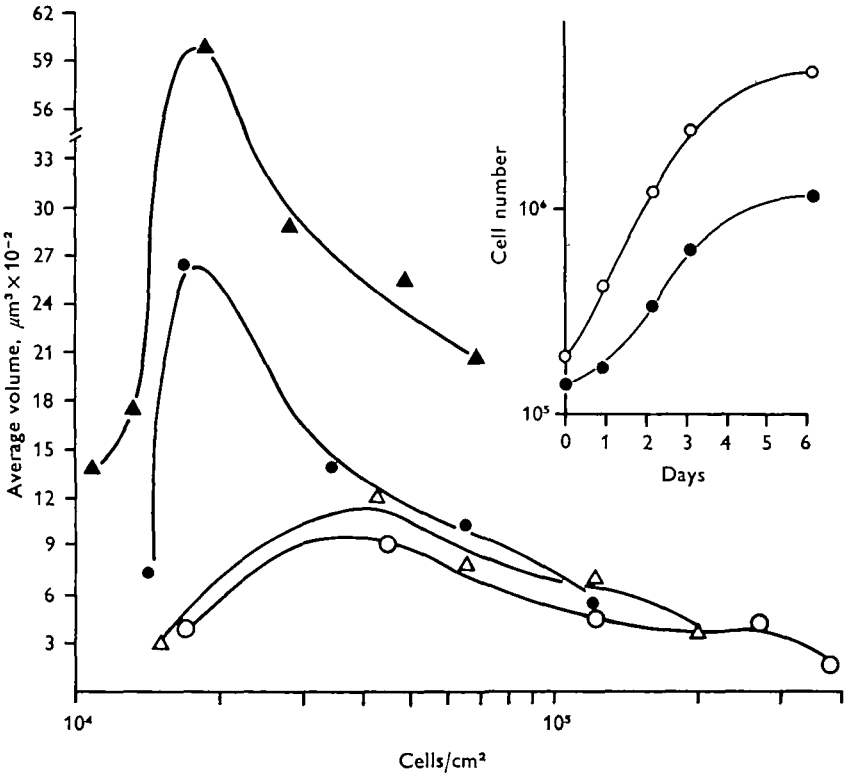

Fig. 3. Average cell volume as a function of population density. The inset represents the growth curve that corresponds to the average volume curve with the same symbols. ▲, 3T3, doubling time T_D 28 h; ●, 3T3, T_D 24 h; △, 3T3T, T_D 19 h; ○, 3T3, T_D 17 h.

between the 3T3 and the 3T3T except for the presence of a significant number of 3T3 with a volume greater than 8000 μm^3. Not evident from Fig. 2 is the fact that only a very small percentage of 3T3T ever exceed 4000 μm^3. The range of cell volume seen in the 3T3 is truly remarkable extending from about 70 to over 24000 μm^3. It is unlikely that any significant number of dead cells is included in these counts since 2 washes from Tris A prior to trypsinization will remove them. The small size of the orifice in the particle counter precludes the possibility of cell clumps being counted as single cells.

In Fig. 3, we have depicted the average cell volume as a function of population density. The growth curves for one set of volume distributions (3T3 and 3T3T) are shown in the inset. In this experiment, at a population density below $2{\cdot}0 \times 10^4$ cells/cm² the cells are still in lag phase. Within 24–48 h, with the onset of exponential growth, both cell types reach their maximum volume and thereafter, it gradually declines. As indicated in Fig. 2, over most of the range of population density, the 3T3 tend to be larger than the 3T3T. It is also quite clear in these 2 experiments and in others not shown, that at least in the case of the 3T3, the average cell volume varies directly with the doubling time. Cells with longer doubling times tend to

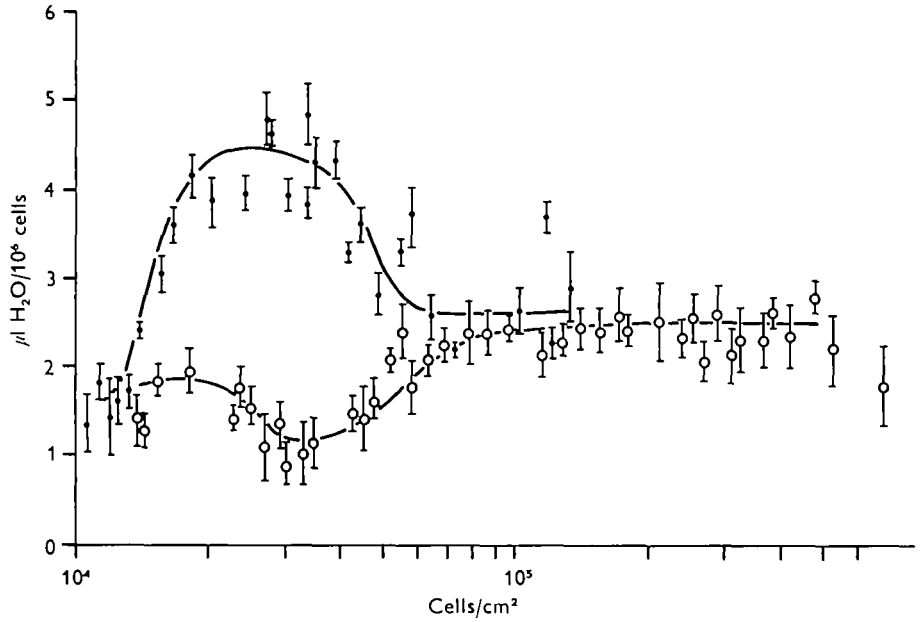

Fig. 4. Cell water variation as a function of population density. Data are pooled from many different experiments. T_D was about 24 h. Cell water was determined 48 h after plating. Each point with standard deviation is calculated from 6 separate coverslips. ●, 3T3; ○, 3T3T.

become much larger and also cannot be stimulated to reach the high cell densities (and smaller volumes) attained with the more rapidly growing cells. In the case of the 3T3T, we have at present no line which grows with a doubling time of more than 20 h or less than 16 h. Since these times tend to vary from experiment to experiment, no definite statement can be made.

On analysis of the frequency distributions shown in Fig. 2, it appears that the determining factor with regard to average size, is the relatively large number of 3T3 cells with volumes of 8000 μm^3 or greater. In an experiment in which the doubling time of the 3T3 was about 30 h, a size distribution of the density showing maximum average diameter ($1{\cdot}53 \times 10^4$ cells/cm^2) revealed that more than 50% of the cells had volumes of 8000 μm^3 or more.

Cell water

Fig. 4 is a composite representation of cell water determinations from many different experiments. As indicated earlier in regard to cell volumes, these experiments were performed on 3T3 whose doubling time was of the order of 22–24 h. We have noted that if for any reason the doubling time is more prolonged, water content tends to be greater. However, the shape of the distribution tends to correspond to that noted in Fig. 3.

The cell water in Fig. 4 of the 3T3T reaches a nadir at densities between 2·5 and $3{\cdot}5 \times 10^4$ cell/cm^2. Thereafter it increases and at population densities of 1×10^5

and higher it remains essentially the same, at a value approximating its maximum. The 3T3 shows a rather striking difference in that at the density at which the 3T3T has reached a minimum water content, the contact-inhibited cell demonstrates a large increase in intracellular water. It is of interest that the lowest values for 3T3 intracellular water occur either at a very low density or at the density which corresponds to that at which no further increase in cell number would occur unless stimulated by daily medium change. Thereafter, the water content tends to remain constant.

The maximum average volume of the 3T3T cell is attained at a point just prior to the population density at which the water content is maximum. The very great variation in intracellular water seen in the 3T3 is not seen in the transformed cell, and by the time densities of 1×10^5 cells/cm^2 are attained, the water tends to remain constant.

RNA and protein content

As indicated in Fig. 5, with increasing cell density, despite daily feeding and maintained exponential growth, the cellular content of stable RNA shows a progressive decrease in both cell types. Since about 90% of this stable cellular RNA is ribosomal (rRNA) (Darnell, 1968), the absorbance at 260 nm of the RNA hydrolysate reflects mainly the cellular content of ribosomes. In the figure (the matching growth curve is shown in Fig. 1), the decline in cellular RNA is plotted as a function of cell density. It should be pointed out, that whether medium change occurs daily or semiweekly, a similar decrease in rRNA is noted (unpublished data). As will be explained in the Discussion, and as shown in Fig. 5, at least for the 3T3, if looked for early enough after plating, there is an initial increase in cellular RNA. In general, the period of increasing stable RNA occurs during the lag phase of growth, at low population density. The magnitude of this increase depends on the degree of crowding achieved in the cultures from which the cells were taken. Dense cultures, in which the content of cellular RNA is initially minimal, show the greatest increase in stable RNA during lag phase.

The content of cellular protein changes in a manner analogous to cellular RNA as is shown in Fig. 6. Again, an early increase during lag phase of growth is evident provided it is looked for soon after cells have been plated. Invariably, the maximum RNA and protein content are reached by the end of lag phase and of course this is just about the time at which the cell has attained its largest volume.

DISCUSSION

It should be clearly understood that the observations reported in this study pertain to the 3T3 and its SV_{40}-transformed variant. While the variations observed may occur in other cell lines, we have not as yet studied them. However, Swaffield & Foley (1960) have shown similar variations during growth in cellular RNA and protein in a mouse fibroblast MF-929 and 2 tumour cell lines, S-180 M and Erhlich ascites. Cell volume and water were not determined. In our experiments, by changing medium daily, we attempted to minimize growth differences between cell lines

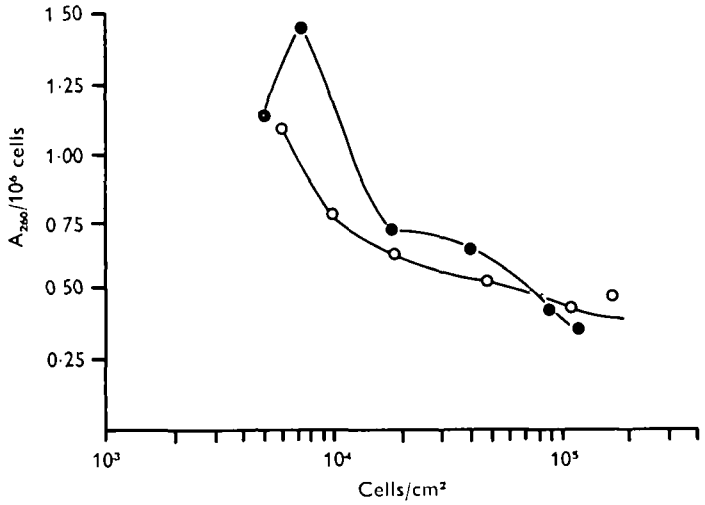

Fig. 5. Cellular RNA variation, determined by absorption at 260 nm, with population density. Each point represents the average of 2 determinations on duplicate plates, performed 24 h after medium change. ●, 3T3; ○, 3T3T.

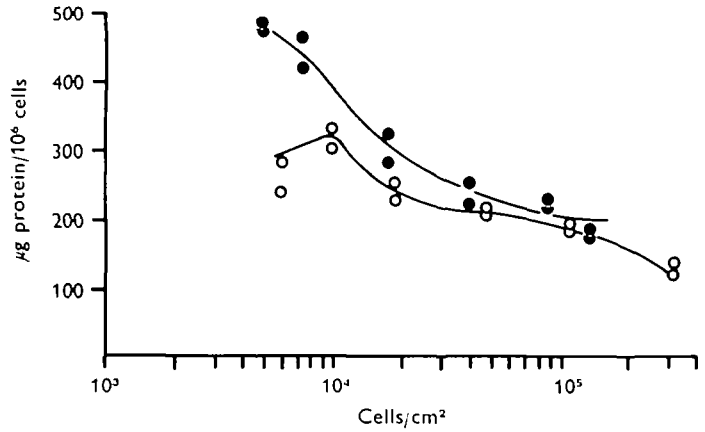

Fig. 6. Cellular protein variation with population density. Each point represents the average of 2 determinations from a single plate of a duplicate set, performed 24 h after medium change. ●, 3T3; ○, 3T3T.

resulting from either depletion of essential nutrilites or accumulation of products of cellular metabolism which might differentially depress biosynthetic processes. For example, it has been shown recently that variation in pH of the medium markedly influences growth and the synthesis of macromolecules (Ceccarini & Eagle, 1971; Perlin & Hallum, 1971).

Cell volume

As indicated in an earlier section, electronic sizing seems to agree reasonably well with results obtained by sizing under the microscope using a micrometer eyepiece.

It is of importance to emphasize that these values may in fact only roughly approximate the actual volumes of growing cells since the cells, on trypsinization and suspension, assume a spheroidal shape; in contrast, during growth, the cells tend to be fibroblastic in contour in all but dense cultures. Furthermore, the washing and trypsinization of the cells may cause segments of the outer membrane to come away from the cell body. Though there is a wide range of volumes, the distribution seen in Fig. 2 is modal and actually most of the cells fall between relatively narrow limits. As was indicated earlier, the higher mean volume of the 3T3 is due to a significant number of cells with volumes exceeding 8000 μm^3. Under the same conditions of random growth the 3T3T show a very much narrower distribution. We feel this is of great importance in emphasizing the heterogeneity of the 3T3 as opposed to the more homogeneous nature of the 3T3T. This heterogeneity must arise by virtue of the fact that the cells grow not as discrete units, but rather in clumps. Such a situation could be expected to affect the potentially contact-inhibited 3T3 to a much greater extent than the 3T3T.

Cell water

The changes in cell water noted in Fig. 4 are complex and not subject to simple interpretation. Results obtained by the isotope methods employed may be misleading, but they tend to be very consistent over a large number of experiments. The validity of using mannitol as a marker for extracellular water has been discussed by Page (1962). At least in an *in vitro* preparation of cat heart muscle, the use of mannitol seems to give greater accuracy than inulin due to more complete equilibration. In our system, determination of extracellular water using inulin gave results which differed by no more than 10% from those using mannitol. We have also shown that for both mannitol and thiourea equilibrations are complete by 30 min (unpublished data).

Thiourea has been used as a marker for total body water for almost 50 years. A useful discussion of the permeability of cells to non-electrolytes is available (Troshin, 1966). Unfortunately, the use of thiourea and urea for this type of measurement is complicated by the fact that these substances are bound to some extent by intracellular protein (Hill, 1930). More recent observations (Bozler, 1959) indicated that the urea space on average was 84 and 88% in *in vitro* preparations of frog sartorius and stomach respectively. We therefore appreciate the fact that Fig. 4 may overestimate cell water by as much as 15%.

It is of interest to compare our results with similar experiments performed by Foster & Pardee (1969). They used a polyoma-transformed 3T3, but otherwise the system was similar to our own. These workers determined cell volume using packed cell volume following centrifugation. This method does not appear to take into account the volume occupied by trapped water. Their cell volumes tend to be greater than ours, and moreover, they did not show a difference between normal and transformed cells. This seems surprising in view of the fact that the size difference can easily be discerned under the microscope. They also did not demonstrate a difference in protein content between the normal and transformed cells with varying population

density, but it did appear that the protein content of their cells decreased with increasing population density.

In their studies of cell water, they demonstrated a simple linear relationship between cell water and cell protein which we are not able to confirm. Their cell water determinations differ from ours in that ^{14}C-labelled urea was used to measure total water. Moreover, their urea and mannitol solutions were made 2·0 mM whereas we added no unlabelled material. Initially, our experimental medium was 0·5 mM in mannitol and thiourea. Further experiments revealed that the same values were obtained whether or not unlabelled material was added. As previously indicated, observations extending over 2 h revealed essentially complete equilibration in 30 min. This is not necessarily to be expected since Bozler's data (1959) indicate a significant osmotic effect by a variety of non-electrolytes including urea and thiourea. For example, 0·2 M urea in Ringer's solution causes an average weight loss of 14 % in frog sartorius after 2 h. This is a very much higher concentration than we have used. In view of the fact that our values for cell water are higher on the average, by a factor of 2 to 3 than those of Foster & Pardee (1969), it would be of importance to know if equilibration had occurred in their cells in the 30-min incubation period.

General remarks

It is apparent that despite exponential growth, the volume, RNA and protein content of the 3T3, and to a lesser extent of the 3T3T, decrease. Parenthetically, it should be noted that although the 3T3 cell is much larger than the 3T3T at most population densities, the stable RNA (ribosome) content is very similar. This point will be elaborated in a later publication. Though the 3T3T cell demonstrates less pronounced unbalanced growth, it would be misleading to imply that this by itself can explain its ability to reach exceedingly high population densities. It is apparent from the data already presented that at higher population densities, the decreases in RNA, protein and volume occur much more gradually, if at all. Despite the relatively low content of ribosomes and protein, significant growth still occurs.

This type of unbalanced growth, which appears to be more marked in 3T3 than in 3T3T, if we use average volume (Fig. 3) as a criterion, is quite unlike that which occurs in the presence of inhibitors of DNA synthesis. In the latter situation, growth ceases but RNA and protein synthesis continue, and cell size increases (Cohen & Studzinski, 1967). Prokaryotic cells, however, provide a possible model for the situation we have observed.

In exponentially growing bacterial cultures, the average mass, RNA, DNA and number of nuclei/cell can be described as exponential functions of the growth rate (Schaechter, Maaløe & Kjeldgaard, 1958). Exponentially growing bacteria are larger than resting cells (Maaløe & Kjeldgaard, 1966). While the latter observation is true for the 3T3 and 3T3T it is also apparent that cell size tends to decrease despite continuing exponential growth. It is also known that as the density of a bacterial culture approaches saturation, the rate of cell division remains unchanged for some time after the rate of mass synthesis has been lowered (Schaechter *et al.* 1958).

During the latter period growth is said to be unbalanced and is felt to be due to depletion of essential nutrients from the medium.

If an analogy exists between the eukaryotic and prokaryotic cells, the model predicts that single or multiple nutritional deficits should be present. In our study, daily medium change as well as growth in continually rotating flasks (unpublished observations) increases the cell yield. Other workers have reported similar findings (Kruse & Miedema, 1965; Griffiths, 1970). On the other hand, medium in which the amino acids and glucose have been increased 5-fold has no such stimulatory effect (unpublished data). Furthermore, to the extent that unbalanced growth is an indication of nutritional deficiency, it would appear that such deficiencies are seen very early during exponential growth. Finally, it is known that medium harvested from confluent cultures of 3T3 is able to support the growth of low population density cultures (Todaro *et al.* 1965).

We suggest that another possible interpretation is that deficiencies do exist, but at intracellular level. Future papers in this series will be directed at demonstrating that the cells undergo a progressive alteration in surface membrane with increasing population density, such that it becomes increasingly difficult for the cell to absorb quantities of nutrients from the medium sufficient to sustain growth. An entirely similar sequence of events ensues for the 3T3T, but the membrane changes occur at higher population densities.

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