Proc. Nat. Acad. Sci. USA
Vol. 72, No. 9, pp. 3551–3555, September 1975
Cell Biology

Central role for magnesium in coordinate control of metabolism and growth in animal cells

(growth regulation/adenine nucleotides/transphosphorylation/divalent cations)

H. RUBIN

Department of Molecular Biology and Virus Laboratory, University of California, Berkeley, Calif. 94720

Communicated by Donald A. Glaser, June 16, 1975

ABSTRACT **The rate of DNA synthesis in cultures of chicken embryo fibroblasts is reduced by deprivation of serum, high population density, and other "physiological" effectors, through a reduction in the number of cells in the S-period of the cell cycle. The same effect can be produced by drastically reducing the concentration of Mg^{++} added to the medium. This effect is erratic, however, and better control of $[Mg^{++}]$ can be achieved with phosphorylated compounds which preferentially bind Mg^{++}. Both ATP and ADP, at concentrations in the medium $\leq [Mg^{++}]$, stimulate DNA synthesis in cultures, and at greater concentrations inhibit DNA synthesis by affecting the proportion of cells in the S-period. Sodium pyrophosphate, which strongly complexes Mg^{++}, causes little stimulation of DNA synthesis at low concentrations, but causes a striking decrease at concentrations exceeding $[Mg^{++}]$ of the medium. The inhibition can be fully reversed by adding an excess of Mg^{++}, and the kinetics of increase in DNA synthesis resemble those which follow the restoration of serum to serum-deprived cultures. Limitation of $[Mg^{++}]$ by pyrophosphate also reduces the rates of RNA and protein synthesis, 2-deoxy-D-glucose uptake, and lactic acid production to an extent comparable to the reduction caused by the removal of serum from the medium. A model for the coordinate control of metabolism, differentiated function, and growth through the activity of divalent cations is described. The compartmentalization of Mg^{++} within the cell serves as the key element in this coordinate control by regulating those metabolic pathways in which the rate-limiting steps are transphosphorylation reactions.**

It is well known that the growth rate of normal fibroblasts in culture depends on such factors as population density, pH, and serum concentration (1). A variety of other cellular activities respond to these effectors in the same way as does growth rate. These activities include transport and metabolism of hexoses (2, 3), biosynthesis of lipids, nucleic acids, and proteins (4), and the execution of differentiated functions such as hyaluronic acid (5) and collagen production (J. Kamine and H. Rubin, unpublished observations). The fibroblast appears to respond to environmental conditions as an integrated unit, accelerating an array of processes in response to stimuli and decelerating them in response to inhibitors. Except for the initiation of DNA synthesis, which depends on the rates of RNA and protein synthesis (1, 6), there is no indication that these processes are directly dependent on one another (4). It is likely, therefore, that they all respond to some common underlying control mechanism.

Identification of the common control mechanism would be advanced if the specific controlling reaction in each of the above processes were established. However, this has only been accomplished in the case of glycolysis, where all the substrates and products in the pathway are readily assayed. Here it has been shown that the enzyme phosphofructokinase is activated in chick embryo fibroblasts following the administration of growth stimulants (3). This is the rate-limiting step for glycolysis in a variety of animal cells, and at least eight effectors of enzyme activity have been found. More recently, it has been reported that in cells which have undergone the malignant transformation, the enzymes hexokinase and pyruvate kinase, in addition to phosphofructokinase, are activated (7).

The term activation is usually taken to mean an increase in the rate at which an enzyme functions as a result of some allosteric change in its configuration (8). The increase in enzyme activity is assumed on the basis of an increase in the ratio of products to substrates of the enzyme reaction. However, such an increase need not depend on allosteric effectors of the enzyme. It could be brought about by the increased availability of a co-factor for the reaction. Mg^{++} is the only co-factor common to the three regulatory steps of glycolysis, and indeed to all transphosphorylation reactions. Since these usually involve large decreases in free energy (9), they are likely to be control points in other metabolic pathways. Indeed, this assumption underlies Atkinson's "energy charge" theory of cellular regulation (10). However, no significant alteration in energy charge has been found in cultured cells under different regulatory conditions (3).

Much of the Mg^{++} in cells is bound to membranes (11) and only a fraction is free, or bound to adenine nucleotides (12). Unfortunately, no simple technique is available to determine the distribution of Mg^{++} among various cell constituents. I approached the question of the role of Mg^{++} in cell regulation by limiting its external supply and observing the effects on cells. Since a simple reduction in external concentration produced erratic effects, it was necessary to use agents which complex Mg^{++} preferentially. These agents produced metabolic effects which are described below.

MATERIALS AND METHODS

Primary cultures of chick embryo fibroblasts (CEF) were made in plastic petri dishes according to established procedures (13, 14). The secondary cultures of CEF used here were initiated with 10^6 cells per 60 mm dish in a medium consisting of mixture 199, 2% tryptose phosphate broth, and 1% chicken serum, designated 199 (2-0-1). Where not otherwise indicated, the medium contained approximately 0.8 mM Mg^{++} and 1.7 mM Ca^{++}.

Procedures for labeling cells with radioactive isotopes, scintillation counting, autoradiography, protein, and lactic acid determinations have been described elsewhere (2, 3, 6). Adenosine diphosphate and triphosphate were obtained

Abbreviation: CEF, chick embryo fibroblasts.

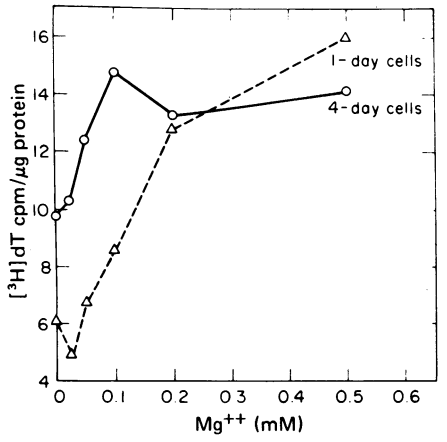

FIG. 1. Effect of [Mg^{++}] on thymidine (dT) incorporation into DNA in sparse and crowded cultures. Medium 199 was prepared with 1.72 mM Ca^{++} and no added Mg^{++}. This was contaminated with approximately 0.02 mM Mg^{++} as measured by atomic absorption spectrophotometry. Tryptose phosphate broth (0.21 mM Mg^{++}) and chicken serum (0.38 mM Mg^{++}) were added to v/v concentrations of 2% and 1%, respectively. The indicated [Mg^{++}] was added to the medium of 1-day old and 4-day old cultures containing about 185 μg and about 800 μg of protein per culture, respectively. The cells were washed twice with Ca^{++} and Mg^{++} deficient 199, and were incubated for 16 hr in complete medium at the indicated [Mg^{++}]. They were then labeled with [^{3}H]dT for 60 min and extracted for scintillation counting and protein determinations.

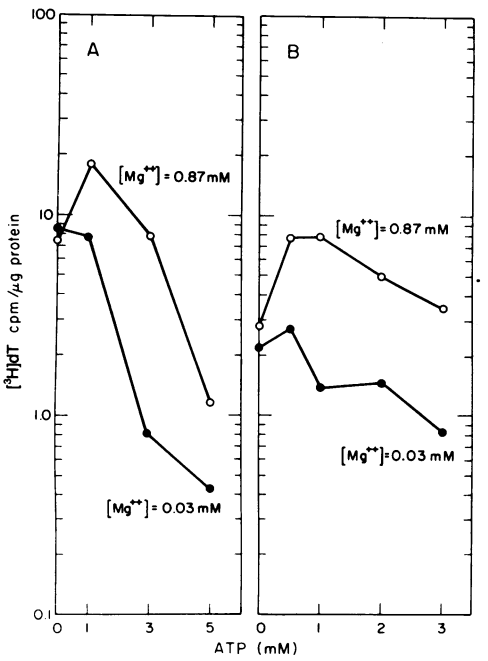

FIG. 2. Effect of [ATP] on DNA synthesis in low and high [Mg^{++}]. Five-day old and 1-day old cultures were used in experiments A and B, respectively. Varying concentration of ATP were added to 199 (2-0-1) containing [Mg^{++}] indicated on the curves. After 16 hr the cells were processed as in Fig. 1.

from Calbiochem and sodium pyrophosphate from Mallinckrodt. [Mg^{++}] and [Ca^{++}] were determined in a Perkin-Elmer model 403 atomic absorption spectrophotometer.

RESULTS

The degree of inhibition of DNA synthesis in CEF produced by lowering [Mg^{++}] in the medium depended on a variety of parameters such as pH, population density, serum quality, and concentration and [Ca^{++}]. The results of a representative experiment with sparse and crowded cultures are shown in Fig. 1. Reduction in [Mg^{++}] was more inhibitory to sparse than to crowded cultures. There was a proportional reduction in the fraction of labeled nuclei when examined autoradiographically. This effect was accomplished without visible damage to the cells. Because of the erratic nature of the response to [Mg^{++}] (see Fig. 2, where there was virtually no inhibition of DNA synthesis in low [Mg^{++}]), an agent was sought which would complex Mg^{++} in preference to Ca^{++}, the only other divalent cation present in comparable amounts, and thereby serve as a Mg^{++} buffer. Phosphorylated compounds seemed to be suitable for this role (15).

The addition of up to 1 mM ATP to chick embryo cultures for 16 hr stimulates DNA synthesis if the conventional concentration of Mg^{++} (0.8 mM) is present (Fig. 2). Higher concentrations of ATP inhibit DNA synthesis. When the Mg^{++} concentration is very low, no stimulation of DNA synthesis by ATP occurs, and marked inhibition is observed with ≥3 mM ATP. Both the stimulation and inhibition of the incorporation of [^{3}H]thymidine into the DNA of the culture are proportional to the percent of cells in the S-period (Fig. 3), which indicates that the length of the S-period remains constant, i.e., the rate of DNA chain elongation is unaffected.

ADP causes stimulation and inhibition of DNA synthesis at the same concentration as does ATP (Fig. 4). Both nucleotides cause a marked vacuolization of chick embryo cells, and the cells are not restored to normal appearance when the nucleotides are withdrawn. Sodium pyrophosphate, which is a much firmer complexor of Mg^{++} than either of the nucleotides, causes little stimulation, but does cause a highly reproducible inhibition of DNA synthesis (Fig. 4). Although pyrophosphate forms a precipitate with Ca^{++}, the

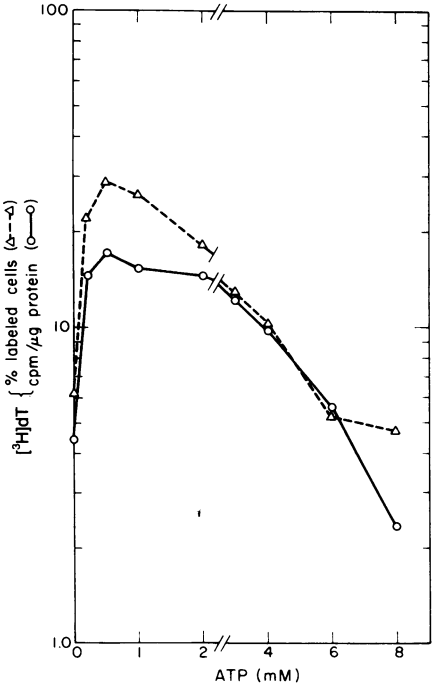

FIG. 3. Comparison of scintillation counting and autoradiography in measuring effects on DNA synthesis of treatment with ATP. The indicated concentrations of ATP were added to fresh medium 199 (2-0-1) of 2-day old cultures for 16 hr. The cultures were labeled with [^{3}H]dT; half of them were processed for scintillation counting and half for autoradiography.

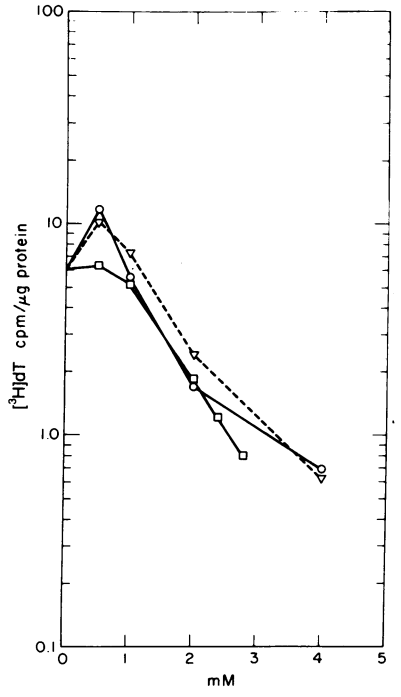

FIG. 4. Comparison of effects of ATP, ADP, and sodium pyrophosphate on the rate of DNA synthesis. The indicated concentrations of ATP, ADP or pyrophosphate were added to the medium 199 (2-0-1) of 3-day old cultures for 16 hr, and the cultures were processed as in Fig. 1. O, ATP; ▽, ADP; □, pyrophosphate.

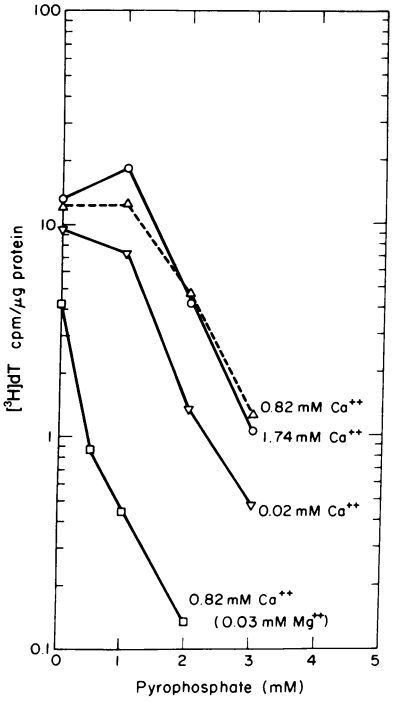

FIG. 5. Relation of [Ca^{++}] and [Mg^{++}] to inhibition of DNA synthesis by pyrophosphate. "Ca^{++}- and Mg^{++}-free medium" contaminated with about 0.01 mM Ca^{++} and about 0.02 mM Mg^{++} was combined with 2% tryptose phosphate broth and 1% chicken serum. Mg^{++} was added to about 0.82 mM in the presence of [Ca^{++}] shown in the top three curves. Ca^{++} was added to about 0.82 mM, and no Mg^{++} was added in the bottom curve. The cultures were incubated for 16 hr and processed as in Fig. 1.

precipitate does not damage cells. The effectiveness of the inhibition of DNA synthesis by pyrophosphate is much more sensitive to [Mg^{++}] than to [Ca^{++}] (Fig. 5). Indeed, the lowest concentration at which pyrophosphate begins to show its inhibitory effect is determined by [Mg^{++}] (Fig. 6). Neither ZN^{++}, Mn^{++}, nor Fe^{+++} relieve the inhibition caused by pyrophosphate.

Unlike the adenine nucleotides, pyrophosphate causes no vacuole formation in cells, although it kills them if present for as long as two days in concentrations three times higher than [Mg^{++}]. If it is removed after 16 hr, the cells return to the control rate of DNA synthesis within 7 hr, and resume multiplication with no overt manifestation of damage (Fig. 7). Upon adding Mg^{++}, but not Ca^{++}, directly to the pyrophosphate-containing medium after inhibition has occurred there is a 2–4 hr delay before the rate of DNA synthesis begins to increase. Within 7 hr it equals and at 10.6 hr exceeds the rate in untreated control cultures (Fig. 8). The relatively slight increase seen in Ca^{++}-treated control cultures is discussed below.

Incorporation of uridine and of proline into acid-insoluble material, the uptake of 2-deoxy-D-glucose, and the production of lactic acid are inhibited by pyrophosphate (Table 1). The effects of pyrophosphate on these parameters are comparable to those produced by either the removal of serum, lowering of pH, or increase in population density (1, 4, 6, 16).

DISCUSSION

A reduction in available Mg^{++} in the medium of cultured cells can be brought about by limiting the amount added to the medium or by specifically complexing it. Reducing the amount added to the medium of crowded cultures from 0.8 to 0.1 mM has little effect on the growth of cells, but further reduction usually decreases the number of cells in the S-period at any given time. This method of [Mg^{++}] control has given erratic results from experiment to experiment and has therefore led to the use of Mg^{++}-complexing agents. Unlike the common chelating agents, various phosphorylated compounds are known to bind Mg^{++} more firmly than Ca^{++} (15). At high concentrations, all of these have been found to inhibit DNA synthesis in cultures of chick embryo fibroblasts. The most reproducible effects are produced by sodium pyrophosphate, which forms much more stable complexes with Mg^{++} than do the adenine nucleotides. The minimal inhibitory concentrations of pyrophosphate are directly proportional to [Mg^{++}]. While there is a slight effect of [Ca^{++}], this is probably caused by competition with Mg^{++} for the pyrophosphate. Other physiologically significant metals do not affect the inhibition. I conclude, therefore, the biological effects of pyrophosphate are caused by a reduction in the Mg^{++} available to the cell.

The inhibition of DNA synthesis caused by 16 hr of treatment with pyrophosphate is fully reversed by adding Mg^{++}. There is a lag period of 2–4 hr before an increase in DNA synthesis becomes detectable, just as there is in the restoration of DNA synthesis by adding serum to serum-deprived cultures. The inhibition of incorporation of uridine into RNA and proline into protein, the uptake of 2-deoxy-D-glucose, and the production of lactic acid are coordinately inhibited by the pyrophosphate-induced deprivation of Mg^{++} to about the same extent, relative to the reduction in DNA synthesis, as they are by serum deprivation and density dependent inhibition (6). Although Zn^{++} deprivation also inhibits the initiation of DNA synthesis and the continuing synthesis of RNA, it affects neither protein synthesis, the up-

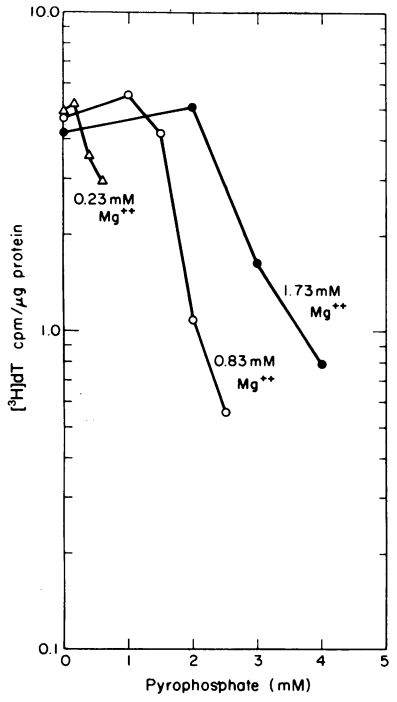

FIG. 6. Minimal inhibitory [pyrophosphate] in the presence of varying [Mg^{++}]. Mg^{++} in concentrations shown on the curves was added to 199 (2-0-1) containing 0.2 mM Ca^{++}, and varying concentrations of pyrophosphate were added for 16 hr. The cultures were then processed as in Fig. 1.

take of 2-deoxy-D-glucose nor the production of lactic acid (refs. 6 and 14 and unpublished), and therefore simulates physiological effectors less faithfully than does Mg^{++} deprivation.

Table 1. Effects of pyrophosphate on various cell functions

	Pyrophosphate-treated cultures/ untreated cultures
Experiment A	
[^{3}H]Thymidine (cpm/μg of protein)	0.23
[^{3}H]Uridine (cpm/μg of protein)	0.31
[^{3}H]Proline (cpm/μg of protein)	0.46
Experiment B	
[^{3}H]Thymidine (cpm/μg of protein)	0.21
2-Deoxy-D-[^{3}H]glucose (cpm/μg of protein)	0.42
Lactic acid (μmol/mg of protein)	0.49

Experiment A: CEF were treated for 16 hr with 0 or 2.2 mM pyrophosphate. The appropriate isotope was added to the medium for 60 min and the cells were extracted for scintillation counting and protein determinations.

Experiment B: CEF were treated for 16 hr with 0 or 2.2 mM pyrophosphate. Two cultures were labeled at this time with [^{3}H]-Thymidine (60 min) or 2-deoxy-D-[^{3}H]glucose (15 min) and the acid-insoluble and acid-soluble fractions, respectively, were extracted for scintillation counting. The medium was removed from the remaining cultures, they were washed free of accumulated lactic acid, and fresh medium of the original composition was added. After a further 6.5 hr incubation, the medium was removed for determination of lactic acid content.

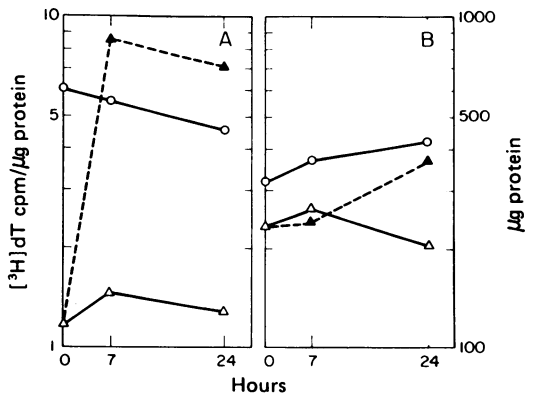

FIG. 7. Reversal of pyrophosphate inhibition of DNA synthesis. Pyrophosphate (2.1 mM) was added to fresh 199 (2-0-1) for 16 hr. Treated and untreated cultures were washed twice and medium without pyrophosphate was added to both groups. In one half of the treated cultures, pyrophosphate was added again. At 0, 7, and 24 hr after the change the cultures were processed as in Fig. 1. O, No pyrophosphate; △, pyrophosphate, 2.1 mM; ▲, pyrophosphate removed after 16 hr (= 0 hr).

The results indicate that Mg^{++} deprivation inhibits the same set of intracellular metabolic pathways as does serum deprivation and density dependent inhibition of growth. This is in accord with the requirement for Mg^{++} in transphosphorylation reactions, such as those shown to be the control points of glycolysis (8, 9), and presumed to be the control points in nucleic acid and protein synthesis (10). It has been established that Mg^{++} is bound by cellular membrane systems (11), and that the amount bound is subject to fluctuations in pH and other ions (11), and presumably by alterations in membrane configuration. Although there is an excess of total Mg over ATP plus ADP in animal cells, only a fraction of the Mg is available to the adenine nucleotides (12). I propose that the rates of the control reactions are determined by the concentration of the complexes of adenine

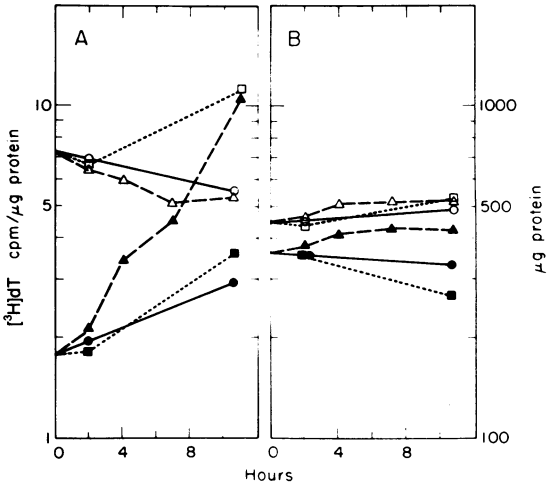

FIG. 8. Direct addition of Mg^{++} or Ca^{++} excess to pyrophosphate-containing medium of inhibited cultures. Pyrophosphate, 0.6 mM, was added to 199 (2-0-1) containing 0.2 mM Ca^{++} and 0.2 mM Mg^{++}. Five-day old cultures were incubated for 16 hr in this medium, and then either 1.0 mM Ca^{++}, or 1.0 mM Mg^{++}, or nothing was added to the cultures. At various times up to 10.6 hr the cells were processed as in Fig. 1. The filled symbols indicate presence of pyrophosphate. O●, No further additions; △▲, Mg^{++} (1.0 mM) added; □■, Ca^{++} (1.0 mM) added.

nucleotide and Mg, which have been shown to be the appropriate substrates in transphosphorylation reactions (9). Since the concentration of the adenine nucleotides remains relatively constant in various states of regulation in cultured cells (1, 3), it is likely that the concentrations of the complexes are determined by the availability of Mg^{++}, which in turn depends on conditions that affect the competitive binding between the nucleotides and membranes. For example, lowering pH would increase protonation of the gamma phosphate of ATP, which has a pK 6.5 (15), and thereby decrease the binding energy of ATP for Mg^{++} (11), while there is no effect of pH in the physiological range on the binding of divalent cations to phospholipid layers (17). Lowering pH might therefore be expected to coordinately inhibit an array of Mg^{++}-dependent pathways in the cell. Treatment with serum which disrupts ordered membrane structure (18) could lower binding affinity for divalent cations (17, 19), including Mg^{++}, and thereby activate nucleotide–Mg dependent pathways. The availability of Ca^{++} and Zn^{++} could be controlled in a similar way and thereby affect specialized activities such as cell movement and metalloenzyme function (14). The inhibition of DNA synthesis which follows the chelation of Ca^{++} by EGTA [ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid] (20) may be caused by the removal of Ca^{++} bound to membranes and its replacement by Mg^{++} (21), thus lowering [Mg^{++}] available to adenine nucleotides. Conversely, stimulation of cell growth by excess Ca^{++} (ref. 22 and Fig. 8) could be caused by displacing Mg^{++} from membrane binding sites (11, 21).

The stimulation of DNA synthesis by low concentrations of ATP or ADP is an unexpected result. The stimulation does not occur unless the concentration of Mg^{++} in the medium is approximately equal to or higher than that of the nucleotides. In the model presented here, the stimulation might result from increased uptake of Mg when it is combined with a nucleotide, since the charge of both components would be reduced.

The expert assistance of T. Koide is gratefully acknowledged. D. Fick and J. Kamine made significant contributions in the discussion and development of this work. This investigation was supported by Public Health Service Research Grant CA 15744 from the National Cancer Institute.

1. Rubin, H. & Fodge, D. (1974) *Control of Proliferation in Animal Cells* (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).
2. Sefton, B. & Rubin, H. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 3154–3157.
3. Fodge, D. & Rubin, H. (1973) *Nature New Biol.* **246,** 181–183.
4. Rubin, H. (1975) *J. Cell. Physiol.*, in press.
5. Moscatelli, D. & Rubin, H. (1975) *Nature* **254,** 65–66.
6. Rubin, H. & Koide, T. (1973) *J. Cell Biol.* **56,** 777–786.
7. Singh, V., Singh, M., August, J. & Horecker, B. (1974) *Proc. Nat. Acad. Sci. USA* **71,** 4129–4132.
8. Larner, J. (1971) *Intermediary Metabolism and Its Regulation* (Prentice-Hall, Englewood Cliffs, N.J.).
9. Lehninger, A. (1970) *Biochemistry* (Worth Publishers, Inc., New York).
10. Atkinson, D. (1968) *Biochemistry* **7,** 4030–4045.
11. Sanui, H. (1970) *J. Cell. Physiol.* **75,** 361–368.
12. Rose, I. (1968) *Proc. Nat. Acad. Sci. USA* **61,** 1079–1086.
13. Rein, A. & Rubin, H. (1968) *Exp. Cell Res.* **49,** 666–678.
14. Rubin, H. (1972) *Proc. Nat. Acad. Sci. USA* **69,** 712–716.
15. Bock, R. (1960) *The Enzymes* (Academic Press, Inc., New York), Vol. II.
16. Bissell, M., Hatié, C. & Rubin, H. (1972) *J. Nat. Cancer Inst.* **49,** 555–565.
17. Dawson, R. & Hauser, H. (1970) *Calcium and Cellular Functions*, ed. Cuthbert, A. (St. Martins's Press, New York).
18. Blaisie, J., Worthington, C. & Dewey, M. (1969) *J. Mol. Biol.* **39,** 407–416.
19. Matthews, E. (1970) *Calcium and Cellular Function*, ed., Cuthbert, A. (St. Martin's Press, New York).
20. Balk, S., Whitfield, J., Youdale, T. & Braun, A. (1973) *Proc. Nat. Acad. Sci. USA* **70,** 675–679.
21. Sanui, H. & Pace, N. (1967) *J. Cell. Physiol.* **69,** 11–20.
22. Dulbecco, R. (1975) *Proc. Nat. Acad. Sci. USA* **72,** 1584–1588.