

# INHIBITION OF DNA SYNTHESIS IN CHICK EMBRYO CULTURES BY DEPRIVATION OF EITHER SERUM OR ZINC

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## ABSTRACT

The rate of DNA synthesis in cultures of chick embryo cells is proportional to the concentration of serum added. The concentration of serum required to stimulate DNA synthesis increases with cell population density and with the duration of culture after trypsinization. The increase of the serum requirement with population density is not caused by the depletion of serum constituents. The requirement of cells for external zinc in DNA synthesis also increases with population density and duration of culture. The kinetics of inhibition of DNA synthesis by deprivation of serum or zinc are similar. Serum deprivation, however, inhibits 2-deoxyglucose uptake and cell movement, but zinc deprivation does not. The deprivation of either serum or zinc inhibits RNA synthesis about twofold. Very low concentrations of actinomycin D prevent the resumption of RNA and DNA synthesis upon restoration of serum or zinc to deprived cultures.

## INTRODUCTION

The rate of DNA synthesis in monolayer cultures of animal cells decreases when the population reaches confluency (15) or when the serum concentration of the medium is reduced (7, 15). There is, in both cases, a reduced rate of uptake of a number of constituents of the medium (15, 19, 22) and a slowdown in cell movement (3).

Zinc deprivation by addition of EDTA to the medium also inhibits the rate of DNA synthesis in animal cells (1, 5, 9, 16). The timing of this decrease and the timing of the increase in the rate of DNA synthesis which occurs after the restoration of zinc resemble those which follow the deprivation and restoration of serum (16, 20). Both serum and zinc appear to be required to initiate DNA synthesis, but not to maintain ongoing synthesis. These similarities suggest that serum and zinc ultimately affect a reaction in the cell cycle antecedent to DNA synthesis,

although there is no reason to suppose that their initial site of action is the same.

This report is a further comparison of the effects of serum and zinc on various aspects of cell behavior. The similarity in effects on DNA synthesis is confirmed, but it is shown that serum, and not zinc, affects cell permeability and movement. The results suggest that the initial site of action of zinc is more proximal to DNA synthesis than is that of serum.

## MATERIALS AND METHODS

### *Tissue Culture*

The methods used for culturing chick embryo cells have been described previously (12). The growth medium most commonly used was 199 containing 2% tryptose phosphate broth and 1% chicken serum and is designated as 199 (2-0-1).

The figures in parentheses signify the concentrations of tryptose phosphate broth, calf serum, and chicken serum, respectively. When the serum concentration was to be varied, the medium was removed, the cell sheet was washed once with medium 199, and medium containing 199 and 2% tryptose phosphate broth plus the appropriate serum concentration was added.

### Chemicals and Radioisotopes

Disodium EDTA (Matheson, Coleman and Bell, Cincinnati, Ohio) was kept as a 10 mM solution in Tris-saline buffer and added directly to the medium as required. Zinc was supplied as  $ZnSO_4 \cdot 7H_2O$  from a 10 mM stock solution in water. When EDTA is added to the medium of animal cell cultures in concentrations less than those of calcium and magnesium, DNA synthesis is inhibited and can be restored only by adding small amounts of zinc (16).

The following radioisotopes were obtained from the New England Nuclear Corp., Boston, Mass.: [*methyl*- $^3H$ ]thymidine (16.7 Ci/mmol); [ $^3H$ ]uridine (5.0 Ci/mmol); [ $2\text{-}^3H$ ]deoxyglucose (6.8 Ci/mmol); and [ $^3H$ ]leucine (38.8 Ci/mmol). Labeling of cells with [ $^3H$ ]thymidine and [ $^3H$ ]uridine was done in medium 199 after removal of growth medium. For scintillation counting, 0.2–0.5  $\mu Ci/ml$  of the appropriate radioisotope was used for 1 h, and for autoradiography 1.0  $\mu Ci/ml$  was used. Labeling with [ $2\text{-}^3H$ ]deoxyglucose and [ $^3H$ ]leucine was done in Hanks' medium minus glucose, using 0.2  $\mu Ci/ml$  for 20 min and 1 h, respectively. The techniques for extraction and scintillation counting of labeled molecules, determination of protein content, and autoradiography of nuclei labeled with [ $^3H$ ]thymidine have been described (16, 6). The fraction of nuclei labeled with [ $^3H$ ]thymidine has been shown to be proportional to total incorporated counts of [ $^3H$ ]thymidine per culture (14, 16), indicating that the latter measures the number of cells synthesizing DNA and not the rate of chain elongation.

## RESULTS

### Effect of Serum Concentration on DNA Synthesis under Varying Culture Conditions

Freshly trypsinized cultures of chick embryo cells were incubated in 199 (2-0-1) for various times and then switched to media of different serum concentrations for 16 h. The media were collected before labeling the cells with [ $^3H$ ]thymidine. The results in Fig. 1 A show that the rate of DNA synthesis was proportional to the concentration of serum present. Freshly trypsinized cultures synthesized DNA at a rapid rate even

in the presence of very low concentrations of serum. In the complete absence of serum, however, the rate of DNA synthesis in the freshly

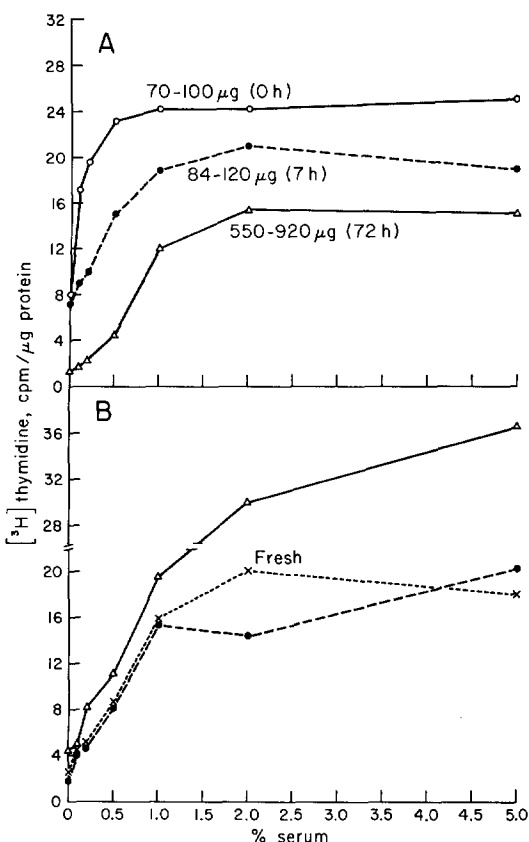


FIGURE 1 Rate of DNA synthesis as a function of serum concentrations added to cultures at various times after trypsinization. (A) Cells in primary cultures were trypsinized and seeded at  $10^6$  per dish. Some were exposed to varying concentrations of chicken serum immediately. Others were incubated for 7 or 72 h in 1% serum, then washed and exposed to varying serum concentrations. After 16 h of exposure, the cultures were labeled with [ $^3H$ ]thymidine. The figures in parentheses on each curve refer to the range of cell protein per dish, the lower figure representing cultures with the lower concentrations of serum. (B) Used media containing varying concentrations of serum were obtained after 16 h incubation on the 7 and 72 h cultures of A. These media were added to cultures which had been in standard medium for 1 day, and the rate of DNA synthesis was determined. (A) Varying serum added at  $\circ$ — $\circ$ , 0 h after trypsinization;  $\bullet$ — $\bullet$ , 7 h after trypsinization;  $\triangle$ — $\triangle$ , 72 h after trypsinization. (B) Used medium from  $\bullet$ — $\bullet$ , 7 hr cultures;  $\triangle$ — $\triangle$ , 72 hr cultures; ( $\times$ — $\times$ ), fresh medium.

trypsinized cultures fell sharply, and was associated with visible cell necrosis.

Cultures which had been exposed to the 1% serum of the standard medium for 7 h after trypsinization were not visibly damaged by the subsequent removal of serum. They required more serum to achieve maximal DNA synthesis than did the cultures which had no previous exposure to serum. After 3 days of growth in 199 (2-0-1), the dependency of DNA synthesis on serum concentration became greater. Even at the highest concentrations of serum used, the maximum rate of DNA synthesis was considerably less than that of the freshly trypsinized cultures.

Fig. 1 B shows the results of testing the residual stimulatory activity of media collected from two groups of cultures of Fig. 1 A. There was no indication that the 16 h incubation had depleted the media of their stimulatory activity. Indeed, the media removed from the cultures of highest population density had greater stimulatory activity than did either fresh medium or the media from the lower density cultures. This is probably due to the release of stimulatory macromolecules by the cells themselves (13, 14).

The influence of population density as distinguished from culture age was studied by seeding cultures at low and high densities and comparing their serum requirements for DNA synthesis. The results in Fig. 2 show that both population density and length of previous exposure to complete medium influence the response to serum concentration. At 1 day, the sparse population required less serum than the dense population to achieve equal rates of DNA synthesis. 2 days of incubation in complete medium increased the serum requirement of the sparse cultures to a level equal to that of the 1 day old dense cultures, although the population density was less than half as great.

The results show clearly that cells require increasing concentrations of serum to maintain a given rate of DNA synthesis as they increase in population density and length of time in culture. This requirement continues to increase with population density and time, even after the cells have become confluent, and it is not caused by a depletion of the stimulatory activity of the medium. It suggests that the state of cells alters progressively with increased crowding and time in culture, to the point where even the highest con-

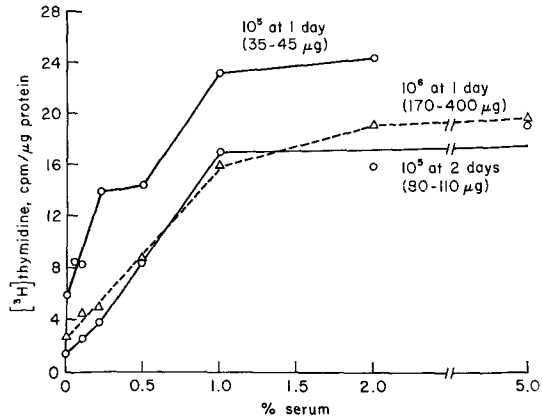


FIGURE 2 Rate of DNA synthesis as a function of serum concentration in cultures seeded at low and high density. Cultures were seeded in 199(2-0-1) at  $1 \times 10^5$  and  $1 \times 10^6$  cells per dish. They were incubated for 1 day and the medium was replaced by medium with tryptose phosphate broth plus varying serum concentrations. They were incubated for 16 h and labeled with [ $^3\text{H}$ ]thymidine. A second group of sparse cultures was incubated in 199(2-0-1) for a second day, and then switched for 16 h to medium with varying serum concentrations and labeled with [ $^3\text{H}$ ]thymidine. The figures in parentheses show the range of protein concentrations present at the time of labeling, the smaller figure representing the cultures with no serum and the larger figure those with the highest serum concentrations.

centrations of serum fail to restore maximal DNA synthesis. Since the inhibitory effects of population density on the rate of DNA synthesis are usually magnified by reducing serum concentration, in some of the experiments to be described serum was removed from high density cultures to accentuate density-dependent inhibition of DNA synthesis.

#### *Effect of Zinc Deprivation on DNA Synthesis under Varying Conditions*

EDTA inhibits DNA synthesis in animal cells, and its effect can only be reversed by zinc (16). Varying concentrations of EDTA were added to cultures at intervals after trypsinization, and to cultures of different population densities. The effects on DNA synthesis are shown in Figs. 3 and 4. Cells were relatively insensitive to inhibition by EDTA if added immediately after trypsinization, but attained almost full sensitivity in 8 h (Fig. 3). Sparse cultures were less sensitive

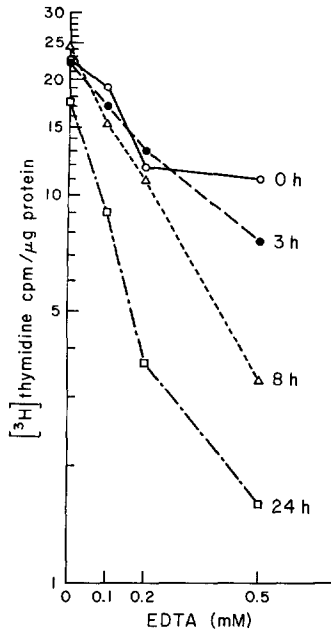


FIGURE 3 DNA synthesis as a function of EDTA concentrations added to cultures at various times after trypsinization. EDTA in the indicated concentrations was added to cultures in 199(2-0-1) at the various times after trypsinization shown with each curve. After a 16 h incubation period, the rate of  $[^3\text{H}]$ thymidine incorporation was determined.

to inhibition by low concentrations of EDTA than were dense cultures, although the latter had already been synthesizing DNA at a slightly reduced rate before the addition of EDTA (Fig. 4). The combined effects of the deprivation of serum and zinc were no greater than the maximal effect of zinc deprivation alone (Fig. 5).

#### *Kinetics of Inhibition of DNA Synthesis in Unsynchronized Cultures by Deprivation of Serum or Zinc*

After either the removal of serum from or the addition of EDTA to the medium of unsynchronized cultures, there is a delay of about 6 h before the rate of DNA synthesis begins a steady decline, and it reaches its minimal level in 12–15 h (16, 20). A detailed comparison was made of the kinetics of inhibition of DNA synthesis between 9 and 13 h after removal of serum or addition of EDTA. In addition, the rates of synthesis of RNA and protein were studied. In each case the radioactivity of the precursor present in the acid-

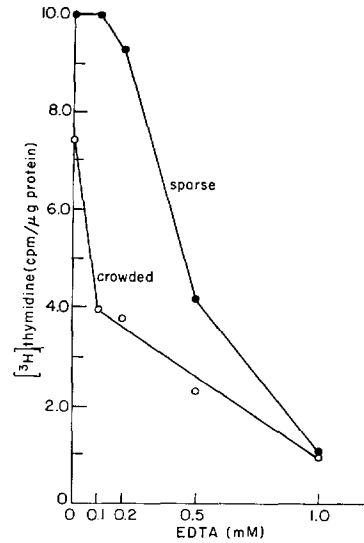


FIGURE 4 Effect of cell population density on sensitivity to EDTA. Cells were seeded in replicate cultures at  $10^5$  (sparse) and  $10^6$  (crowded) cells per dish in 199(2-0-1). 1 day later, EDTA at the indicated concentrations was added for 16 h and the rate of  $[^3\text{H}]$ thymidine incorporation was determined.

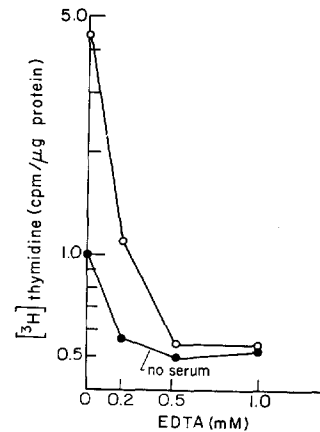


FIGURE 5 Combined effects of serum and zinc deprivation on DNA synthesis. EDTA at the indicated concentrations was added to serum containing [199(2-0-1)] and serum-deprived [199(2-0-0)] cultures, and the rate of  $[^3\text{H}]$ thymidine incorporation was determined at 16 hr.

soluble pool was measured. The results in Fig. 6 show that the decrease in the rate of DNA synthesis occurred in the same time interval after either serum removal or EDTA addition. This decrease was accompanied by a similar decrease

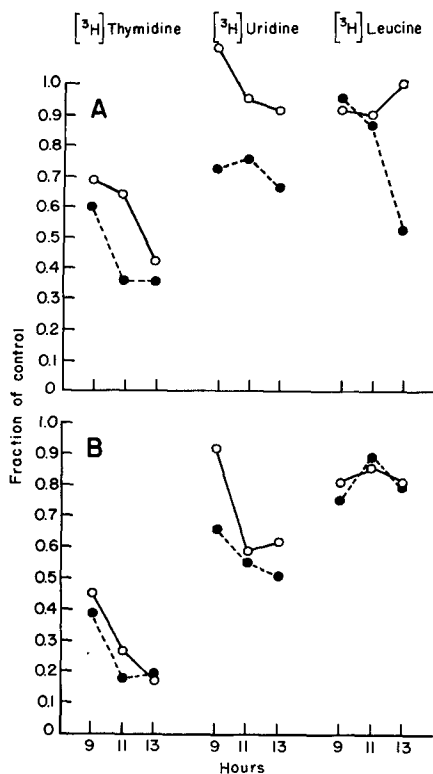


FIGURE 6 Kinetics of effects of serum- and zinc-deprivation on DNA, RNA and protein synthesis. Serum-free medium or EDTA 0.5 mM was added to cultures for the indicated times, and the uptake into (A) acid-soluble and (B) acid-insoluble materials of  $[^3\text{H}]$ thymidine,  $[^3\text{H}]$ uridine, and  $[^3\text{H}]$ leucine was compared with that of control cultures.  $\circ$ — $\circ$ , 0.5 mM EDTA;  $\bullet$ — $\bullet$ , serum-free.

in the radioactivity of the acid-soluble pool labeled with  $[^3\text{H}]$ thymidine.

Serum-deprived cells and EDTA-treated cells differed with respect to the incorporation of  $[^3\text{H}]$ uridine. Both the acid-soluble and -insoluble fractions were decreased by 9 h in the serum-deprived cultures. In the EDTA-treated cultures, the acid-insoluble fraction was decreased to the same extent as that of the serum-deprived cultures, but the acid-soluble fraction was not significantly decreased. Only serum deprivation produced a significant effect on the uptake of  $[^3\text{H}]$ leucine, and then only at 13 h. Neither serum nor zinc deprivation significantly altered the rate of incorporation of  $[^3\text{H}]$ leucine into the acid-insoluble fraction.

A comparison was made of the effects of serum deprivation and EDTA treatment on the transport of  $[2\text{-}^3\text{H}]$ deoxyglucose. Measurements of the effects on the uptake and incorporation of  $[^3\text{H}]$ uridine were also made. The results in Table I show that the uptake of  $[2\text{-}^3\text{H}]$ deoxyglucose was detectably inhibited by 6 h after serum deprivation and that the inhibition increased with time. At no time was there any effect of EDTA on  $[2\text{-}^3\text{H}]$ deoxyglucose uptake.  $[^3\text{H}]$ Uridine uptake into the acid-soluble pool also decreased within 6 h after serum deprivation, as did the incorporation into the acid-insoluble fraction. EDTA-treated cultures showed no effect on  $[^3\text{H}]$ uridine uptake and incorporation 6 h after the treatment was begun, but did show a significant decrease at 16 h.

TABLE I  
Effect of EDTA-Treatment and of Serum-Deprivation on the Uptake and Incorporation of Various Precursors

Isotope	EDTA		(-) serum	
	6 h	16 h	6 h	16 h
$[^3\text{H}]$ Thymidine	0.90	0.16	0.63	0.43
$[^3\text{H}]$ Uridine: pools	1.10	0.79	0.56	0.61
RNA	1.06	0.49	0.56	0.38
$[2\text{-}^3\text{H}]$ Deoxyglucose	1.0	1.0	0.57	0.41

0.5 mM EDTA was added to one group of cultures. Serum-free medium was added to a second group of cultures, and a third group was maintained in standard medium. At 6 and 16 h after treatment, the uptake for one hour of  $[2\text{-}^3\text{H}]$ deoxyglucose and  $[^3\text{H}]$ uridine into acid-soluble pools, and the incorporation of  $[^3\text{H}]$ uridine and  $[^3\text{H}]$ thymidine into acid-insoluble material were measured. Results are expressed as fractions of the values for cultures in standard medium.

*Effects of Serum Deprivation and EDTA Treatment on Cell Movement and on DNA Synthesis*

Confluent cultures were maintained for 1 day in the absence of serum, or in the presence of serum plus EDTA. A swath of cells was then removed with a rubber policeman, the cultures were washed in medium 199, and fresh medium of the appropriate composition was replaced on the cultures. They were incubated for 24 h, exposed to [<sup>3</sup>H]thymidine for 1 h, and prepared for autoradiography. As seen in the legend to Fig. 7, the fraction of cells in the confluent region synthesizing DNA was about the same in the serum-deprived and EDTA-treated cultures. The fraction synthesizing DNA in the denuded region was doubled with respect to the confluent region in the serum-deprived cultures, and tripled in the EDTA-treated cultures, confirming that the effect of both treatments is dependent on the cell population density. Fig. 7 shows that the deprivation of serum was more strongly inhibitory to cell movement than was treatment with EDTA.

*EDTA Inhibition in Partially Synchronized Cultures*

Cultures were partially synchronized by withdrawing serum for 16 h and then restoring the serum (20). EDTA was added to some of the cultures to determine how quickly its inhibitory effect could be detected. It can be seen in Fig. 8 that EDTA inhibited the increase in DNA synthesis which ordinarily is seen at 4 and 6 h after restoring serum. The inhibition is not always complete, however, as seen in the rising rate of DNA synthesis at 8 and 10 h.

The question arose whether the time required for inhibition of DNA synthesis by EDTA could be shortened by adding it at different times after restoring serum to partially synchronized cultures. It can be seen in Fig. 9 that at least 4 h are required for a detectable inhibition of DNA synthesis after the addition of EDTA, regardless of when it is added. Presumably, a zinc-requiring step must precede DNA synthesis by about 4 h.

An experiment was done in which EDTA was added at various times after serum had been restored to deprived cultures, and each set of cultures was incubated for 16 h after addition of EDTA. Fig. 10 A shows the kinetics of DNA synthesis after restoration of serum, and Fig.

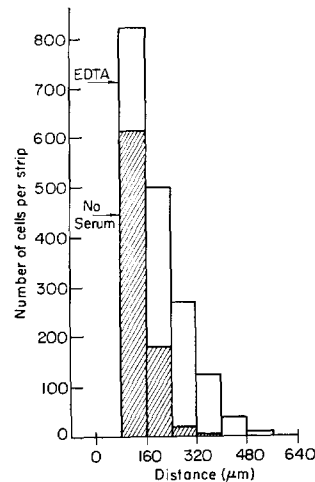


FIGURE 7 Effects of serum and zinc deprivation on the rate of cell movement. Confluent cultures were deprived of serum or exposed to 0.5 mM EDTA for 24 h. A swath of cells was removed with a rubber policeman, the cultures were washed and the appropriate medium without serum or with EDTA was added. The cultures were incubated for another 24 h and exposed to 1 μCi/ml [<sup>3</sup>H]thymidine for 1 h and prepared for autoradiography. The distance moved was measured with the aid of an ocular reticule.

The proportion of labeled cells was:

Cultures	Fraction of labeled nuclei in	
	Confluent region	Denuded region
Serum deprived	0.072	0.15
EDTA treated	0.067	0.22

10 B the extent of inhibition of DNA synthesis after 16 h incubation in EDTA which had been added at the times indicated on the abscissa. It is evident that the sensitivity of DNA synthesis to inhibition by EDTA depends on the time during the growth cycle at which the EDTA was administered. If EDTA was added at the same time as the serum, the extent of inhibition was no greater after 16 h in EDTA than it was after 8 h (see Figs. 8 and 9) when the cells were approaching their maximum rate of DNA synthesis. From this, it appears that cells in the S period are unsusceptible to inhibition by EDTA. This is in accord with the observation that those cells which manage to enter the S period, despite the presence of EDTA, synthesize DNA at a normal rate (16). If EDTA was added 8 h after serum and was present before

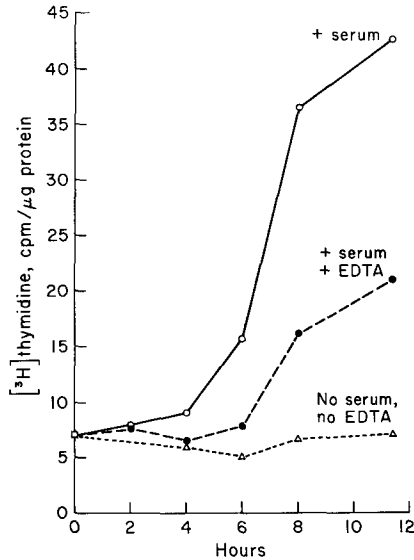


FIGURE 8 Early effects of EDTA on DNA synthesis in partly synchronized cultures. Cultures were placed in serum-free medium 199(2-0-0) for 16 h. They were then divided into three groups to which 2% chicken serum, 2% chicken serum and 0.5 mM EDTA, or nothing was added and the rate of incorporation of  $[^3\text{H}]$ thymidine was determined at various times as noted on the abscissa.

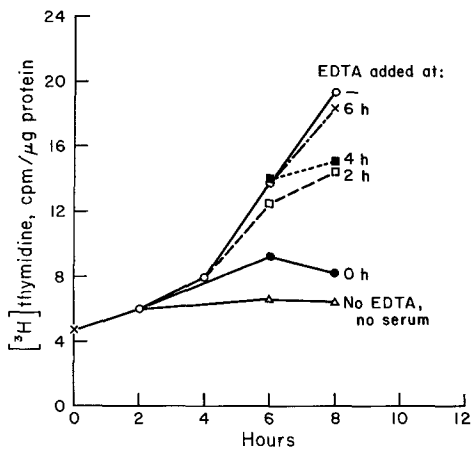


FIGURE 9 Short-term effects of adding EDTA at various times after serum stimulation of partly synchronized cultures. Cultures were placed in serum-free medium 199(2-0-0) for 16 h. 2% chicken serum was then added to the cultures and EDTA was added at 0, 2, 4, and 6 h. The rate of  $[^3\text{H}]$ thymidine incorporation in the serum-stimulated cultures was determined at these intervals, and in the EDTA-treated cultures at 6 and 8 h.

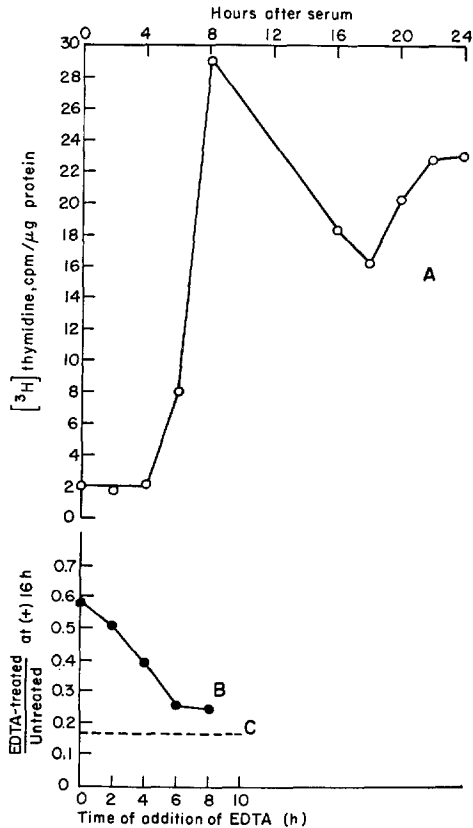
the second, less synchronized round of DNA synthesis began, the inhibitory effect of EDTA was enhanced. The sensitivity to EDTA was greatest if the medium was not changed at all (Fig. 10 C). The results stress once again the dependence of the EDTA effect on the physiological state of the cell.

#### *Actinomycin D Effect on the Stimulation of DNA Synthesis by Serum or Zinc*

The delay in the inhibition of DNA synthesis after the removal of serum or the addition of EDTA, plus the delayed onset of full DNA synthesis after restoration of serum or zinc, indicated that the reaction requiring serum and zinc occurred several hours before DNA synthesis. To determine if RNA synthesis was involved, cultures which had been deprived of serum or zinc were treated with actinomycin D at the same time as these components were restored. We used extremely low concentration of actinomycin (0.01  $\mu\text{g}/\text{ml}$ ) which inhibits ribosomal RNA synthesis without affecting significantly the synthesis of other classes of RNA (10). The results in Table II show that this low concentration of actinomycin reduced both RNA and DNA synthesis in control cultures by a factor of about 2 after a 7 h exposure. It blocked the twofold increase of RNA synthesis normally seen after the restoration of serum or zinc. This moderate reduction in the overall rate of RNA synthesis by actinomycin completely blocked the increase in the rate of DNA synthesis in the restored cultures. The results suggest that ribosomal RNA synthesis is required to initiate DNA synthesis after restoration of serum or zinc.

#### DISCUSSION

The inhibition of DNA synthesis in chick embryo cells caused by deprivation of serum is similar in several respects to that caused by EDTA chelation of zinc. Dense populations of cells are more sensitive than sparse populations to inhibition by both treatments, except when the dense populations are already strongly inhibited by virtue of their crowding alone. The sensitivity of cells to serum and zinc deprivation increases with time after trypsinization. In both cases, there is a delay of about 6 h in the inhibition of DNA synthesis after removal of the requisite component, and a delay in the resumption of full DNA synthesis after the deficient component is restored. RNA synthesis is required after restoration before DNA



**FIGURE 10** Long-term effects of adding EDTA at various times after serum-stimulation of partly synchronized cultures. Cultures were placed in serum-free medium 199(2-0-0) for 16 h. 2.0% chicken serum was then added to the cultures, and 0.5 mM EDTA was added at 0, 2, 4, 6, and 8 h. The rate of [<sup>3</sup>H]thymidine incorporation was determined at various times after addition of serum ([A] upper panel) and at 16 h after addition of EDTA to the serum-treated cultures (relative rate of [<sup>3</sup>H]thymidine incorporation in [B] lower panel). The broken line (C) in the lower panel shows the relative rate of DNA synthesis 16 h after addition of EDTA to cultures which had not had serum removed or supplemented, i.e., nonsynchronized cultures.

synthesis can be resumed. Both treatments decrease only the fraction of cells synthesizing DNA at any given time without affecting the rate of synthesis in a cell once it has entered the S period (16, 20).

There are in addition, however, several cell properties affected by serum which are not affected by zinc. These properties seem to be associated with the cell membrane. Deprivation of serum causes a marked decrease in the rate of

**TABLE II**  
*Effect of Actinomycin D on the Stimulation of DNA and RNA Synthesis by Zinc and Serum*

Pretreatment	Addition	Actinomycin D	[ <sup>3</sup> H]-Thymidine	[ <sup>3</sup> H]-Uridine
—	—	—	6.95	15.6
—	—	+	2.95	6.35
EDTA	—	—	1.0	4.25
	—	+	0.65	2.9
	Zn <sup>++</sup>	—	3.6	8.65
	Zn <sup>++</sup>	+	0.65	3.7
Serum removal	—	—	2.6	8.1
	—	+	1.1	2.7
	Serum	—	8.6	18.8
	Serum	+	1.0	9.4

Cultures were divided in three groups which were: (a) untreated, (b) treated with 0.5 mM EDTA, or (c) cultured in serum-free medium for 16 h. At this time, half the EDTA-treated cultures received 0.02 mM Zn SO<sub>4</sub>·7H<sub>2</sub>O, and half the serum-deprived cultures received 2.0% serum. At the same time, half the cultures in each group and subgroup received 0.01 μg/ml actinomycin D. After 7 h further incubation, the cultures were labeled with [<sup>3</sup>H]thymidine or [<sup>3</sup>H]uridine. The results are expressed as the average of counts per minute per microgram protein in two cultures.

uptake of [2-<sup>3</sup>H]deoxyglucose by the cell and the restoration of serum causes rapid and extensive increase in the rate of uptake, but complexing of zinc by EDTA has no measurable effect. (Actually we have observed that massive increases in the concentration of zinc—and other metals—to the threshold of toxicity do stimulate the uptake of 2-deoxyglucose, but this involves concentrations far beyond those used here.) The rate of uridine uptake decreases rapidly after serum deprivation, but decreases slowly after zinc deprivation, and then only in parallel with a decrease in the rate of RNA synthesis. Finally, cell movement is responsive to serum concentration but is independent of zinc.

The stimulation of 2-deoxyglucose uptake by serum occurs in cells without any evidence of a lag period (19), and the same appears to be true of its effect on cell movement. Since both of these effects are likely to be consequences of alteration in the activity of the cell membrane, they add to the evidence that the primary effector site of serum is the cell membrane. Zinc



deprivation, however, gives no evidence for membrane alteration, its major detected effect being on the rate of DNA synthesis, with a lesser effect on the overall rate of RNA synthesis. It might, however, have a more profound effect on the synthesis of a particular class of RNA such as ribosomal RNA which constitutes only a fraction of the RNA synthesized during the 1-h labeling period used in these experiments (10). The similarities between zinc and serum in their effects on DNA synthesis raise the possibility that serum stimulates DNA synthesis by acting on the membrane to make zinc available within the cell. An analogy for such a relationship is the electro-mechanical coupling, in striated muscle, of membrane depolarization to muscular contraction. A variety of stimuli cause depolarization of the myofibrillar membrane, thereby liberating calcium which initiates the contractile response (17). Similarly, we suggest that prolonged depolarization which is induced in tissue culture cells by low population densities (15) or the addition of serum (8) initiates the release of sequestered zinc which triggers a process prerequisite to DNA synthesis.

Our results are consistent with the idea that the zinc-requiring step is RNA synthesis. The resumption of DNA synthesis in zinc-deficient cells is blocked if actinomycin D is added when zinc is restored. The DNA-inhibitory concentration of actinomycin D is 0.01  $\mu\text{g/ml}$  which suppresses the synthesis of ribosomal and not of other classes of RNA (10). Ribosomal RNA synthesis is depressed in density-inhibited chick embryo fibroblasts (4). It is very rapidly stimulated in quiescent lymphocytes upon the addition of phytohemagglutinin (2). The activity of RNA polymerase I, the ribosomal RNA polymerase, is increased without a lag period after phytohemagglutinin stimulation of the lymphocytes (11).

These findings when taken together are consistent with the idea that ribosomal RNA synthesis is required to initiate DNA synthesis. In the case of stimulated lymphocytes, the increased activity of RNA polymerase does not require protein synthesis (11) which indicates that the stimulatory event involves the activation of pre-existing enzymes. Since the RNA polymerase of *Escherichia coli* has been shown to require zinc for the initiation of RNA synthesis (18), it is possible that the controlling event for DNA synthesis in animal cells is the liberation of zinc, to be used in

the initiation of ribosomal RNA synthesis. It cannot, however, be ruled out that some other reaction requires zinc, and that both this reaction and ribosomal RNA synthesis are required to initiate DNA synthesis. In this regard it is noteworthy that several enzymes of glucose catabolism require zinc (21) and that we have recently detected rapid changes in glucose catabolism after stimulation of cell multiplication (D. Fodge and H. Rubin, unpublished).

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