results. It may be that the washing of the eggs and the dilution of semen in Loeb's experiments were not sufficient to remove most of the magnesium ions.

We also checked the effect of removal of magnesium ions on sperm motility. Semen was diluted to 1 : 50,000 with ASW containing various concentrations of calcium and magnesium. The swimming speed was estimated by measuring the tracks made by the spermatozoa on dark-field micrographs. The results revealed that there is no significant difference in speed among the different sperm suspensions. In other words, the reduction in the fertilization rate observed above is not attributable to any adverse effects of the absence of magnesium ions on sperm motility.

Takahashi and Sugiyama (*6*) showed that, when sea urchin spermatozoa were treated with dissolved egg jelly, their acrosome reaction was induced and these reacted spermatozoa could fertilize the eggs even in the virtual absence of calcium if insemination was carried out within 30 seconds after the treatment with egg jelly. Their results indicated that calcium ions in seawater are not necessary for the processes following the acrosome reaction. Therefore, as the next step, we examined whether or not magnesium ions are needed for the subsequent processes at fertilization.

For this purpose, eggs of *S. intermedius* were washed and suspended as described above. Spermatozoa were treated with dissolved egg jelly to induce the acrosome reaction as described by Dan *et al.* (*7*). Insemination was accomplished by mixing the egg suspensions with the jelly-treated sperm suspension within 30 seconds after the spermatozoa were exposed to the jelly solution. The fertilization rate was determined as described above.

The results revealed that, in the calcium-free ASW that contained 50 m*M* magnesium chloride, the fertilization rate was 100 percent, whereas when the magnesium chloride concentration was reduced to less than 10 m*M*, almost no fertilization occurred (Fig. 2). The same was true in *H. pulcherrimus* and *A. crassispina*.

It appears to be paradoxical that more magnesium ions were needed to cause fertilization with the acrosome-reacted spermatozoa treated with jelly than with the normal spermatozoa, because the treated spermatozoa conducted only a part of the whole fertilization process. In this respect, we found that the spermatozoa treated with jelly showed higher fertilizability when incubated prior to insemination in calcium-free ASW that contained 50 m*M* magnesium chloride, as compared with those incubated in calcium-free ASW that contained 1 m*M* magnesium chloride. The result implies that the presence of a high concentration of magnesium ions is favorable to preserving the structure and function of the reacted acrosome. In fact, the reacted spermatozoa lose their fertilizability rapidly, and do not seem to be equivalent to the nonreacted ones.

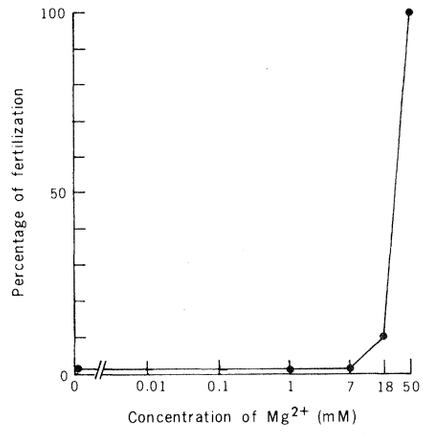

Fig. 2. Fertilization rate of *S. intermedius* as a function of magnesium ion concentration in the absence of calcium. The spermatozoa had been treated with dissolved egg jelly to induce acrosome reaction immediately before insemination.

In addition, we found that treatment with butyric acid of unfertilized eggs, washed four times with Ca, Mg-free ASW, caused the formation of fertilization membrane in the absence of calcium or magnesium ions, or both. Therefore, it is conceivable that the membrane formation itself does not require these divalent cations, the exogenous ones at least. This result is consistent with those obtained by Steinhardt and Epel (*8*) with the artificial activator A23187.

From these results we suggest that magnesium ions in seawater are indispensable for fertilization of sea urchins, probably at some step or steps between the acrosome reaction and the formation of fertilization membrane, such as fusion between sperm acrosomal membrane and egg plasma membrane or penetration of the spermatozoon into the egg, including membrane lysin and the like.

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Bone Cells in Culture: Morphologic Transformation by Hormones

Abstract. *Hormones and purine nucleosides and nucleotides induced cultured bone cells to transform transiently from a spherical to a stellate shape. Cytochalasin B also induced the transformation. The change was blocked by colchicine and vinblastine, but not by lumicolchicine or cycloheximide. This morphologic transformation may provide a dynamic model of hormone action and bone cell modulation in vitro.*

We report here that bone cells in culture undergo spontaneous and hormone-induced transient morphologic transformation from spherical to stellate shape.

Bone cells were isolated from fetal Holtzman rat calvariae (21 days gestation) and cultured in Falcon tissue culture flasks (3×10^5 cells added to each flask; growth area, 25 cm^2) by modification of the method of Peck *et al.* (*1*) and were observed intermittently for 12-hour periods through an inverted phase-contrast microscope. The bone cells in culture reached monolayer confluence in 7 to 10 days and appeared spherical in shape.

A low level of transient morphologic transformation accompanied cell maturation in culture. As they reached their 7th day in monolayer, cells became stellate in sporadic areas of some flasks and formed circumscribed, lacelike networks

that constituted less than 2 percent of the total monolayer areas. The cells reverted to the spherical shape within 6 hours (*2*). Serum inhibited the transformation, just as it is known to inhibit the morphologic differentiation of other cells in culture (*3*). Removal of fetal calf serum from the culture medium caused the change to occur earlier and resulted in transient (6-hour) transformation of up to 30 percent of 48-hour monolayer cultures and up to 100 percent of 7-day monolayer cultures (quantitation method, Table 1). Reintroduction of serum after 1 hour caused reversion to the spherical shape within the following hour.

Hormones, particularly parathyroid hormone (PTH), are regulators of bone cell differentiation in vivo (*4*). For studies of the morphologic response of cultured bone cells to hormones, we minimized spontaneous and maximized induced transformation by using monolayer cultures less than 48 hours old and removing serum from the medium 12 hours before study (Table 1). The percentage of transformation in control flasks was 0 to 16 percent.

The biologically active synthetic tetratriacontapeptide of bovine parathyroid hormone [bPTH(1-34)] (gift of Beckman Bioproducts) caused the spherical cultured bone cells to become stellate and form a lacelike network of cells (Fig. 1). Transformation induced by bPTH(1-34) began within 30 minutes and peaked in 60 to 90 minutes, and the cells reverted to their shape prior to treatment in 3 to 6 hours (*5*). Transformation was dose-dependent (Table 1, experiment A); it began at near physiologic doses of bPTH(1-34) of between 5 and 10 ng/ml and reached 100 percent with doses of between 10 and 100 ng/ml. The lowest dose of bPTH(1-34) that caused the morphologic change was in a range similar to that which activated adenylate cyclase in cultured bone cells (*6*). At comparable or higher doses biologically inactive, oxidized bPTH(1-34) (*7*) (which did not activate adenylate cyclase in cultured bone cells) induced no morphologic transformation (Table 1, experiment A) (*8*).

Epinephrine also both stimulates bone cell adenylate cyclase (*9*) and induces the morphologic transformation (Table 1, experiment E). The β-adrenergic blocking agent propranolol inhibited the response to epinephrine, but not the response to bPTH(1-34) or to dibutyryl cyclic adenosine monophosphate (DBcAMP), implying that this response was specific to epinephrine (Table 1, experiments D and E). Other agents, which do not activate adenylate cyclase in this bone cell culture system (*9*), did not induce transformation; these agents include synthetic adrenocorticotropic hormone (Cortrosyn, Organon), 100 ng/ml; synthetic salmon calcitonin (Armour), 1 to 1000 ng/ml; and crystalline porcine insulin (Lilly), 1 to 1000 ng/ml. Also, neither synthetic human gastrin (Imperial Chemical Industries) (500 ng/ml) nor cholecystokinin-pancreozymin (Dr. V. Mutt, Karolinska Institute) (100 ng/ml) induced transformation of the cultured bone cells.

Cyclic AMP (cAMP) mediates many actions of the hormones tested, and cyclic nucleotides may play central roles in cell differentiation (*10*). We therefore studied the morphologic response of cultured bone cells to other agents that increase the intracellular concentrations of these nucleotides (Table 1, experiments B and C). The cAMP analogs, DBcAMP and 8-bromo-cAMP, and cyclic guanosine monophosphate (cGMP) caused transformation (*11*). Theophylline and purine nucleosides adenosine and guanosine also increase purine nucleotide concentrations within cells (*12*) and in-

Table 1. Quantitation of cultured bone cell transformation. We first incubated 24-hour monolayer cultures for 12 hours in additive-free culture medium, then removed this medium, and added 2 ml of culture medium with test agents (medium alone added to controls). Flasks were randomized before treatment and observed by one observer (S.S.M.) unaware of the treatment agent, 60 to 90 minutes (peak effect) after treatment. For quantitating the percentage of transformation, each flask was placed on the movable stage, graduated in millimeters, of a Nikon model MS inverted phase-contrast microscope and observed at each of 15 designated coordinates (corresponding to locations with vertical coordinates 110, 115, and 120 mm and horizontal coordinates 10, 20, 30, 40, and 50 mm) through a four-quadrant camera eyepiece (Nikon model PFM) (15 coordinate positions × four quadrants of eyepiece = 60 areas per flask) at a magnification of × 50; there were approximately 75 cells per area. Percentage of transformation is the percentage of areas containing at least one stellate cell. Results of observations of two flasks per experimental point were averaged. Presented here are results of these and other experiments; two different observers qualitatively confirmed results of representative experiments. When included, inhibitors were added simultaneously with agents and also to preincubation medium 30 minutes prior to DBcAMP experiments. Vehicles that accompanied treatment agents [sodium bisulfite for epinephrine (Epin), absolute alcohol for cytochalasin B, and acetic acid for parathyroid hormone] did not alter cell morphology when administered in the same concentrations and volumes to control flasks. The results of all seven experiments were tested extensively with the methods of analysis of variance and χ^2. Abbreviation bPTH refers to bPTH(1-34).

Treatment	Transformation (%)	Treatment	Transformation (%)
Experiment A		*Experiment D (cont'd.)*	
Control	6	bPTH + cycloheximide ($10^{-5}M$)	88§
bPTH (5 ng/ml)	13*	bPTH + cytochalasin B ($10^{-7}M$)	100§
bPTH (10 ng/ml)	58†	bPTH + colchicine ($10^{-5}M$)	2‡
bPTH (100 ng/ml)	100†	bPTH + vinblastine ($10^{-5}M$)	0‡
bPTH (1000 ng/ml)	100†	*Experiment E*	
bPTH (oxidized) (1000 ng/ml)	13*	Control	10‡
bPTH (oxidized) (4000 ng/ml)	8*	DBcAMP (1 mM)	100§
Experiment B		DBcAMP + propranolol ($4 \times 10^{-6}M$)	100§
Control	16‡	Epin ($10^{-5}M$)	96§
cAMP ($10^{-3}M$)	17‡	Epin + propranolol ($4 \times 10^{-6}M$)	6‡
DBcAMP ($10^{-3}M$)	100§	Epin + cytochalasin B ($10^{-7}M$)	100§
8-Bromo-cAMP ($10^{-3}M$)	100§	Epin + colchicine ($10^{-5}M$)	2‡
cGMP ($10^{-3}M$)	89§	Epin + vinblastine ($10^{-5}M$)	1‡
Dibutyryl cGMP ($10^{-3}M$)	8‡	*Experiment F*	
Experiment C		Control	3‡
Control	0‡	Epin ($10^{-5}M$)	84§
Uridine ($10^{-3}M$)	8‡	Epin + cycloheximide ($10^{-5}M$)	78§
Cytidine ($10^{-3}M$)	1‡	*Experiment G*	
Guanosine ($10^{-3}M$)	35‖	Control	3‡
Adenosine ($10^{-3}M$)	95§	DBcAMP (1 mM)	100§
Theophylline ($5 \times 10^{-3}M$)	100§	DBcAMP + cycloheximide ($10^{-5}M$)	100§
Experiment D		DBcAMP + cytochalasin B ($10^{-7}M$)	100§
Control	4‡	DBcAMP + colchicine ($10^{-5}M$)	0‡
bPTH (20 ng/ml)	100§	DBcAMP + vinblastine ($10^{-5}M$)	0‡
bPTH + propranolol ($4 \times 10^{-6}M$)	98§		

*Not significantly different from control. †Significantly different from control ($P < .001$). In experiments B through G the percentage of transformations caused by treatments differed significantly ($P < .002$). These responses clustered tightly with a set of responses falling into one cluster if no two responses differed significantly ($P = .05$); responses fell into a second cluster if they satisfied this definition and differed from each response in the first cluster with $P < .01$ (experiment C) and $P < .001$ (experiments B and D through G). In all six experiments, no response in one cluster differed from a response in another cluster with $P > .01$; thus the clusters were well separated. ‡All responses marked with this symbol were in the same cluster. §All responses with this symbol were in the same cluster. ‖All responses with this symbol were in the same cluster.

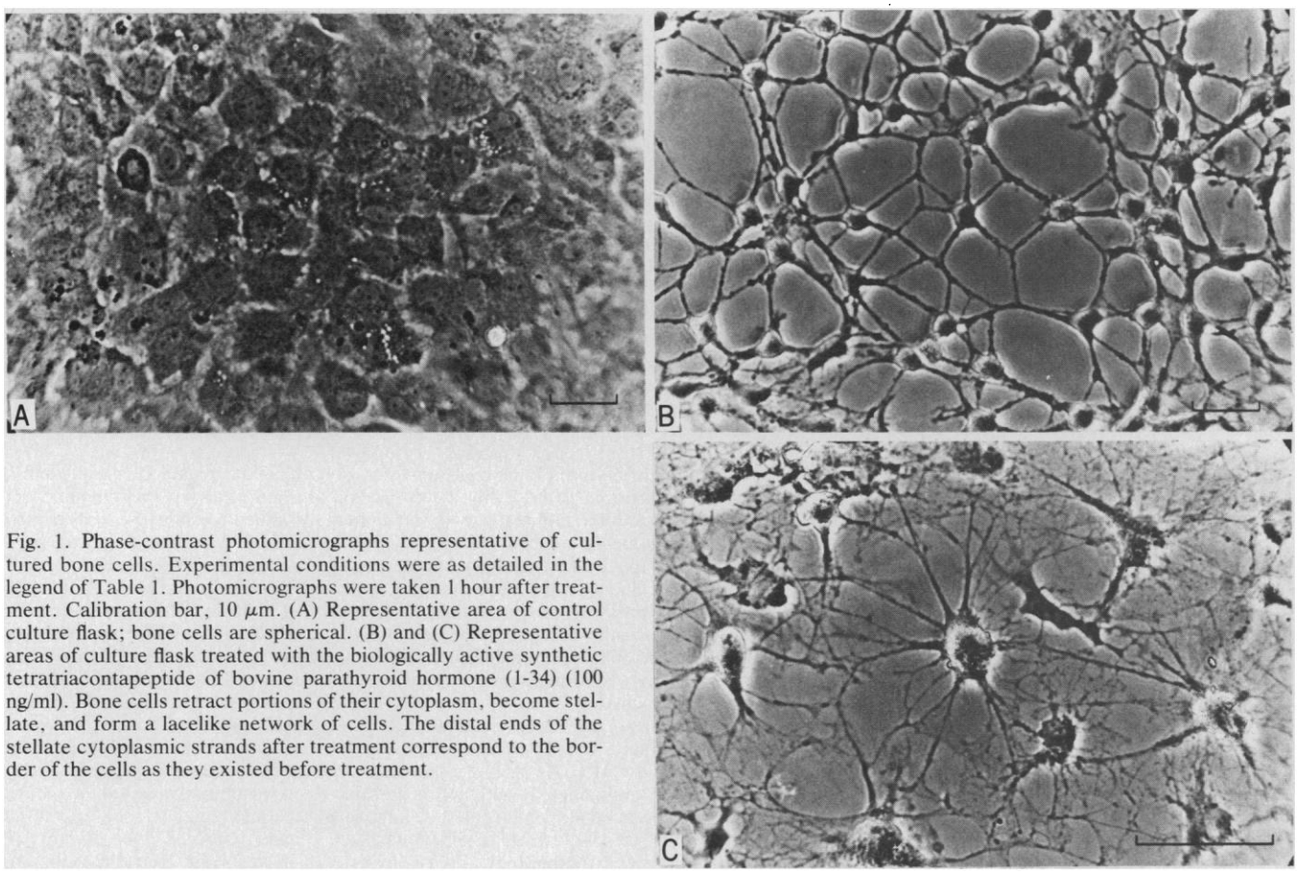

Fig. 1. Phase-contrast photomicrographs representative of cultured bone cells. Experimental conditions were as detailed in the legend of Table 1. Photomicrographs were taken 1 hour after treatment. Calibration bar, 10 μm. (A) Representative area of control culture flask; bone cells are spherical. (B) and (C) Representative areas of culture flask treated with the biologically active synthetic tetratriacontapeptide of bovine parathyroid hormone (1-34) (100 ng/ml). Bone cells retract portions of their cytoplasm, become stellate, and form a lacelike network of cells. The distal ends of the stellate cytoplasmic strands after treatment correspond to the border of the cells as they existed before treatment.

duced the morphologic change; the pyrimidine nucleosides cytidine and uridine produced no transformation. These studies suggest that purine, but not pyrimidine, nucleotides mediate transformation induced by hormones.

We probed the subcellular anatomic basis of the morphologic change (Table 1, experiments D to G): bPTH(1-34), epinephrine, or DBcAMP induced transformation when each agent was added alone. Concurrent addition of microtubule inhibitors vinblastine or colchicine blocked the transformation caused by these agents, but neither cytochalasin B (cyto B) nor cycloheximide inhibited the response to bPTH(1-34), epinephrine, or DBcAMP. In separate experiments, lumicolchicine (*13*) ($10^{-5}M$) did not block transformation induced by bPTH(1-34) (25 ng/ml) or DBcAMP ($10^{-3}M$); in addition, lowering the temperature to 4°C [which reversibly depolymerizes microtubules (*13*)] blocked, and returning the temperature to 37°C restored, the transformation response to bPTH(1-34) (50 ng/ml) and DBcAMP ($10^{-3}M$). Independent observations—that colchicine blocked the appearance of bone cell microtubules, PTH-induced "ruffle border" changes in osteoclasts, and PTH-induced ^{45}Ca release from organ cultures of bone (*14*)—also suggest that regulation of bone cell structure and function by hormones may require microtubules.

In separate experiments, higher doses ($2 \times 10^{-7}M$) of microfilament inhibitor cyto B caused transformation. Cytochalasin B also caused transformation of cells from a spheroid to a stellate shape in organ cultures of bone (*15*). In contrast to spontaneous transformation of cultured bone cells induced by nucleosides, nucleotides, and hormones, that caused by cyto B was permanent, and cell death (vacuolization and peeling of cells from flask surfaces) followed within 18 hours of continuous exposure to cyto B. On the other hand, cyto B–induced transformation could be reversed, cell viability maintained, and transformation response to PTH restored by washing the cells with additive-free medium 1 hour after treatment with cyto B. Cytochalasin B may have induced transformation of bone cells in organ and cell cultures as a result of effects other than, or in addition to, inhibition of microfilaments (*16*). However, if inhibition of microfilament function caused transformation, and inhibition of microtubule assembly blocked transformation, these studies raise the question whether bone cell structure depends upon a regulated balance between opposing microfilament and microtubular activities. A similar relation may exist between microfilaments and microtubules of pancreatic beta cells during glucose-induced insulin secretion in vitro (*17*).

While transformed bone cells in culture may resemble osteocytes in vivo, our data do not demonstrate whether cultured bone cell transformation in vitro and bone cell modulation in vivo are comparable phenomena. Other types of cells in culture undergo permanent or transient morphologic changes in response to agents that augment intracellular cyclic nucleotide concentrations and whose actions are blocked by microtubule disrupting agents. In many cases the transformed cells assume functional as well as morphologic characteristics of their in vivo counterparts (*18*). Whether biochemical changes also accompany the hormone-induced morphologic modulation of cultured bone cells must await further investigation.

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Ribonucleotide Reductase in Blue-Green Algae: Dependence on Adenosylcobalamin

Abstract. *Ten species of freshwater blue-green algae exhibit an adenosylcobalamin-dependent ribonucleotide reductase, thus explaining the requirement for cobalt by these organisms. The evidence suggests a phylogenetic affinity between the cyanophytes and bacteria, such as* Clostridium *and* Rhizobium, *and the euglenoid flagellates, which also use the cofactor-dependent reductase. In contrast, the ribonucleotide reductase reaction in the few green algae surveyed shows no dependence on cobalamins.*

The importance of vitamin B_{12} in algal nutrition has been widely documented (*1*). In contrast to a group such as the dinoflagellates, however, very few blue-green algae need an exogenous source of this vitamin. Only a small number of marine species have been shown by van Baalen to require vitamin B_{12} for growth (*2*). Nevertheless, the requirement for cobalt by these organisms is universal (*3*) and various corrinoids have been extracted from species such as *Anabaena cylindrica* (*4, 5*).

The nutritional requirement for vitamin B_{12}, either exogenous or synthesized by the organism, could be established by demonstrating a cobalamin-dependent metabolic reaction. Although several enzymatic reactions have been shown to require a coenzyme form of vitamin B_{12}, only three appear to be widespread. Methylcobalamin is used as a cofactor in methionine synthesis in some strains of *Escherichia coli* (*6*). This enzyme may also be of importance in mammalian cells (*7*). The methylmalonyl coenzyme A isomerase reaction uses adenosylcobalamin as cofactor. This enzyme has been isolated from various sources—from bacteria to sheep liver (*8*). However, it may be of limited importance in some cells, especially microorganisms. Ribonucleotide reductase also requires an adenosylcobalamin cofactor in some organisms. The reaction catalyzed by this enzyme is crucial to cell division, as it supplies the deoxyribonucleotide precursors for DNA synthesis. Two forms of the enzyme exist; as isolated from *E. coli* the enzyme contains two atoms of nonheme iron per molecule. It requires no B_{12} cofactors (*9*). A similar enzyme has been demonstrated in mammalian cells (*10, 11*). The adenosylcobalamin-dependent type of reductase was first demonstrated in *Lactobacillus leichmannii* (*12*). Although this type of reductase may be considered the more esoteric form of the enzyme, a survey of literature indicates that it is probably more common among prokaryotes than the iron-containing reductase. A ribonucleotide reductase that requires a cobalamin cofactor is found in *Clostridium* (*13*), *Rhizobium* (*14*), various denitrifying organisms (*15*), and *Bacillus megaterium* (*16*). In addition, the adenosylcobalamin-dependent reductase has been demonstrated in one class of algae, the euglenoid flagellates (*17*). In this report, we show the occurrence of this enzyme in various blue-green algae. Using the sensitive assay procedure of monitoring the exchange of ^3H from [5′-$^3H_2$]adenosylcobalamin to water during the enzymatic reaction (*13, 18*), we have demonstrated that the B_{12}-dependent type of enzyme is widespread among the cyanophytes.

Axenic cultures of blue-green algae were obtained from various sources and grown in media indicated in Table 1. Cell growth was monitored by optical density at 650 nm or direct counting in a Petroff-Hauser counting chamber. Cells were harvested in the logarithmic phase of growth in centrifugation at 7000*g* in a DuPont-Sorvall RC-5 refrigerated centrifuge. The cells were resuspended in a buffer containing 0.01*M* dimethylglutarate buffer (*p*H 7.2) and 0.001*M* mercaptoethanol. They were washed once and resuspended in the same buffer (1 g of cells, wet weight, per 5 ml of buffer). One gram of charcoal (neutral Norit A) was added per 10 ml of cell suspension. The suspension was then sonicated for 10 minutes with a Heat Systems Branson Sonifer. The sonicated extract was centrifuged at 27,000*g* for 20 minutes, and the supernatant was removed and assayed for ribonucleotide reductase activity as soon as possible. Protein was determined by the biuret (*19*) method, with bovine serum albumin as a standard.

The assay mixture contained the following: 0.12*M* 3,3-dimethylglutarate buffer, *p*H 7.2; 1.2 m*M* ethylenediaminetetraacetic acid; 26 m*M* dithiothreitol; 2 m*M* adenosine triphosphate (P-L Biochemicals); 0.01 m*M* [5′-$^3H_2$]adenosylcobalamin (specific activity, 15 μc/μmole (*20*); and 1 to 5 mg of protein of crude algal extract in a final volume of 0.5 ml. Coenzyme was added in dim light and the reaction initiated by addition of