Proc. Natl. Acad. Sci. USA
Vol. 75, No. 9, pp. 4379–4383, September 1978
Cell Biology

Magnesium reverses inhibitory effects of calcium deprivation on coordinate response of 3T3 cells to serum

(growth regulation/DNA synthesis/uridine uptake)

A. H. RUBIN, M. TERASAKI, AND H. SANUI

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

Contributed by A. Harry Rubin, June 19, 1978

ABSTRACT **Deprivation of Ca^{2+} in crowded cultures of 3T3 cells inhibits the onset of DNA synthesis. By raising $[Mg^{2+}]$ to 15 mM the inhibition produced by Ca^{2+} deprivation can be fully overcome. Sparse cultures are not inhibited by a similar deprivation of Ca^{2+}, and therefore are not stimulated by supranormal $[Mg^{2+}]$. The time course of stimulation of the onset of DNA synthesis by supranormal $[Mg^{2+}]$ in low $[Ca^{2+}]$ is the same as that produced by serum in physiological concentrations of Ca^{2+} and Mg^{2+}. Concentrations of $Mg^{2+} > 20$ mM in low $[Ca^{2+}]$ reverse the stimulation, and $[Mg^{2+}] \geq 30$ mM kills many cells. In contrast to the stimulation by 15 mM Mg^{2+}, supranormal $[Ca^{2+}]$ has no effect on the onset of DNA synthesis in cultures inhibited by Mg^{2+} deprivation, if the formation of insoluble Ca–P_i complexes is prevented. Neither Na^+ nor K^+ reproduces the effects of Mg^{2+}. The uptake of uridine is another parameter of the coordinate response of 3T3 cells to serum stimulation that is inhibited by Ca^{2+} deprivation, and supranormal $[Mg^{2+}]$ also reverses this inhibition. The results support the thesis that the coordinate response of growth and metabolism to external effectors is regulated by the availability of Mg^{2+} within the cell and that the inhibitory effects of Ca^{2+} deprivation are indirect and caused by a reduction in the availability of Mg^{2+}.**

When animal cells are stimulated to multiply by serum, hormones, or various other unrelated agents added to their culture medium, several early events occur, including acceleration in transport and phosphorylation of substrates (1, 2), energy metabolism (3), and synthesis of differentiated cell products (4, 5). After an extended lag period, the accelerated onset of DNA synthesis becomes manifest (6). This array of events has been designated the coordinate response to distinguish it from the "pleiotypic response," which explicitly excludes differentiated functions (7). We have shown that all the elements of the coordinate response can be inhibited in chick embryo cells in a reversible manner by lowering the $[Mg^{2+}]$ of the medium (8, 9). From these results and several other considerations, we have proposed that variations in the availability of Mg^{2+} for transphosphorylation reactions within the cell mediate the coordinate response of cells to external effectors (8). Others have proposed that Ca^{2+} plays a central role in mediating the growth response of cells to external effectors (10). Support for a mediating role for Ca^{2+} is chiefly based on the observation that drastically lowering the $[Ca^{2+}]$ of the medium inhibits the onset of DNA synthesis and thereby limits the multiplication of cells (11). Additional support seemed to come from the finding that supranormal concentrations of Ca^{2+} stimulate the growth of quiescent 3T3 cells (12), but this has proven to be a nonspecific effect caused by insoluble complexes of Ca^{2+} and inorganic orthophosphate (P_i) (13, 14). It has also been shown that Ca^{2+} deprivation in chick embryo cells causes a marked increase in the passive permeability of cells (15) and causes other changes that do not occur when cells are inhibited in a more physiological way by the withdrawal of serum or addition of cortisol (15, 16). Here we show that the inhibition of the onset of DNA synthesis in 3T3 cells by Ca^{2+} deprivation can be prevented by adding supranormal concentrations of Mg^{2+}. Indeed, under some conditions, treatment with supranormal Mg^{2+} causes a greater acceleration of onset of DNA synthesis than is obtained by simple serum stimulation. This reinforces our earlier conclusions that Ca^{2+} deprivation produces its growth inhibitory effects by reducing the availability of Mg^{2+}, and that the latter is the direct mediator of the coordinate response.

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MATERIALS AND METHODS

Cell Culture and Labeling. BALB/c3T3 mouse cells were obtained from J. Bartholomew and maintained in monolayer culture on Falcon plastic petri dishes in Dulbecco's modification of Eagle's medium with 10% calf serum (17). The cells were removed from the dish for transfer by treatment with 0.01% crystalline trypsin in Tris-buffered saline containing 0.5 mM EDTA. The line was maintained by seeding 5×10^4 cells per 100-mm dish and transferring them every 4–5 days. For most experiments, 5×10^4 cells were seeded on 60-mm dishes and grown to confluency. They were usually used 5–7 days after seeding, but in some cases they were used later.

Cultures were labeled with [^{3}H]thymidine by exposing them to 1 μCi of [^{3}H]thymidine per ml (specific activity 20 Ci/mmol) in modified Eagle's medium for 1 hr. They were then processed for scintillation counting of acid-insoluble material and for protein determination (18), or for fixation and autoradiography (19). Cultures were labeled with 3 μCi of [^{3}H]uridine per ml (37 Ci/mmol) in the appropriate experimental medium for 10 min at 37.5°C, washed three times with ice-cold Tris-buffered saline, and extracted for 15 min with cold 5% trichloroacetic acid for scintillation counting of acid-soluble material. The cultures were then dissolved in 0.1 M NaOH for protein determination.

Modified Eagle's medium was prepared without Ca^{2+}, Mg^{2+}, or P_i and the appropriate amounts were added as indicated in each experiment. $[P_i]$ was 1.0 mM, except in the experiment of Table 1. Ca^{2+} was added as $CaCl_2 \cdot 2H_2O$, and P_i as Na_2HPO_4, both from 100 mM aqueous solutions. Mg^{2+} was usually added as $MgCl_2 \cdot 6H_2O$, except when Specpure $MgSO_4 \cdot 7H_2O$ was used, as noted. Mg^{2+} was added from a 150 mM solution containing 10% dialyzed calf serum, in order to maintain the osmolarity and serum concentrations constant despite the addition of relatively large volumes of solution. Dilution of nutrients in the medium did not affect results. Calf serum used in experiments was extensively dialyzed against physiological saline free of Ca^{2+} and Mg^{2+}.

The procedures for measuring cation contents of cells by atomic absorption spectrophotometry were previously reported (20). Culture dishes (100 mm) were used in the cation determination experiments. Briefly, the cultures were washed five times with 10 ml per wash of CO_2-free 0.25 M sucrose solution,

approximately pH 7. For determination of surface-bound cations, they were exposed for 10 sec to either CO_2-free or carbonated (pH 4) sucrose solutions. Protons in the latter displaced the externally bound cations, and the difference in cation content of the pH 4 and pH 7 washes is referred to as surface-bound cation content of the cells (21). The free $[Ca^{2+}]$ of the medium was determined at pH 7.4 with an Orion Research digital ion analyzer, model 801A, with a Radiometer Ca^{2+} selective electrode.

RESULTS

Effects of Varying $[Ca^{2+}]$ and $[Mg^{2+}]$ on [^{3}H]Thymidine Incorporation. $[Ca^{2+}]$ was varied in modified Eagle's medium with 10% dialyzed serum over the range 0.02–1.7 mM, and each $[Ca^{2+}]$ was combined with $[Mg^{2+}]$ in the range 0.01–20.0 mM. Cultures were incubated for 17 hr in these media and labeled with [^{3}H]thymidine. In the presence of physiological $[Mg^{2+}]$ of 1.0 mM, there was only a slight inhibitory effect on [^{3}H]-thymidine incorporation of reducing $[Ca^{2+}]$ from 1.7 to 0.2 mM, but further reduction to 0.02 mM Ca^{2+} killed most of the cells in the culture (Fig. 1*a*). The cells in 0.02 mM Ca^{2+} remained viable when $[Mg^{2+}]$ was raised to 2.0 mM, but the rate of [^{3}H]thymidine incorporation was about 1/10th that in the same $[Mg^{2+}]$ with $[Ca^{2+}] \geq 0.2$ mM. Increasing $[Mg^{2+}]$ to 20 mM increased the rate of [^{3}H]thymidine incorporation about 3-fold in $[Ca^{2+}] \geq 0.2$ mM. A much more dramatic increase in the rate of [^{3}H]thymidine incorporation was seen in 0.02 mM

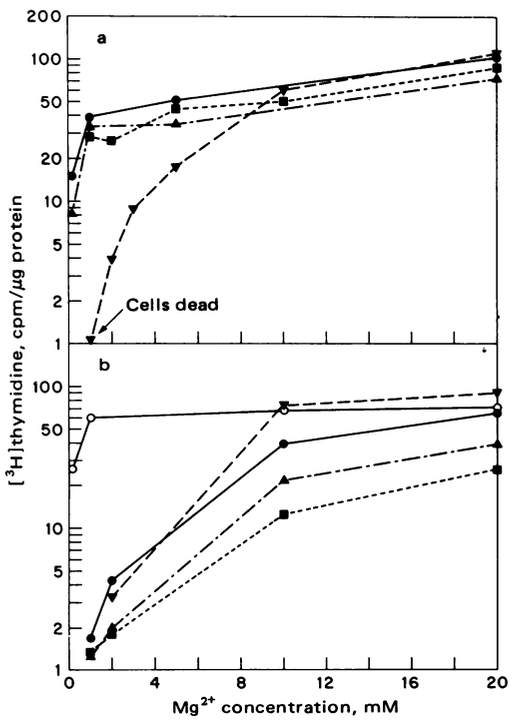

FIG. 1. Effects of varying $[Ca^{2+}]$ and $[Mg^{2+}]$ in the medium on [^{3}H]thymidine incorporation. (*a*) Five-day-old cultures were washed twice with modified Eagle's medium free of Ca^{2+} and Mg^{2+}. Media containing the appropriate concentrations of Ca^{2+} and Mg^{2+} in modified Eagle's medium with 10% dialyzed calf serum were added. Actual $[Ca^{2+}]$ and $[Mg^{2+}]$ of the media were determined by atomic absorption spectrophotometry. Cultures were incubated for 17 hr at 37.5°C, labeled with [^{3}H]thymidine for 1 hr, then extracted for scintillation counting and protein determinations. $[Ca^{2+}]$, mM: ▼, 0.02; ■, 0.2; ▲, 0.5; ●, 1.7. (*b*) Procedure was the same as in *a*, except that various [EGTA] were added to incubation media of four groups containing 1.7 mM Ca^{2+} to reduce free Ca^{2+}. ([EGTA], mM: ○, 0; ●, 1.8; ▲, 2.0; ■, 2.2.) A fifth group contained 0.02 mM Ca^{2+} and no EGTA (▼). Specpure $MgSO_4 \cdot 7H_2O$ was used as the source of Mg^{2+} to minimize the possibility of contamination by Ca^{2+}.

Ca^{2+} when $[Mg^{2+}]$ was increased to 20 mM, since it then equalled or exceeded the highest rates in the cultures containing higher $[Ca^{2+}]$. A reduction of $[Mg^{2+}]$ to 0.01 mM, the amount contaminating the medium, inhibited [^{3}H]thymidine incorporation to some extent even in 1.7 mM Ca^{2+}.

The minimum $[Ca^{2+}]$ we could achieve in the medium by simply omitting it, and using dialyzed serum, was about 0.02 mM. Since we wished to observe the effects of even lower concentrations of Ca^{2+}, we added the Ca^{2+}-chelating agent ethylene glycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) in concentrations exceeding those of the usual $[Ca^{2+}]$ of the medium. Measurements with the Ca^{2+} electrode showed that free Ca^{2+} was reduced to less than 0.01 mM by 1.8 mM EGTA, and to less than 0.001 mM by the larger amounts of EGTA. Each reduction in $[Ca^{2+}]$ resulted in a lowered rate of [^{3}H]thymidine incorporation at 17 hr, but raising Mg^{2+} to concentrations greatly in excess of the physiological value greatly stimulated that rate in every case (Fig. 1*b*). The stimulation of the cultures by supranormal $[Mg^{2+}]$ in the conventional $[Ca^{2+}]$ without EGTA was less pronounced than that in the previous experiment. Thus, Ca^{2+} deprivation not only accentuates the effects of varying $[Mg^{2+}]$, but makes them more reproducible from experiment to experiment.

Effects of Cell Population Density on Response to $[Ca^{2+}]$ and $[Mg^{2+}]$. Sparse and crowded cultures were prepared by seeding 5×10^4 cells per dish and allowing them to multiply for 1 or 5 days. They were then exposed to a wide range of $[Mg^{2+}]$ in low $[Ca^{2+}]$ or to a range of $[Ca^{2+}]$ in 1 mM or 15 mM Mg^{2+}. The rates of thymidine incorporation per unit protein were much higher in the sparse than in the crowded cultures at all $[Ca^{2+}]$ and $[Mg^{2+}]$, and there were only minor effects in the sparse cultures of varying either ion in the range of concentrations tested (Fig. 2). In the crowded cultures, however, 15 mM Mg^{2+} stimulated thymidine incorporation 16-fold above the rate in 1 mM Mg^{2+} (Fig. 2*a*). Further increases in $[Mg^{2+}]$ caused a marked inhibition of thymidine incorporation, and 30 mM Mg^{2+} caused foci of dead cells to appear. Raising $[Mg^{2+}]$ to 15 mM increased the rate of thymidine incorporation to approximately the same maximum in the crowded cultures at

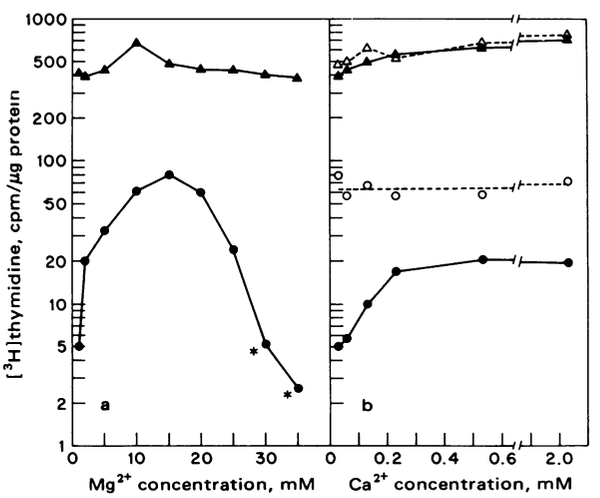

FIG. 2. Effects of cell population density on response to $[Ca^{2+}]$ and $[Mg^{2+}]$. Dishes were seeded with 5×10^4 cells, either 5 or 1 day before the experiment, thus constituting crowded and sparse cultures. They were then switched to media with the indicated combinations of $[Ca^{2+}]$ and $[Mg^{2+}]$, and processed as in the legend of Fig. 1 to determine the rate of [^{3}H]thymidine incorporation. Circles represent crowded, and triangles, sparse cultures. Asterisks, foci of damaged cells. (*a*) Effects of variable $[Mg^{2+}]$ in constant $[Ca^{2+}]$ (0.03 mM). (*b*) Effects of variable $[Ca^{2+}]$ in two constant $[Mg^{2+}]$: (△, ○) 15 mM; (▲, ●) 1 mM.

all [Ca^{2+}] tested (Fig. 2*b*). As in the experiment of Fig. 1*a*, there was about a 3-fold stimulation by 15 mM Mg^{2+} in the presence of near physiological [Ca^{2+}] of 2.0 mM. The stimulation of thymidine incorporation by supranormal [Mg^{2+}] in physiological [Ca^{2+}] occurred in all experiments, but its extent varied from about 1.2- to 4-fold. By contrast, the stimulation by supranormal [Mg^{2+}] in [Ca^{2+}] ~ 0.02 mM was always 10-fold or greater with this line of cells. Such marked stimulation did not occur in transformed 3T3 cells that had escaped density-dependent inhibition since thymidine incorporation was not inhibited in the first place by Ca^{2+} deprivation. In this sense, crowded cultures of transformed cells behaved like sparse cultures of the untransformed cells. Early passage cultures of mouse embryo fibroblasts behaved in a manner intermediate between the untransformed and transformed 3T3 cells.

Scintillation Counting Versus Autoradiography in Measuring Time Course of [Ca^{2+}] and [Mg^{2+}] Effects on Thymidine Incorporation. The medium of quiescent cultures was changed to fresh medium with varying [Ca^{2+}] and [Mg^{2+}], and thymidine incorporation was measured at intervals by scintillation counting of solubilized cells and by autoradiography of fixed cells. For 10 hr there was no change in the rate of thymidine incorporation as measured by either technique (Fig. 3). By 18 hr, a sharp increase had occurred in total incorporated thymidine of extracts, and in the proportion of labeled cells both in the cultures with physiological [Ca^{2+}] and [Mg^{2+}] and in those with low [Ca^{2+}] and 15 mM Mg^{2+}. Incorporation remained at a high rate, as measured by both techniques, until 24 hr in these two sets of cultures and then declined. In the cultures with 0.02 mM Ca^{2+} and only 1.0 mM Mg^{2+}, there was a much smaller increase in thymidine incorporation before the decline set in. It is apparent that total incorporation by scintillation counting is a reasonably accurate indicator of the number of labeled cells, and therefore measures the rate at which cells progress into the S period. The results also show that 15 mM Mg^{2+} stimulates the progression through G1 into the S-period of cultures in low [Ca^{2+}] with about the same lag and peak periods as are produced by medium change with physiological [Ca^{2+}] and [Mg^{2+}].

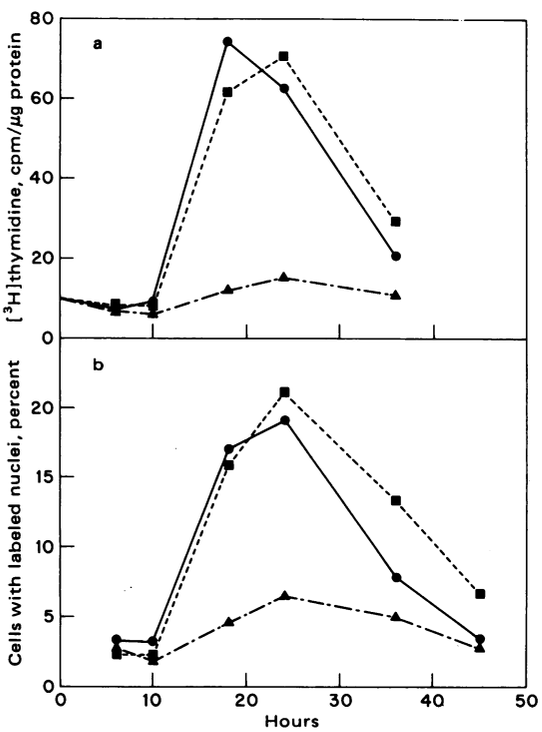

FIG. 3. Scintillation counting versus autoradiography in measuring kinetics of [^{3}H]thymidine incorporation in various [Ca^{2+}] and [Mg^{2+}]. Five-day-old confluent cultures were washed and switched to modified Eagle's medium plus 10% dialyzed calf serum containing various [Ca^{2+}] and [Mg^{2+}]. At the indicated times they were labeled with [^{3}H]thymidine for 1 hr and processed for scintillation counting (*a*) or autoradiography (*b*). The radioactivity of at least 2000 nuclei was measured for each autoradiographic point. ■, 0.02 mM Ca^{2+} and 15.0 mM Mg^{2+}; ●, 1.7 mM Ca^{2+} and 1.0 mM Mg^{2+}; ▲, 0.02 mM Ca^{2+} and 1.0 mM Mg^{2+}.

Effects of Supranormal Concentrations of Ca^{2+}, Na^+, and K^+ on Thymidine Incorporation. Supranormal concentrations of Ca^{2+} have been reported to stimulate thymidine incorporation in 3T3 cells. Although this has been shown to be caused by insoluble complexes of Ca^{2+} and P_i (Ca–P_i) (13, 14), it was of interest to determine whether cells inhibited by Mg^{2+} deprivation in physiological [Ca^{2+}] could be stimulated by 15 mM Ca^{2+} under conditions in which the insoluble Ca–P_i complexes do not occur. This could be achieved by lowering [P_i] in the medium to 0.1 mM. We found that cells inhibited by Mg^{2+} deprivation were not stimulated by 15 mM Ca^{2+} in 0.1 mM P_i (Table 1). In 1.0 mM P_i, where 15 mM Ca^{2+} did form insoluble complexes with P_i, there was a further inhibition of thymidine incorporation already inhibited by Mg^{2+} deprivation (Table 1). (Stimulation by Ca–P_i complexes is erratic when [Ca^{2+}] > 10 mM (13) and usually does not occur in Mg^{2+}-deprived cultures.) In no case did 15 mM Ca^{2+} produce stimulation that even approached the magnitude of the effect produced by supranormal Mg^{2+} in low [Ca^{2+}]. Nor did the addition of 50 mM Ca^{2+} produce the severe inhibition and cell death caused by 50 mM Mg^{2+}.

When [K^+] was increased to 15 mM in low [Ca^{2+}], there was no increase in thymidine incorporation (Table 2). When [K^+] was increased to 65 mM, there was no significant inhibition of the heightened thymidine incorporation produced by 15 mM Mg^{2+}.

Increasing [Na^+] from 140 to 200 mM, which necessitated increasing the osmolarity of the medium, had a slight inhibitory effect on thymidine incorporation (Table 2). At no [Na^+] between 140 and 200 mM was there an increase in thymidine

Table 1. Effects of supranormal [Ca^{2+}] on [^{3}H]thymidine incorporation in Mg^{2+}-deprived cultures

Ca^{2+}, mM	Mg^{2+}, mM	P_i, mM	[^{3}H]Thymidine, cpm/μg protein
	[P_i] effects		
1.7	1.0	0.1	43.49
1.7	1.0	1.0	36.26
	[Mg^{2+}] effects in low [Ca^{2+}]		
0.017	1.0	1.0	12.53
0.017	15.0	1.0	133.98
0.017	50.0	1.0	0.57*
	[Ca^{2+}] effects in low [Mg^{2+}]		
1.7	0.004	0.1	15.81
1.7	0.004	1.0	12.30
15.0	0.007	0.1	9.33
15.0	0.007	1.0	4.19†
50.0	0.011	0.1	9.48

Seven-day-old cultures were washed with modified Eagle's medium free of Ca^{2+}, Mg^{2+}, and P_i and incubated in modified Eagle's medium with the indicated concentrations of Ca^{2+}, Mg^{2+}, and P_i plus 10% dialyzed calf serum for 17 hr. They were then labeled with [^{3}H]thymidine and processed for scintillation counting and protein determination. Medium in this experiment was treated with Chelex resin to reduce [Mg^{2+}] to less than 0.015 mM present as contaminant, which was necessary to inhibit [^{3}H]thymidine incorporation in the presence of physiological [Ca^{2+}].

* Widespread cell damage.

† Ca–P_i precipitate in medium.

Table 2. Effects of supranormal [K+] or [Na+] on [3H]thymidine incorporation in Ca^{2+}-deprived cultures

Ca^{2+}, mM	Mg^{2+}, mM	Na^+, mM	K^+, mM	[3H]Thymidine, cpm/μg protein
1.7	1.0	140	5	78.6
0.024	1.0	140	5	13.8
		Supranormal [Mg^{2+}]		
0.024	15.0	126	4.5	224.9
0.024	50.0	94	3.35	0.32*
		Supranormal [K^+]		
0.024	1.0	140	15.0	9.73
0.024	15.0	70	65.0†	172.9
		Supranormal [Na^+]		
0.024	15.0	200‡	4.7	97.31

Six-day-old cultures were washed in modified Eagle's medium free of Ca^{2+} and Mg^{2+} and incubated in modified Eagle's medium plus 10% serum containing the indicated concentrations of cations for 17 hr. They were then labeled with [3H]thymidine and processed for scintillation counting and protein determination.

* Widespread cell damage.

† Osmolarity maintained ~300 mosM. If increased to ~500 mosM, 65 mM K^+ damaged cells.

‡ Osmolarity of medium increased to ~400 mosM.

incorporation (data not shown). Thus, changes in the three other major cations of the cell could reproduce neither the stimulatory nor the inhibitory effects of progressive increases in [Mg^{2+}].

Ion Concentrations in Cells Subjected to Variations in Extracellular Ca^{2+} and Mg^{2+}. Reduction of [Ca^{2+}] in the medium from 1.52 to 0.02 mM without a change in [Mg^{2+}] caused about a 40% reduction of intracellular [Ca^{2+}] and a 75% reduction in Ca^{2+} bound to the external surface of the cells (Table 3). It caused no significant change of intracellular Mg^{2+} but doubled the surface-bound Mg^{2+}, indicating that Mg^{2+} replaced much of the Ca^{2+} that had been removed from the surface. When the [Mg^{2+}] was raised to the stimulatory concentration of 13.4 mM in low Ca^{2+}, there was no effect on intracellular Ca^{2+}, but there was a significant increase of both intracellular and surface-bound Mg^{2+}.

Effects of Mg^{2+} on Uptake of Uridine. Serum stimulates the uptake of uridine by 3T3 cells as well as the incorporation of thymidine into DNA (22). We confirmed that 3T3 cells increase their rate of uridine uptake between 10 and 20 min after fresh medium containing 10% dialyzed serum was added (Fig. 4). The initial increase in uptake occurred when only Ca^{2+} was omitted and also occurred, although to a lesser extent, when both Ca^{2+} and Mg^{2+} were omitted. However, uridine uptake declined after 30 min in cultures deprived of both Ca^{2+} and Mg^{2+} and reached a rate even lower than that of cultures without serum at 180 min. In the presence of 1.0 mM Mg^{2+}, uridine uptake leveled off after its initial rise in the Ca^{2+}-deprived cultures, and then began to decline after 40 min. In the presence of 15 mM Mg^{2+}, the Ca^{2+}-deprived cultures reached a rate of uridine uptake about equal to that of the controls and remained close to the control levels to the end of the 3-hr experimental period.

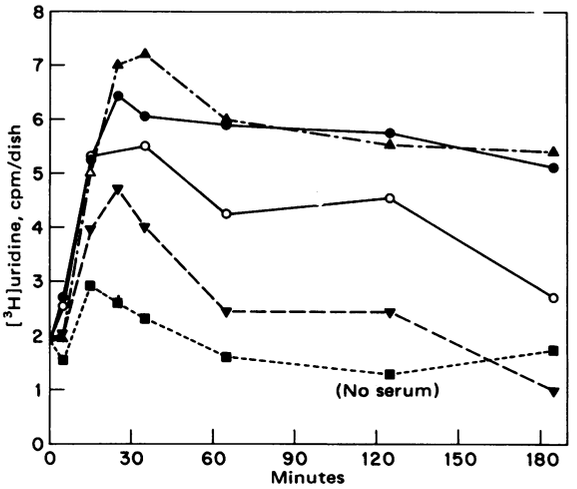

FIG. 4. Kinetics of [3H]uridine uptake in various [Ca^{2+}] and [Mg^{2+}]. Seven-day-old cultures were incubated in modified Eagle's medium with 1% serum for 24 hr. They were then washed twice with modified Eagle's medium free of Ca^{2+} and Mg^{2+}, and media of the appropriate composition with 10% dialyzed serum (top four curves) or no serum (bottom curve) were added. Beginning at this time, and at various times afterwards the medium was withdrawn and replaced with medium of the same composition containing 3.0 μCi of [3H]uridine per ml for 10 min. The cultures were processed to extract acid-soluble radioactivity. (●) 0.02 mM Ca^{2+}, 15.0 mM Mg^{2+}; (▲) 1.7 mM Ca^{2+}, 1.0 mM Mg^{2+}; (○) 0.02 mM Ca^{2+}, 1.0 mM Mg^{2+}; (▼) 0.02 mM Ca^{2+}, 0.015 mM Mg^{2+}; (■) 1.7 mM Ca^{2+}, 1.0 mM Mg^{2+}.

DISCUSSION

Other investigators have proposed that the availability of Ca^{2+} controls the proliferation of cells, and that it does so by activating a "master reaction" which leads to initiation of DNA synthesis and cell division (10, 11). The proposal is based on the finding that lowering of [Ca^{2+}] in the medium inhibits the initiation of DNA synthesis in rat lymphoblasts, chick fibroblasts, and 3T3 cells. We have previously shown that in chicken embryo fibroblasts there is mutual potentiation of DNA synthesis and growth by Ca^{2+} and Mg^{2+} (23). In the present work we show that the inhibitory effects of Ca^{2+} deprivation on the initiation of DNA synthesis and on the stimulation of uridine uptake in mammalian cells can be prevented by raising the Mg^{2+} of the medium to concentrations far above the physiological levels. The maximal stimulation in very low [Ca^{2+}] is produced with 15 mM Mg^{2+}. This maximum in some experiments exceeds the rate of DNA synthesis in control cultures containing the physiological concentrations of both Ca^{2+} and Mg^{2+} (Tables 1 and 3). However, an increase in cell number did not parallel the increase in DNA synthesis with supranormal Mg^{2+} in low Ca^{2+} (data not shown). This may be related to the moderate cell detachment that occurs in low [Ca^{2+}].

Table 3. Divalent cation content of cells in media with varying [Ca^{2+}] and [Mg^{2+}]

Medium, mM		Protein,	[3H]Thymidine,	Ca^{2+}, nmol/μg protein		Mg^{2+}, nmol/μg protein	
Ca^{2+}	Mg^{2+}	μg/dish	cpm/μg protein	Intracellular	Surface-bound	Intracellular	Surface-bound
1.52	0.98	640	4.90	0.007	0.008	0.053	0.003
0.02	0.96	520	2.17	0.004	0.002	0.052	0.006
0.02	13.40	540	28.64	0.004	0.002	0.072	0.008

Cells (1×10^5) were seeded on 100-mm Falcon dishes in modified Eagle's medium and 10% calf serum and incubated for 11 days. The medium was replaced with modified Eagle's medium and dialyzed calf serum with various [Ca^{2+}] and [Mg^{2+}], as shown, and incubated for 17 hr. Two cultures in each group were labeled with [3H]thymidine. The remaining cultures were processed to determine the concentrations of surface-bound and intracellular Ca^{2+} and Mg^{2+}.

The accumulated evidence indicates that Mg^{2+}, and not Ca^{2+}, is the direct mediator of the growth response of the cells. We have suggested previously that derpivation of Ca^{2+} produces inhibitory effects on metabolism indirectly by making less Mg^{2+} available for critical regulatory reactions in the cell (15, 23). Here we show that the inhibition of the initiation of DNA synthesis produced by drastic Mg^{2+} deprivation in the presence of physiological $[Ca^{2+}]$ is not relieved by supranormal Ca^{2+} (Table 1). In contrast, the inhibition produced by drastic Ca^{2+} deprivation in physiological $[Mg^{2+}]$ is fully overcome by supranormal $[Mg^{2+}]$. Increased concentrations of Na^+ or K^+ do not stimulate the onset of DNA synthesis in low $[Ca^{2+}]$. Recent evidence shows that wide variations in the intracellular concentration of either of these cations fails to affect the onset of DNA synthesis (unpublished data).

Stimulation by supranormal $[Mg^{2+}]$ produces approximately equal increases in total thymidine incorporation and the percent of labeled nuclei (Fig. 3). This correlation shows that high $[Mg^{2+}]$ stimulates the progress of cells through the G1 period into the S period, and not merely the uptake of thymidine or the rate of DNA chain elongation. After addition of medium with low $[Ca^{2+}]$ and supranormal $[Mg^{2+}]$ there is a lag period of about 10 hr before any change is seen in the proportion of cells in the S period, there being a sharp increase at 18 hr and then a decrease at 36 hr. These changes parallel those which occur in physiological concentrations of the divalent cations and lend support to the idea that the processes leading to the onset of DNA synthesis are controlled by the intracellular availability of Mg^{2+}.

We found that the rate of uridine uptake by 3T3 cells varies with $[Mg^{2+}]$. Since uridine uptake also varies with serum and insulin concentrations (22), it can be considered part of the coordinate response of these cells to external effectors, as it is in chicken embryo cells (8, 9, 24). Phosphorylation rather than transport is the limiting factor in uridine uptake by 3T3 cells (2). Since all transphosphorylation reactions require Mg^{2+}, and many are inhibited by free ATP^{4-}, the availability of Mg^{2+} could play a direct role in regulating uridine uptake. It does not play a direct role in regulating DNA chain elongation, since a lag of 10 hr occurs before DNA synthesis responds to alterations in $[Mg^{2+}]$ or to [serum]. Indeed, it is only the rate of progress through the G1 period (probability of onset of DNA synthesis) that is affected by these variables and not the length of the S period (rate of DNA chain elongation). The probability of onset of DNA synthesis is affected by many biochemical pathways, including those concerned with energy metabolism, RNA synthesis, and protein synthesis (6). The availability of Mg^{2+} could control the onset of DNA synthesis by regulating one or more of these pathways. The unresponsiveness of DNA chain elongation to changes in $[Mg^{2+}]$ of the magnitude used here shows that the effects are selective if not highly specific. The selectivity is probably provided by such factors as the quantitative requirements of transphosphorylating enzymes for Mg^{2+}, their sensitivity to inhibition by free ATP^{4-}, and their location within the cell.

Decreasing Ca^{2+} in the medium to 20 μM in 1 mM Mg^{2+} does not cause a decrease of total intracellular Mg^{2+}, as measured by atomic absorption spectrophotometry. Since the inhibitory effects of Ca^{2+} deprivation are reversed by Mg^{2+}, we assume that free $[Mg^{2+}]_i$ is reduced by binding to sites formerly occupied by Ca^{2+}. In this sense Ca^{2+} might play some role in regulation through its effect on the availability of Mg^{2+}. There is, however, no need to invoke such a role for Ca^{2+}, since Mg^{2+} could as well be directly controlled. Addition of supranormal $[Mg^{2+}]$ to such cultures does raise total $[Mg^{2+}]_i$ and presumably increases both free and bound Mg^{2+}. Sparse cultures are unaffected by reductions in $[Ca^{2+}]$ that profoundly inhibit crowded cultures. This finding recalls the report that transformed cells are more resistant to the inhibitory effects of Ca^{2+} deprivation than are normal cells (25), and suggests that the proportion of divalent cations in the free state is higher in sparse or transformed than in crowded cultures. This could result from a reduced affinity for divalent cations of sequestering sites such as membranes in sparse and transformed cultures, due to configurational changes in those sites or to changes in the internal milieu which reduce ion binding to the sites.

An unanticipated finding is the marked inhibition of onset of DNA synthesis by $[Mg^{2+}] > 20$ mM in low Ca^{2+}. It results in a bell-shaped curve for the onset of DNA synthesis as a function of $[Mg^{2+}]$, which resembles those seen for some Mg^{2+}-dependent enzyme reactions (26, 27). This suggests that the reaction that regulates the onset of DNA synthesis has a similar Mg^{2+} dependency, and that it might be possible to identify it by this property.

The excellent technical assistance of Mrs. Berbie Chu is gratefully acknowledged. Dr. T. Okazaki provided the information on the response of secondary cultures of mouse embryo fibroblasts to variations in $[Ca^{2+}]$ and $[Mg^{2+}]$. This work was supported by National Institutes of Health Research Grant CA 15744 awarded by the National Cancer Institute.

1. Rubin, H. & Koide, T. (1975) *J. Cell. Physiol.* **86,** 47–58.
2. Rozengurt, E., Stein, W. & Wigglesworth, N. (1977) *Nature (London)* **267,** 442–444.
3. Fodge, D. & Rubin, H. (1973) *Nature (London) New Biol.* **246,** 181–183.
4. Moscatelli, D. & Rubin, H. (1977) *J. Cell. Physiol.* **91,** 79–88.
5. Kamine, J. & Rubin, H. (1977) *J. Cell. Physiol.* **92,** 1–12.
6. Rubin, H. & Fodge, D. (1974) in *Control of Proliferation in Animal Cells,* eds. Clarkson, B. & Baserga, R. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 801–816.
7. Tomkins, G. & Gelehrter, T. (1972) in *Biochemical Actions of Hormones,* ed. Litwack, G. (Academic, New York), pp. 1–20.
8. Rubin, H. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 3551–3555.
9. Rubin, H. (1976) *J. Cell. Physiol.* **89,** 613–625.
10. Whitfield, J., MacManus, J., Rixon, R., Boynton, A., Youdale, T. & Swierenga, S. (1976) *In Vitro* **12,** 1–18.
11. Boynton, A., Whitfield, J., Isaacs, R. & Morton, H. (1974) *In Vitro* **10,** 12–17.
12. Dulbecco, R. & Elkington, J. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 1584–1588.
13. Rubin, H. & Sanui, H. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5026–5030.
14. Barnes, D. & Colowick, S. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5593–5597.
15. Bowen-Pope, D. & Rubin, H. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 1585–1589.
16. Rubin, A. H. & Chu, B. (1978) *J. Cell. Physiol.* **94,** 13–20.
17. Vogt, M. & Dulbecco, R. (1963) *Proc. Natl. Acad. Sci. USA* **49,** 171–179.
18. Rubin, H. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 712–716.
19. Tobey, R., Petersen, D., Anderson, E. & Puck, T. (1966) *Biophys. J.* **6,** 567–581.
20. Sanui, H. & Rubin, H. (1977) *J. Cell. Physiol.* **92,** 23–32.
21. Sanui, H. & Rubin, H. (1978) *J. Cell. Physiol.* **96,** 265–278.
22. Rozengurt, E. & Stein, W. (1977) *Biochim. Biophys. Acta* **464,** 417–432.
23. Rubin, H. & Koide, T. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 168–172.
24. Rubin, H. (1977) *J. Cell. Physiol.* **91,** 249–260.
25. Balk, S. (1971) *Proc. Natl. Acad. Sci. USA* **68,** 271–275.
26. Garner, P. & Rosett, T. (1973) *FEBS Lett.* **34,** 243–246.
27. Roberts, B. & Patterson, B. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 2330–2334.