There is no evidence to support genetic drift or alteration as the factor responsible for the dramatic decrease in tumor incidence. The question of possible loss of Bittner mammary tumor virus was studied in group C mice. Both blood and mammary tumor extracts from these mice were active in producing hyperplastic alveolar nodules; this activity is indicative of MTV presence (*7*). This evidence, together with ultimate mammary tumor production, indicated that MTV was present as a persisting infection in these animals. Such assays may not answer conclusively, however, the more quantitative question of MTV attenuation. In respect to the possibility of gaining a new passenger virus that might repress mammary tumor development, serological tests for the spectrum of known murine viruses did not reveal any provocative contamination in these C3H mice (*13*); however, these tests are not conclusive evidence for the absence of latent infections, particularly those of more common strains of mouse encephalomyelitis viruses (*14*).

Additional possibilities for explaining the effect of stress on tumor induction include the enhanced cellular release or synthesis of MTV. Injected cortisol increases the concentration of intracytoplasmic A and extracellular B particles (MTV) in mouse mammary tumors (*15*). A variety of corticosteroids have been reported to stimulate MTV synthesis in cell cultures (*16, 17*). Moore (*17*) has suggested that sociological stress in women may be a factor in the etiology of breast cancer in view of the finding that corticosteroids can increase MTV production in mice and in cell culture. Such in vivo data are consistent with the hypothesis associating the adverse effects of stress with impairment of immunological surveillance and the consequential escape of transformed cells; however, the in vitro observations suggest that the corticoid influences may cause an increase in mammary tumor virus production as well as an impairment in the immunological control of transformed cells.

If physiological stress is assumed to be an auxiliary potentiating factor in the early appearance and high mammary tumor incidence of groups A and B, several mechanisms of action are possible. For example, does the intact surveillance defense mechanism act against the transformed malignant cell, against the oncogenic virus, against the neoplastic transformation process, or against some combination of these? The impaired surveillance hypothesis provides a simplistic but logical rationale to explain the multiple observations if it is assumed that viral transformation of normal cells occurs regularly but is continuously negated as long as there is a constant and effective host surveillance. When immunological competence is compromised, even temporarily, by loss or inactivation of T cells or other vital defense elements following stress-induced corticoid hormone elevation, the host surveillance fails to destroy the transformed malignant cells during their immunologically vulnerable stage. The data further imply that once a cancer cell escapes to an organizational state beyond the limited defensive abilities of immunological surveillance, the production of a lethal tumor may then be inevitable and not reversible by natural host defenses.

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Cyclic AMP and Cyclic GMP: Mediators of the Mechanical Effects on Bone Remodeling

Abstract. *Compressive forces of physiological magnitude (60 grams per square centimeter) reduce the adenosine 3',5'-monophosphate and guanosine 3',5'-monophosphate content of the epiphyses of tibiae from 16-day-old chick embryos. An equivalent hydrostatic pressure applied directly to cells isolated from this tissue also affects cyclic nucleotide accumulation. The tissue response is uniform throughout the epiphysis, whereas the cell response varies according to the area of origin.*

Living bone responds to mechanical forces by "adaptive" changes in internal architecture (*1*). The mode of conveyance of the mechanical signal to the bone remodeling process is still unknown. In this study we present evidence for the involvement of adenosine 3',5'-monophosphate (cyclic AMP) and guanosine 3',5'-monophosphate (cyclic GMP) in the response of chick-embryo long bone to compressive forces. There were several reasons for considering the cyclic nucleotides as potential messengers in the transduction of the physical stimuli into biochemical signals. Hormones that affect bone remodeling—parathyroid hormone and thyrocalcitonin—modulate the cyclic AMP level in bone cells through inter-

action with the cell membrane (*2*). There is evidence demonstrating cyclic AMP and cyclic GMP involvement in the control of cell growth and cell division in fibroblast cultures (*3*); and in a recent review McMahon has hypothesized that cyclic nucleotides play a central role in development and cytodifferentiation in general (*4*). In an experimental system designed to deliver a quantitated force to bone in tissue culture, we have found that a pressure of physiological magnitude (60 g/cm^2) reduces the glucose consumption of tibiae from 16-day-old chick embryos to 50 percent that of controls and stimulates the incorporation of [^{14}C]thymidine into DNA (*5*). Carbohydrate metabolism and cell division are known to be subject to the control of cyclic nucleotides. Therefore, using this recent method of pressure application to bone in culture, we proceeded to look for pressure-induced changes in cyclic nucleotide levels (*6*).

In the first set of experiments we determined the time course of cyclic AMP accumulation in tibiae exposed to a pressure of 60 g/cm^2. Significantly less cyclic AMP was found in the epiphyses of bones exposed to pressure, relative to matched controls (Table 1). In the same experiments, the cyclic AMP content of the diaphyses was not significantly different from controls [88.6 ± 14.8 (S.E.M.) pmole per diaphysis at 15 minutes]. Further experiments were directed to the response of the epiphyses to pressure and to answers to the following questions: (i) the role of the matrix, (ii) the role of cellular specificity, and (iii) the relation between the changes in cyclic AMP and cyclic GMP. To answer these questions we have taken advantage of the cellular arrangement in the epiphyseal cartilage. The epiphyses can be divided into three segments: a distal segment rich in cells with proliferative potential; a middle segment of growing, nondividing cells; and a proximal segment (close to the diaphysis), which contains the hypertrophied nongrowing, nondividing cartilage cells and a number of bone-forming cells.

In one set of experiments we first exposed the bones to pressure in the presence and absence of theophylline, then we dissected the epiphyses into the three segments and determined contents of cyclic AMP and cyclic GMP in each segment. The results (Table 2) show that a pressure of 60 g/cm^2 affected all three segments equally. The pressure caused a reduction in accumulation of cyclic GMP and cyclic AMP, which, relative to matched controls, was more pronounced in the absence of theophylline. The drop in cyclic GMP was larger than that of cyclic AMP. These effects fit the features of the Ca^{2+} and protein-modulator-dependent phosphodiesterase found in bovine heart and porcine and rodent brain (*7*). If a similar enzyme is present in this tissue, its activation, through an increased availability of calcium, could be the mode by which pressure modulates the levels of cyclic nucleotide.

In the next experiments we first dissected the epiphyses into segments, then separated the cells from each segment by enzyme digestion, applied a hydrostatic pressure of 60 g/cm^2 to the isolated cells, and measured the cyclic AMP and cyclic GMP (*8*). The results presented in the last two lines of Table 2 show that a hydrostatic pressure of 60 g/cm^2 applied directly to the isolated cells alters the cellular cyclic nucleotide content. The separation unmasked differences among the cells obtained from the three tissue segments. The pressure-induced changes were also different from those produced in the intact tissue. In cells from the distal segment the pressure caused an increase in cyclic GMP; in cells from the middle segment both cyclic AMP and cyclic GMP were elevated; and in cells originating from the proximal segment the concentration of cyclic GMP was reduced. The latter effect matched that produced in the tissue. Knowing that the

Table 1. The effect of compressive forces on cyclic AMP accumulation in the epiphyses of 16-day-old chick embryo tibiae. The tibiae were aseptically dissected free of muscle and were kept in culture medium (*6*) at 37°C until all bones were dissected. One pair of tibiae at a time was then incubated for 15 minutes in 10 m*M* theophylline at 23°C. One bone of each pair was thereafter exposed to a continuous compressive force of 60 g/cm^2, in the same medium, at 37°C, for the time periods indicated in the table. The contralateral tibia was incubated without pressure. At the end of the incubation period, the bones were frozen in a mix of Dry Ice and methanol. The epiphyses were cut from the diaphyses under a dissecting microscope in the cold and were processed and analyzed (*13*). The ratio of cyclic AMP in the compressed bone to that in the control was computed for each pair. The mean of the ratios of all pairs for each time period was then calculated. Statistical significance was calculated by Student's *t*-test for paired samples.

Time (min)	Cyclic AMP in control bones (pmole/epiphyses)	*N*	Cyclic AMP (exp/control)*	*P*
0	38.5 ± 13.2	6		
2	64.4 ± 4.9	15	0.98	†
4	78.5 ± 3.9	6	1.06	†
8	84.3 ± 7.1	6	0.80	< .01
12	92.7 ± 1.2	6	0.67	< .01
15	93.5 ± 10.6	6	0.72	< .01
18	82.2 ± 6.3	12	0.65	< .01
20	66.8 ± 8.4	4	0.87	†

*Mean ratio of experimental to control. †Difference is not statistically significant.

Table 2. The effect of pressure on accumulation of cyclic AMP and cyclic GMP in different segments of chick tibia epiphyses and in the cells isolated therefrom. For the examination of the tissue segments, tibiae from 16-day-old chick embryo were obtained and exposed to pressure for 15 minutes as described in legend to Table 1. After the experiment, when the epiphyses were separated from the diaphyses, they were divided into three equal (by eye) segments of about 0.8 mm each. Histologic examination showed that the distal segment contained fusiform fibroblast-like cells, that the middle segment contained small round cartilage cells, and that the proximal segment contained larger cartilage cells embedded in matrix and a small amount of newly formed bone. The results represent the mean and standard error of the mean of the number of experiments indicated in the last line. Statistical significance was estimated using Student's *t*-test for paired samples.

Segment	Accumulation in tissue (pmole/ μg DNA)				Accumulation in cells (pmole/10^6 cell)	
	10 m*M* theophylline		No theophylline		10 m*M* theophylline	
	Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP
Distal						
Pressure	4.25 ± 0.4	0.87 ± 0.2*	1.42 ± 0.1*	0.32 ± 0.06*	61.7 ± 11.2	13.4 ± 1.8*
Control	4.05 ± 0.2	1.36 ± 0.2	2.04 ± 0.2	0.54 ± 0.03	88.0 ± 9.0	7.9 ± 1.7
Middle						
Pressure	3.55 ± 0.7	0.81 ± 0.2*	1.44 ± 0.3*	0.27 ± 0.07*	105.5 ± 18.7*	5.5 ± 0.5*
Control	5.02 ± 0.8	1.46 ± 0.2	2.56 ± 0.6	0.54 ± 0.04	77.0 ± 13.5	3.6 ± 0.7
Proximal						
Pressure	3.02 ± 0.3	0.87 ± 0.1*	1.52 ± 0.1*	0.29 ± 0.05*	106.2 ± 16.5	6.3 ± 0.7*
Control	3.97 ± 0.3	1.48 ± 0.1	2.30 ± 0.4	0.53 ± 0.07	104.3 ± 17.2	12.6 ± 1.9
No. experiments	9		5		15	

*$P < .05$.

isolation procedure necessarily modifies the cell membrane, we performed these experiments in order to assess the contribution of the tissue environment to the evocation of the cellular response. The observations indicate that the perturbation produced by the small hydrostatic pressure applied directly to the isolated cells alters the amounts of cyclic nucleotides in cells and that these changes are cell specific and dependent on the matrix (or cell environment).

In this context, we view the tissue as a stimulus-receptor system in which the distortion of the cell membrane produced by a mechanical, electrical (*9*), or chemical (*10*) perturbation initiates specific events in the bone remodeling process through cyclic nucleotide modulation. The meaning of the reduction in cyclic AMP can be inferred from observations in other experimental systems. In fibroblast cultures low levels of cyclic AMP are correlated with growth stimulation, whereas addition of cyclic AMP inhibits growth (*3*). Similarly, the growth-stimulating hormone, somatomedin, inhibits the adenylate cyclase activity of chondrocyte and chick-embryo cartilage preparations in vitro (*11*). The concurrent drop in cyclic GMP observed in the tissue does not fit the hypothesis of reciprocal changes in the two cyclic nucleotides (*12*). In contrast, the response of the isolated cells (from the distal segment) seems to follow this pattern. A deeper understanding of these differences could come from the study of the mechanism by which pressure modulates the cyclic nucleotide levels. Regardless of mechanism, however, our results demonstrate that, under the experimental conditions described, the immediate response of long bone in vitro to a pressure of physiological magnitude includes a change in the levels of cyclic AMP and cyclic GMP. The cyclic nucleotides may play the role of messengers in the conveyance of the mechanical perturbation to the biochemical machinery of the cell, serving as regulatory molecules in the cytodifferentiation required for bone remodeling.

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Membrane Protein Analysis by Two-Dimensional Immunoelectrophoresis

Abstract. *Water-insoluble membrane proteins may be analyzed by a new, rapid technique that combines electrophoresis on high-resolution sodium dodecyl sulfate (SDS) polyacrylamide gels and immunoelectrophoresis. After separation in the first dimension by electrophoresis in SDS, the proteins are subjected to a second electrophoresis at right angles through a two-layered buffered agarose gel. They first pass through a layer containing Lubrol PX which forms complexes with free SDS and then into an antiserum layer where antigen-antibody precipitates form. Precipitin arcs appear at positions corresponding to the antigens separated in the first dimension. The effectiveness of the technique was demonstrated with frog and cattle opsins, human erythrocyte membrane proteins, and their rabbit antiserums and for several water soluble proteins. By this method two fundamental parameters, molecular weight and antigenicity, may be readily used for analysis of membrane proteins.*

Antiserums to cellular membrane constituents are valuable for studying the spatial arrangement of antigens on cell surfaces, their distribution among various cell types, and the effects of antibody binding on membrane-mediated cell functions. However, the water insolubility of many membrane macromolecules has made it difficult to confirm the specificity of the corresponding antiserums or to identify the antigens unequivocally.

Membrane proteins are now routinely separated and analyzed by electrophoresis on sodium dodecyl sulfate (SDS) polyacrylamide gels, a method that furnishes reproducible polypeptide patterns for several membrane systems (*1*–*3*). No other system has comparable advantages of simplicity, sensitivity, and resolution in the analysis of the water-insoluble proteins of biological membranes. We therefore designed a compatible immunochemical assay by combining SDS polyacrylamide gel electrophoresis in the first dimension (*4*) with immunoelectrophoresis into antiserum in the second dimension.

The procedure is an extensive modification of the techniques of "rocket," and