

# INHIBITION OF CELL GROWTH IN THE G<sub>1</sub> PHASE BY ADENOSINE 3',5'-CYCLIC MONOPHOSPHATE

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## ABSTRACT

Incorporation of tritiated thymidine into acid-precipitable material was used to measure the rate of DNA synthesis in secondary cultures of human diploid fibroblasts. Confluent cultures of human diploid fibroblasts, which are synchronized in the G<sub>1</sub> phase due to contact inhibition, were released from growth inhibition either by the addition of fresh medium to the cultures or by trypsinization and replating at nonconfluent densities. Either treatment resulted in a synchronous wave of DNA synthesis beginning 10–15 h after treatment and peaking at 20–25 h. In confluent cultures stimulated by fresh medium, either the addition of 0.25 mM N<sup>6</sup>, O<sup>2</sup>-dibutyryl-adenosine 3',5'-cyclic monophosphate (db-cAMP) to the medium in the interval 4–8 h after stimulation or the replacement of the fresh medium in that same 4 h interval with the depleted medium present on the cells for the 2 day period before stimulation delayed the synchronous onset of DNA synthesis in the cultures by about 4 h. In nonconfluent cultures freshly seeded from trypsinized confluent cultures, this same depleted medium obtained after a 2 day incubation of fresh medium on confluent cultures is shown to support the progress of the cells into S phase; however, the addition of 0.25 mM db-cAMP to the medium 3½ h after replating still partially prevented the initiation of DNA synthesis in the cultures. The results are discussed in terms of the role of serum and cAMP in the control of cell growth in fibroblast cultures.

## INTRODUCTION

A number of lines of evidence are available to suggest a role for adenosine 3',5'-cyclic monophosphate (cAMP) in contact inhibition of cell division. The growth of a number of different cell types can be inhibited by exogenous cAMP. These include L cells and HeLa cells (25), and cells from a number of tumorigenic cell lines (14). Recently, N<sup>6</sup>, O<sup>2</sup>-dibutyryl-cAMP (db-cAMP) has been shown to synchronize Chinese hamster ovary cells (24) and human diploid fibroblasts (13) in the G<sub>1</sub> phase. Finally, in normal fibroblast cell lines, cAMP levels are found to be higher in contact-inhibited cultures than in nonconfluent, rapidly growing cultures (19, 20).

In a previous report from this laboratory (13), evidence was presented to indicate that the stimulation of cell growth in confluent cultures of human diploid fibroblasts by fresh serum required a decrease in the intracellular level of cAMP. The results indicated that there is a cAMP-sensitive interval of about 7 h duration in the G<sub>1</sub> phase of human diploid fibroblasts during which events necessary for the cells to progress through G<sub>1</sub>, and subsequently enter into S phase, are inhibited by high intracellular levels of cAMP. It was also shown that serum stimulation of contact-inhibited cells decreased intracellular cAMP during this entire interval.

Several investigations have shown that treatment with proteolytic enzymes, such as trypsin or Pronase, can also stimulate cell growth in contact-inhibited cultures (4, 5, 26). In a recent report (6), a model was proposed which suggested that agents which stimulate cell growth in contact-inhibited cultures, such as proteases or serum, do so by causing a surface alteration leading to a decrease in cAMP. This model implied that this decrease in cAMP only needed to last for a very short time, perhaps 10 min, in order to allow the cells to resume growth; this short interval of decreased intracellular cAMP would be sufficient as the initial "signal" leading to the subsequent entry into S phase many hours later.

In the present series of experiments, evidence is presented to indicate that this model is not applicable to serum stimulation of confluent cultures of human diploid fibroblasts. Furthermore, the effect of db-cAMP on the synchronous growth of non-confluent cultures freshly seeded from trypsinized confluent monolayers is inconsistent with this model. Possible mechanisms by which serum and trypsin might stimulate cell growth in contact-inhibited fibroblast cultures are discussed.

## MATERIALS AND METHODS

### *Cell Cultures*

Strains of human diploid fibroblasts obtained from skin biopsies were kindly supplied by Dr. Henry Nadler, Department of Pediatrics, Northwestern University Medical School. Standard tissue culture techniques were used and no strain was carried in culture for more than 4 or 5 wk. All cells were grown on 35 mm or 60 mm plastic tissue culture Petri dishes (Falcon Plastics, Div. B-D Laboratories, Inc., Los Angeles, Calif.) in a CO<sub>2</sub> incubator (5% CO<sub>2</sub>, 95% air) at 37°C. The culture medium, Eagle's Minimal Essential Medium (EMEM) (12) supplemented with 10% fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.), was changed every 2-3 days and always 2 days before an experiment was started. Because of its greater buffering capacity, Earle's balanced salt solution was used in the culture medium rather than Hanks' balanced salt solution. The sodium salt of db-cAMP (Sigma Chemical Co., St. Louis, Mo.) was used when required. The trypsin used in this study was purchased from General Biochemicals, Div. Mogul Corp., Chagrin Falls, Ohio, and was dissolved in GIBCO solution A, which contains 6.8 g NaCl, 0.4 g KCl, 1.0 g glucose, 2.2 g NaHCO<sub>3</sub>, and 5 mg phenol red per liter of solution. GIBCO solution A has essentially the same formulation as Puck's saline A (21)

except that the GIBCO solution contains a larger amount of NaHCO<sub>3</sub>.

### *Incorporation Studies with Tritiated Thymidine*

As previously described (13), uptake of [<sup>3</sup>H]thymidine (<sup>3</sup>H]TdR) into acid-precipitable material was used to measure the rate of DNA synthesis, and therefore the proliferative activity, in cell cultures. Cultures were incubated for 30 min in fresh culture medium containing 1.0 μCi [<sup>3</sup>H]TdR/ml (New England Nuclear, Boston, Mass.; sp act, 6 Ci/mmol). After 30 min, the medium was discarded and the cells were washed three times with cold 0.15 M NaCl. Each culture was then dissolved in a known amount of 1.0 N NaOH. A sample of this was used to determine the protein content of the culture using the method of Lowry (15), while the remainder was neutralized with 1.0 N HCl and precipitated with trichloroacetic acid (TCA) at a final concentration of 5%. The precipitate, after being washed three times with 5% TCA, was dissolved in 1.0 ml of 0.05 N NaOH, and 0.1 ml was counted in a Packard Liquid Scintillation Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) with an efficiency of 15%. The rate of DNA synthesis was always expressed as TCA-precipitable counts per minute per milligram cell protein, and in some experiments this rate was then normalized to be a percentage of an appropriate control value.

## RESULTS AND

## PERTINENT DISCUSSION

### *Effect of Fresh Medium*

### *on DNA Synthesis in*

### *Contact-Inhibited Cultures*

Human diploid fibroblasts in culture were previously shown to be contact inhibited when the density of the culture exceeded 50 μg cell protein/cm<sup>2</sup> of culture dish (13). In the first experiment to be described here, three groups of contact-inhibited cultures were refed 2 days after their last medium change with fresh EMEM containing 10% fetal calf serum (F+S). One group remained untreated for the remainder of the experiment. The second group was refed again, after 5 h in F+S, with the depleted medium which was on the cells for the 2 day period before the initial medium change. Before this medium was added back to the cultures, however, it was replenished with glutamine, amino acids, and vitamins. This replenished medium will hereafter be referred to as contact-inhibited (CI) medium. The third

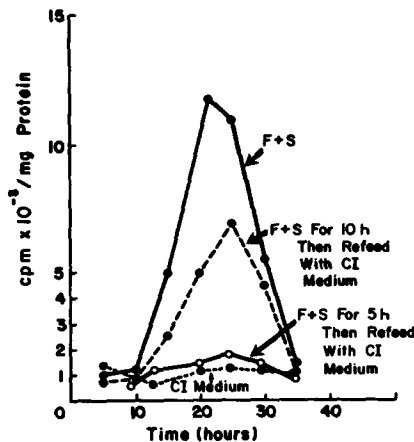


FIGURE 1 Kinetics of commitment to DNA synthesis after refeeding with fresh medium. Three groups of contact-inhibited cultures were refed 2 days after their last medium change with fresh EMEM containing 10% fetal calf serum (F + S). Two of the groups were refed with CI medium either 5 or 10 h after stimulation, while the third group remained untreated for the remainder of the experiment. A fourth group of cultures was refed at the beginning of the experiment with CI medium. DNA synthesis in all four groups was followed as a function of time after stimulation as described in Materials and Methods. CI medium is medium which has been incubated on confluent cultures for 2 days and is then replenished with amino acids, glutamine, and vitamins. Each point in the figure is the average of two determinations.

group of cultures was refed with CI medium 10 h after the initial medium change. In both groups of cultures which were refed with CI medium at either 5 or 10 h, the cultures were washed twice with serum-free EMEM, preheated to 37°C, just before the addition of the CI medium in order to remove as much of the fresh serum from the cells as possible.

A fourth group of cultures remained unstimulated and instead was refed with CI medium at zero time. In all four groups DNA synthesis was followed as a function of time after the initial refeeding.

In the top curve in Fig. 1, it can be seen that stimulation for the entire experiment with F+S resulted in a synchronous wave of DNA synthesis beginning 10–15 h and peaking 20–25 h after refeeding. These data indicate that at least some of the cells were released from their G<sub>1</sub> phase arrest of growth, which is characteristic of contact inhibition (17), and were allowed to progress

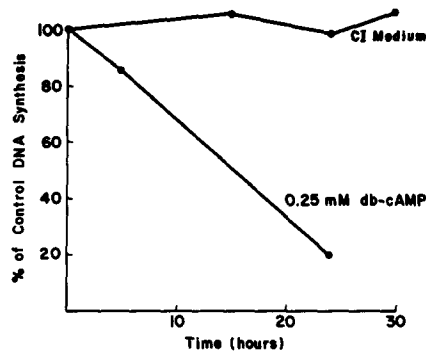
synchronously through G<sub>1</sub> and initiate DNA synthesis simply by adding fresh medium. Autoradiographic experiments not shown here indicated that about 10% of cells in confluent cultures were actually stimulated to initiate DNA synthesis after a medium change.

This partial release from growth inhibition by refeeding has been demonstrated before (20, 30) and is thought to be due to a macromolecular substance in fresh serum which desensitizes a small percentage of cells to the inhibitory effects of cell contact (30). The macromolecule involved appears to be depleted from fresh medium after incubation on confluent cultures for 2 days, as such depleted medium had no stimulatory effect on confluent cultures, even when replenished with vitamins, amino acids, and glutamine (Fig. 1, bottom curve).

Examination of the other two curves in Fig. 1 reveals that 10 h of stimulation with F+S followed by incubation with CI medium did not appreciably affect the time of peak synthesis but decreased the size of the peak by about 50%. However, stimulation with F+S for only 5 h was not sufficient to result in any appreciable peak of DNA synthesis, indicating that almost none of the cells were able to progress through G<sub>1</sub> and enter S phase under these conditions. In a previous paper (13), it was reported that the initiation of DNA synthesis in confluent cultures refed with fresh medium could be almost completely inhibited if those cultures were given CI medium as late as 7 h after the fresh medium change.

#### DNA Synthesis in Nonconfluent Cultures

Our previous data indicated that when the cell protein density of human fibroblast cultures was less than 10 μg cell protein/cm<sup>2</sup> of culture dish, DNA synthesis was maximal and independent of culture density (13). In the following experiment, all cultures had protein densities less than 10 μg/cm<sup>2</sup> and are referred to as nonconfluent cultures. Three sets of nonconfluent cultures were refed with F+S, CI medium, or with F+S containing 0.25 mM db-cAMP. This concentration of db-cAMP has been shown to partially synchronize nonconfluent human diploid fibroblast cultures by inhibiting the growth of the cells early in the G<sub>1</sub> phase (13). DNA synthesis was determined in all three sets of cultures as a function of time after refeeding. At any particular time after refeeding, the synthesis determined for the



**FIGURE 2** Effect of CI medium on DNA synthesis in nonconfluent cultures. Three groups of nonconfluent cultures were refed with F + S, CI medium, or F + S containing 0.25 mM db-cAMP. DNA synthesis was then determined as a function of time after refeeding. The values obtained at each time were then normalized to be a percentage of the synthesis in the control cultures refed with F + S. Each point represents two or three determinations. At no time during the course of the experiment did the density of any culture exceed 8  $\mu\text{g}$  cell protein/cm<sup>2</sup>. CI medium is defined in the legend to Fig. 1.

cultures refed with either CI medium or with F+S containing 0.25 mM db-cAMP was expressed as a percentage of the synthesis in the control set refed with F+S.

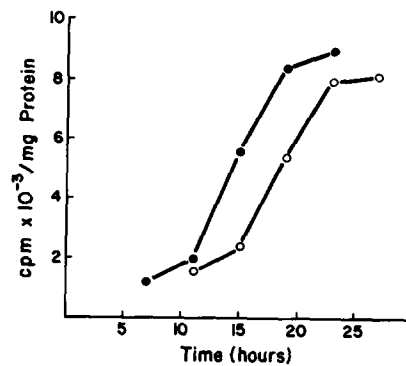
The results in Fig. 2 demonstrate that during at least a 30 h period, DNA synthesis does not decrease in the group refed with CI medium but does decrease in a time-dependent fashion in cultures treated with a growth-inhibitory concentration of db-cAMP. Because it supports the proliferation of nonconfluent cells, the inability of CI medium to stimulate confluent cultures (Fig. 1) was probably not due to the presence of toxic substances or the absence of nutrients essential for growth.

The data of Ceccarini and Eagle (8, 9) suggest that the pH of the medium may be an important determinant of cell proliferation in fibroblast cultures. Measurements of pH indicated that the CI media used in the experiments reported here, either before or after being replenished with vitamins, amino acids, and glutamine, had pH's approximately 0.25–0.30 pH units lower than the F+S medium they were derived from. This decrease in pH, although small relative to the pH changes noted by Ceccarini and Eagle (9), nonetheless confirms their finding that pH fluctuations do occur in the culture systems utilizing the bicarbonate-CO<sub>2</sub> buffer. However, since the re-

sults in Fig. 2, as well as the results in Fig. 5 to be described later, demonstrate that CI medium fully supports cell proliferation in nonconfluent cultures, the inability of CI medium to stimulate cell proliferation in contact-inhibited cultures probably cannot be accounted for on the basis of pH. Furthermore, alkalization of CI medium back to the pH of F+S does not restore the ability of that medium to stimulate DNA synthesis in confluent cultures (data not shown), a result also found by Ceccarini and Eagle (9).

### *Effect of cAMP on Events Occurring Early after Stimulation of Confluent Cultures with Fresh Medium*

From Fig. 1 and previous observations in a similar experiment (13) it appears that as late as 5–7 h after serum stimulation of confluent cultures intracellular events occur which are necessary for cells to subsequently enter into DNA synthesis and which require fresh serum in order to occur. An alternative explanation to the one just proposed is that perhaps after stimulation of confluent cultures with F+S, there is a period of at least 5 h during which the cells are not making any preparations to initiate DNA synthesis. This would mean that the 5–7 h interval needed for the cells to become committed to DNA synthesis after serum addition would represent a "lag" period during which events related to the progress



**FIGURE 3** Effect of 0.25 mM db-cAMP on early events occurring after stimulation of confluent cultures. Two groups of confluent cultures were refed 2 days after their last medium change with F + S (●—●) or F + S containing 0.25 mM db-cAMP (○—○). 4 h later, the group treated with db-cAMP was refed with F + S. DNA synthesis was followed in both groups as a function of time after stimulation. Each point represents duplicate determinations.

of the cells through  $G_1$  were not occurring. That this is not true is demonstrated by the following experiment.

Two groups of confluent cultures were refed either with F+S or with F+S containing 0.25 mM db-cAMP, the minimal concentration of db-cAMP previously required to inhibit completely the initiation of DNA synthesis in stimulated confluent cultures (13). After 4 h, the cultures treated with db-cAMP were refed with F+S not containing db-cAMP. DNA synthesis was then followed in both groups of cultures as a function of time after the initial medium change.

The results in Fig. 3 show that treatment with 0.25 mM db-cAMP in the interval 0–4 h after stimulation delayed the onset of DNA synthesis by 4 h with respect to the onset of synthesis in the control group.

This result leads to two conclusions. One, events occur very early after stimulation with F+S which are necessary for the eventual initiation of DNA synthesis in the cultures, and two, those early events are inhibitable by db-cAMP. If either of these two conclusions were not correct the delay in Fig. 3 would have to be less than 4 h.

#### *Necessity for the Continual Presence of Fresh Serum after Stimulation*

Three sets of contact-inhibited cultures were refed with fresh medium. One of the sets served as the control group and was left untreated after stimulation. The cultures in the other two sets, however, were refed at 4 h after stimulation either with CI medium or with fresh medium containing 0.25 mM db-cAMP. The cultures which were to be refed with CI medium were washed twice with preheated serum-free EMEM just before the addition of CI medium. After 4 h of further incubation, the cultures in these two sets were washed with serum-free EMEM and were refed again, 8 h after the initial stimulation, with fresh medium. DNA synthesis in all three sets was then followed as a function of time after the initial medium change.

Fig. 4 demonstrates that treatment with CI medium or with F+S containing 0.25 mM db-cAMP during the 4 h interval 4–8 h after stimulation displaced the kinetics curve for DNA synthesis by somewhat more than 4 h to the right of the curve describing the synthesis in the control group. That the delay in synthesis was somewhat more than 4 h suggests that the cells originally

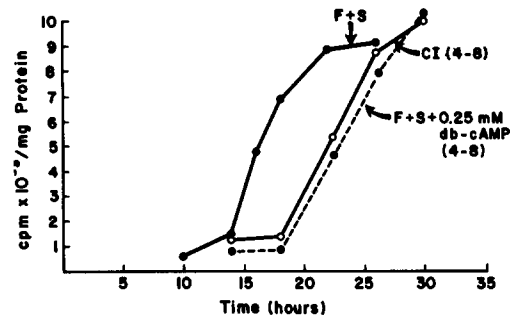


FIGURE 4 Requirement for the continuous presence of fresh serum after stimulation of confluent cultures. Three sets of confluent cultures were refed with F + S. One of the groups was refed after 4 h with F + S containing 0.25 mM db-cAMP (●---●) while another was refed with CI medium (○---○). After 4 more h, these two groups were refed again with F + S. The third group remained untreated after the initial refeeding and served as the control group (●—●). DNA synthesis was determined in all three groups as a function of time after the initial medium change. All points are the average of two determinations.

stimulated for 4 h with F + S not only were unable to progress any further toward the initiation of DNA synthesis during the 4 h interval they were incubated with CI medium or with F+S containing 0.25 mM db-cAMP, but that, in addition, some of them had reverted back to an earlier point in the  $G_1$  phase of the cycle.

Whether or not some of the cells actually did revert back to an earlier stage in  $G_1$  during the 4 h incubation with CI medium or with F+S containing 0.25 mM db-cAMP, the experiment in Fig. 4, nonetheless, shows that the progress of the cells toward S phase, after a medium change on confluent cultures, requires the continuous presence of medium containing fresh serum; removal of F+S after only 4 h and refeeding with CI medium abruptly halts the progression of the cells through  $G_1$  for the period of time the CI medium is on the cells. The fact that 0.25 mM db-cAMP has the same effect as the CI medium in Fig. 4 is consistent with previous studies which suggest that the stimulation of DNA synthesis in confluent cultures by fresh medium requires a decrease in intracellular cAMP (6, 13, 20, 27, 28).

#### *Effect of cAMP on DNA Synthesis in Trypsinized Cells*

It has been shown previously that treatment with trypsin or Pronase can stimulate cell growth in con-

fluent cultures of normal fibroblasts (4, 5, 26). Since trypsin attached covalently to nonphagocytized beads has this effect also (5), it was felt that trypsin action was at the surface membrane. In a recent report, a brief 10 min treatment of confluent 3T3 cell monolayers with Pronase followed by incubation in CI medium was shown to release some of the cells in the cultures from contact-inhibition (6). Moreover, the stimulation of growth in those cultures could be inhibited by db-cAMP if 0.5 mM db-cAMP was added to the cultures during the 10 min period of proteolytic treatment. On the basis of these results and on the basis of the sharp decreases in intracellular cAMP observed to occur after brief treatments of contact-inhibited 3T3 cell monolayers with various proteases, including trypsin and Pronase, or with fresh serum, a model was proposed suggesting that high intracellular cAMP levels are inhibitory to cell growth only at the point in early  $G_1$  at which the cells are arrested at confluence. Furthermore, the model implied that cAMP levels only had to be decreased for a very short period of time in contact-

inhibited cultures, perhaps 10 min, in order to allow the cells in those cultures to progress past this point and into DNA synthesis. Accordingly, this model predicts that cells in confluent cultures should become insensitive to the growth inhibitory properties of cAMP within just a few minutes after proteolytic treatment.

The following experiment was carried out to test this prediction. Contact-inhibited cultures were treated with 0.05% trypsin dissolved in GIBCO solution A for 5 min at 37°C. The cells obtained from the cultures were then replated at approximately a 1:20 dilution in F+S. About 1 h was required for cell attachment. After a total of 3.5 h in F+S, the cultures were refed either with CI medium or with CI medium containing 0.25 mM db-cAMP. DNA synthesis was then followed as a function of time after the trypsin treatment.

The results of this experiment (Fig. 5) lead to several conclusions. First, the synchronous wave of DNA synthesis in the upper curve in Fig. 5 indicates that CI medium does support cell growth in nonconfluent cultures, a result consistent with the data in Fig. 2. In addition, the time of peak synthesis in the culture approximates the time of peak synthesis in confluent cultures stimulated with F+S (Fig. 1). Thus, there appears to be no significant increase in the length of the  $G_1$  phase during growth in CI medium compared to growth in F+S.

Finally, the 68% inhibition of the wave of DNA synthesis observed in the lower curve in Fig. 5 demonstrates that even 3.5 h after trypsinization, cells can be prevented from entering into S phase by the addition of 0.25 mM db-cAMP to the medium. This result does not fulfill the prediction that the cells should become insensitive to the growth-inhibitory properties of cAMP very quickly after trypsinization. The wave of DNA synthesis which did occur in the lower curve indicates that at least some of the cells were able to initiate DNA synthesis in the presence of db-cAMP. However, a small amount of synthesis was expected since previous data from this laboratory (13) indicated that 0.25 mM db-cAMP only partially synchronized nonconfluent cultures in the  $G_1$  phase, hence permitting some cell growth to occur.

The experiment just described was performed on  $G_1$ -synchronized cells obtained by removal of the cells from a confluent monolayer. Unlike the situation with the 3T3 cell system (6), we were not

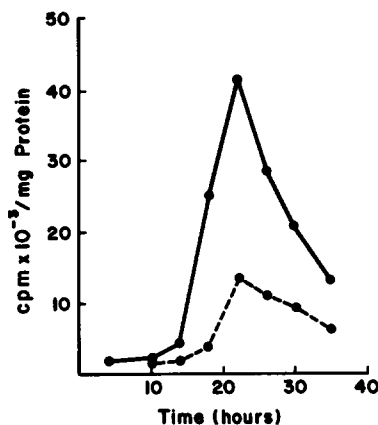


FIGURE 5 Kinetics of DNA synthesis in nonconfluent cultures freshly seeded from trypsinized confluent monolayers. At  $t = 0$ , confluent cultures were trypsinized for 5 min with 0.05% trypsin dissolved in GIBCO solution A. After 5 min, the cells detached from the plates, which are synchronized early in the  $G_1$  phase, were plated out at about  $\frac{1}{20}$  of the density of the confluent cultures they were trypsinized from. After 3.5 h, these freshly seeded cultures were refed with CI medium (●—●) or with CI medium containing 0.25 mM db-cAMP (●---●). DNA synthesis was determined in both groups of cultures as a function of time after trypsinization. Each point is the average of duplicate cultures.

able to stimulate cell division in confluent cultures of human diploid fibroblasts with any brief proteolytic treatment followed by incubation in CI medium, unless trypsinization was extensive enough to disrupt the monolayer, thus creating nonconfluent areas in the cultures. A similar inability to stimulate confluent human fibroblast cultures with proteolytic enzymes was reported by Ceccarini and Eagle (9).

In addition, the following experiment was performed. Confluent cultures were treated with 0.2 ml of 0.01% trypsin dissolved in GIBCO solution A for 5 min. This treatment resulted in an obvious detachment of many of the cells from the culture dish. However, instead of pouring off the detached cells, the cells were allowed to reattach to the dish by adding to the cultures 5 ml of the CI medium present on the cells before trypsin treatment. Subsequent measurements of DNA synthesis indicated that even though the trypsin had caused detachment of cells from the dish, creating obvious nonconfluent patches, very little stimulation of DNA synthesis occurred in the cultures if the cells were allowed to reattach, thus reestablishing confluency. This indicates that brief trypsin treatments can only stimulate DNA synthesis in confluent human diploid fibroblast cultures by creating nonconfluent areas in the culture. Possible implications of this result will be discussed.

#### DISCUSSION

A model has been proposed in which it was suggested that cAMP levels in contact-inhibited fibroblasts only had to be decreased for a very short period of time after treatment with serum or proteases, perhaps 5–10 min, in order for the cells to be released from growth inhibition and progress through the cell cycle (6). Evidence contradicting this is available. Previously (13), 0.25 mM db-cAMP was shown to inhibit the initiation of DNA

synthesis in serum-stimulated cultures of confluent human diploid fibroblasts if added as late as 7 h after stimulation. Furthermore, addition of 0.25 mM db-cAMP to the medium 4 h after stimulation abruptly halted the progress of the cells through  $G_1$  (Fig. 4). Finally, cells arrested early in the  $G_1$  phase due to contact inhibition which are stimulated to resume growth by trypsinization and replating at nonconfluent densities could be prevented from initiating DNA synthesis by adding 0.25 mM db-cAMP to the medium 3.5 h after trypsinization (Fig. 5).

The situation concerning the role of cAMP and serum in the control of fibroblast proliferation is represented in Fig. 6. In that figure, A is the point in early  $G_1$  at which the cells in contact-inhibited cultures are arrested at confluence. Recent data suggest that db-cAMP may arrest the growth of nonconfluent human fibroblasts (13) and Chinese hamster ovary cells (24) near this point also. In addition, Fig. 3 indicated that 0.25 mM db-cAMP prevented contact-inhibited cells stimulated with F+S from progressing beyond point A for the entire period of time it was present in the medium.

Point B is the point in  $G_1$  after serum stimulation of confluent human fibroblast cultures at which the cells stimulated to grow lose their fresh serum requirement for the subsequent initiation of DNA synthesis and progress into S phase even in CI medium. From Fig. 1 and from previous results (13), B is at least 5–7 h from A for practically all the cells stimulated by serum. In an experiment similar to that described in Fig. 1 here, Todaro et al. (30) demonstrated that at least 2–3 h of stimulation with fresh serum was required before any appreciable number of cells in confluent 3T3 cell cultures became committed to DNA synthesis. Point B in the 3T3 system thus appears to be at least 2–3 h from A. Although the reason for this time difference in the interval A to B for human fibroblasts and 3T3 cells is not clear, the important point is that a distinct period of serum stimulation is required in both cases. Furthermore, similar work performed by Burk (7) on stationary baby hamster kidney (BHK) cells and by Temin (29) on stationary chick fibroblasts suggests that point B in these cells is also 3–4 h from A.

Point B has another significance. Previously (13), we demonstrated that just as soon as the cells in serum-stimulated cultures reached point B, addition of 0.25 mM db-cAMP to the medium

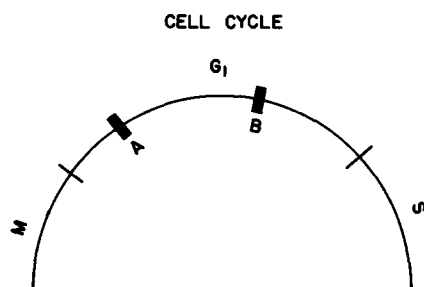


FIGURE 6 Points in the  $G_1$  phase important to the regulation of cell growth in human diploid fibroblasts. Points A and B are defined in the text.

could no longer prevent the initiation of DNA synthesis in the cells. This means that B is also the point after which serum-stimulated cells are refractory to the inhibitory effects of db-cAMP on the progress of the cells through  $G_1$ .

In Fig. 4, it was shown that if serum-stimulated cultures were refed either with F+S containing 0.25 mM db-cAMP or with CI medium after only 4 h of stimulation, the cells continued no further through  $G_1$  until F+S was given back to the cells. Thus, in the entire 7 h interval A to B, removal of fresh serum from the cells or the addition of db-cAMP to the medium abruptly halts the progress of the cells through  $G_1$ .

Since cAMP levels in confluent cultures were shown to decrease within 1 h after serum stimulation and to remain decreased for at least 10 h after stimulation (13, 20), and since the cAMP phosphodiesterase inhibitor, theophylline, prevented this decrease in intracellular cAMP at exactly the same concentration of theophylline required to inhibit cell growth in serum-stimulated cultures (13), it appears that the growth-stimulatory effect of fresh serum on confluent cultures requires a decrease in intracellular cAMP in the interval A to B.

On the basis of the situation depicted in Fig. 6 and on the basis of the report by Burger et al. (6) showing that a brief 10 min Pronase treatment can stimulate cell growth in confluent 3T3 cell monolayers without actually disrupting the monolayer, the following proposal is offered to explain how serum and proteases may stimulate cell growth in contact-inhibited cultures. Furthermore, this proposal may explain why we were unable to stimulate cell growth in confluent human diploid fibroblast cultures with any brief trypsin treatment which did not disrupt the monolayer.

We believe that fresh serum contains a factor, depleted from CI medium, which interacts with the surface membrane, inducing a change in membrane state which is readily reversible upon withdrawal of the fresh serum. This serum-induced change in membrane state then somehow leads to a decrease in intracellular cAMP, allowing the cells to progress through  $G_1$ . This last statement should not be interpreted to mean that a decrease in cAMP is the only change brought about by serum but rather that such a decrease may be at least one of many changes brought about by serum which are required for stimulation of contact-inhibited cells.

Continuing further, the readily reversible nature of the postulated alteration in membrane state induced in contact-inhibited cells by fresh serum means that removal of the fresh serum anywhere in the interval A to B will result in the restoration of the prestimulatory state of the membrane, causing the cells not to progress any further through  $G_1$ .

Proteases we feel also cause a membrane alteration. However, this alteration probably involves considerable chemical modification which may not be readily reversible. If this alteration is not readily reversible, then removal of the protease or stoppage of protease action before point B will not result in the restoration of the prestimulatory membrane state necessary for the inhibition of growth before protease treatment. Thus, the cells would be allowed to progress into S phase without the addition of fresh serum.

If proteases remove or at least modify surface structures necessary for cell-to-cell recognition and thus for contact inhibition, then confluent cells treated with proteases would behave like nonconfluent cells, which grow and multiply in CI medium. Since trypsin treatment is one way commonly used to remove surface carbohydrates (2, 3, 18) and since surface sugars are thought to play an important role in cell recognition and cell growth control (10, 11, 22, 23, 31) this idea is certainly possible, though not proven.

The comments just made may account for our inability to stimulate cell growth in confluent cultures in this study by any brief trypsin treatment which did not actually cause cell detachment, creating in the culture patches of nonconfluent cells which do not require fresh serum in the interval A to B (Figs. 2 and 5). Perhaps in human fibroblast cultures, the 5–7 h interval between A and B is sufficient to repair the surface structures altered by trypsin. If this repair process were sufficiently completed before point B, then the membrane would be able to reinstitute the cell-to-cell interactions necessary to convert the membrane back to its prestimulatory state. This would restore the fresh serum requirement for the cells to progress through  $G_1$  from A to B, thus preventing any further progress of the cells toward S phase in CI medium. Conversely, in the 3T3 cell system, since points A and B appear to be only 2–3 h apart, it may be that this interval of time is not sufficient to repair the alterations caused by brief proteolytic treatments, thus allowing the



cells to progress past point B before contact inhibition could be reinstated. Thus, the cells could continue on into S phase in CI medium, thereby accounting for the ability of a brief 10 min Pronase treatment to induce cell growth in confluent 3T3 cells (6) without disrupting the monolayer.

Recent evidence with trypsin-treated BHK cells indicates that the restoration of surface proteins iodinated by lactoperoxidase after trypsinization is a time-dependent process requiring at least 10 h to complete (16). In addition, Borek et al. (1) have shown that after trypsin treatment of normal fibroblasts, about 6 h was required for the cells to return to their normal nonagglutinable state with wheat germ agglutinin or concanavalin A. Thus, as suggested, repair of cell surfaces acted upon by proteases is not immediate.

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