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THE EFFECTS OF CYCLIC 3'5'ADENOSINE MONOPHOSPHATE AND CYCLIC 3'5'GUANOSINE MONOPHOSPHATE UPON SUTURAL EXPANSION BY AN ORTHOPEDIC FORCE

by

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Abstract

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Local control mechanisms that mediate the growth and remodeling in skeletal tissue of the craniofacial apparatus, and thereby their response to externally applied orthopedic forces, are complex and at present not fully understood. One control pathway, however, that has received growing attention has been the action of the cyclic nucleotides. Results of previous research with bone tissue have demonstrated that both cAMP and cGMP are important mediators in the alveolar remodeling response to orthodontically induced tooth movements. However, the specific nature of the effects that externally applied orthopedic forces have on the growth and remodeling of bones in the craniofacial complex is still unclear.

The present study was performed to examine the effects of an orthopedic stimulus upon cyclic nucleotide levels of the involved bones and how these levels relate to growth and remodeling changes that occur at the various sutures. To study this effect,

an expansile force was applied across the sagittal suture in young growing rats and corresponding cyclic nucleotide levels were experimentally altered by the use of theophylline, a phosphodiesterase inhibitor.

The sample consisted of 22 female Sprague Dawley rats which were divided into four groups, with one group acting as a control. The remaining three experimental groups consisted of:

1. T group - animals force fed theophylline (1 mg/100g body weight - 4 times/day);
2. S group - expansion spring implanted across sagittal suture to provide an orthopedic stimulus;
3. T + S group - animals received theophylline as well as the implanted spring.

Cranial implants were placed in all animals to measure dimensional changes of the calvaria over the experimental period. In addition, one animal from each group was injected with a vital dye for subsequent histological evaluation.

Following a 21 day experimental period, animals were decapitated and the bone around the sagittal suture removed and analyzed in four categories.

A. Sutural expansion and surface area were determined from cranial implant measurements to assess sutural response to different experimental variables.

B. Levels of cAMP and cGMP were individually quantitated by by specific radioimmunoassay procedures to evaluate variable dependent changes in concentration.

C. Analysis of non-collagen sample protein content to provide a standard unit by which to compare the levels of cAMP/cGMP.

D. Histological evaluation of remodeling changes that occurred at the suture during the experimental period.

The most significant findings of the present study were:

1. Orthopedic forces have a definite effect on intracellular cyclic nucleotide levels in sutural areas of the involved bones, resulting in a significant rise in cAMP and decrease in cGMP;

2. Theophylline, when used alone, resulted in a similar increase although of lower magnitude in cAMP levels compared to that of the mechanical stimuli with an additive effect when both were combined;

3. Cyclic GMP decreased 50% and remained constant relative to the control regardless of the variable(s) involved;

4. A positive association was demonstrated between the amount of expansion observed and the level of cAMP present in the bone around the suture.

5. Sutural expansion forces resulted in extensive remodeling changes primarily at the sagittal suture with very little expansile effects on the rest of the craniofacial complex as a whole.

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DEDICATED

TO

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INTRODUCTION

Growth, particularly that of the craniofacial complex, has received a great deal of attention in the field of orthodontics, primarily because of its importance in treatment outcome. Ever since the advent of cephalometric radiography, which made the serial examination of the craniofacial structure in growing children possible, there has been a flourishing of studies in the orthodontic literature dealing with this subject. A great deal of effort has been put forth by researchers attempting to determine the parameters of growth, such as where (Brash, 1924), how (Sicher, 1955) and why growth occurs (Moss, 1960; Scott, 1958). These parameters have been investigated both from the normal aspect through studying growing individuals and the abnormal through redirecting existing patterns and observing resultant deviations (Sarnat, 1955).

Much of the success of orthodontic treatment relies on the determinants of these parameters and to what degree they can be manipulated through treatment. Deformation of bone as a result of alterations in growth patterns has been practiced for various reasons in different societies for centuries. Perez-Martinez (1960) illustrated a variety of cranial deformities induced by the Colombian Indians. Morphological changes in these skulls were produced as a result of binding the head with strips of cloth and thereby applying external forces to the growing cranium in order to alter

existing growth and remodeling processes. Likewise, in orthodontics, externally applied orthopedic forces (e.g., headgear, chin-cup, etc.) are often utilized to alter bone relationships in the growing craniofacial complex. Orthopedic forces, when applied to these various bones, have been shown to affect growth and remodeling at their various sutures (Meldrum, 1975; Haas, 1961).

Investigators have established rather conclusively that orthopedic forces can alter growth and remodeling in the craniofacial complex, but the sequential events triggered by this force and leading to the observed change in form at the cellular level is not fully understood. Many local control mechanisms governing growth determinants have been suggested, but what actually occurs to activate the biological machinery within the various bone cells or controls their direction remains obscure. Some light was shed into this area, however, when Sutherland and Rall (1960) alluded to the role of adenosine 3', 5'-cyclic monophosphate (cAMP) in mediating the effects of extracellular stimuli upon their target cells. Since this advance, cyclic AMP has been demonstrated as a "second messenger" in many important hormonal and drug interactions which have significent effects on many tissues, including bone.

Soon after the identification of cAMP, another important cyclic nucleotide was found. This nucleotide, cyclic GMP, was thought to be another intracellular second messenger which antagonized the effects of cAMP. An early mechanism of action of cGMP, although very similar to that of cAMP, seemed to have totally oppo-

site cellular effects; the so-called yin-yang hypothesis (Lehninger, 1975). More recent investigations, however, have shown that this strict reciprocal interplay between cAMP and cGMP is not true. Furthermore, it is now thought that the levels of the cyclic nucleotides, as well as their stimulation, are cell and tissue specific.

Bone tissue investigations by Davidovitch (1980a, b), Burrows (1978), and Roden (1975), among others, have shown that orthodontically applied mechanical forces can affect intracellular cyclic nucleotide levels. In addition, these investigators have provided evidence that indicates cAMP and cGMP may have some function in the mediation of alveolar bone remodeling. The role that levels of these nucleotides have in the scenario of sutural growth, however, has never been conclusively shown.

This present investigation attempts to alter growth and remodeling by artificially inducing changes, at the cellular level, of both cAMP and cGMP. These induced changes were brought about by the use of theophylline, a drug known to be a powerful inhibitor of the phosphodiesterase enzyme that breaks down both cAMP and cGMP, once they have been formed. These drug induced effects were examined in the cranial sutures, across which an expansile force had been placed, of growing Sprague Dawley rats. Sutural growth was analyzed by: (1) direct measurement between cranial implants and (2) ground histological sections. Cyclic nucleotides were measured utilizing a radioimmune assay technique specific for each nucleotide.

BACKGROUND REVIEW

Theories dealing with the growth and development of the craniofacial complex have fluctuated from a totally genetic aspect (Brodie, 1923), to more recent theories involving a much greater environmental aspect. As emphasized by Moss and Salentyn (1964) the craniofacial complex performs a variety of vital functions such as respiration, olfaction, vision, and ingestion. All such functions are carried out by what they term "functional cranial components." Each of these components consists of a skeletal unit that supports and/or protects its specifically related functional matrix. Moss proposed the existence of two general types of functional matrices: a periosteal matrix consisting of the vascular, neural and muscular tissues, and a capsular matrix composed of bony enclosed soft tissue compartments such as the brain and orbit. These matrices differ in their growth roles. Periosteal matrices act on the skeletal units directly by influencing deposition and resorption, thereby altering their size and morphology. Capsular matrices act through growth and a resultant increase in volume, to cause a passive outward movement of the surrounding skeletal units.

Growth thus involves both enlargement and remodeling. It has been established that the bones of the craniofacial complex do not merely increase in size, but also change in shape as

growth progresses (Enlow, 1975). According to Enlow both the endosteal and the periosteal membrane surfaces of any of the bones of the head and face are fairly evenly divided between regional fields of deposition and resorption. These fields allow for specific remodeling changes as the individual bones enlarge.

The growth of the brain precedes general body and facial growth, and thus will establish early the template for lateral and anterioposterior dimensions of the cranium, and in turn, the facial skeleton (Enlow 1975). As with Moss' capsular matrix, the bones of the calvaria are passively translated outward as the brain grows. This expansion creates tension in the sutural membranes which immediately respond by deposition of new bone along the sutural edges (Enlow, 1975). As these bones are enlarging along their circumference, the diploic spaces are also widening by a process of cortical drift. This movement apart of the cortical plates comes about as a result of the actions of periosteal deposition and endosteal resorption. Overall, remodeling (e.g. resorption/deposition) is not as complex in the flat bones that make up the calvaria due to their relatively simple morphology, although reversals of deposition and resorption do occur at the sutures, thus allowing for a flattening in the changing curvature of the skull concomitant with growth (Enlow, 1975).

On the cellular level, the processes of bone deposition and resorption are mediated by osteoblastic and osteoclastic cells respectively contained in the periosteal/endosteal membranes. The

intercellular substances of bone consist of two fundamentally different components. One is organic and the other is inorganic. Studies on calcified bone have shown that by dry weight around 70-77% of bone substance is inorganic and the balance organic (Ham, 1974). In addition to the collagen, the organic matrix also contains some sulfated mucopolysaccharides and glycoproteins.

Observations by Pugliarella, et al. (cited by Herring, 1964) conducted on bovine bone indicates that the bone matrix is not homogeneous, but varies in degree of mineralization with progressive age. The collagen content which is initially high during the less calcified, immature period, decreases with the stage of mineralization. Herring (1964) found the collagen content to vary from 31.99% dry weight in osteoid tissue (e.g. uncalcified bone matrix) to 23.63% in a fully calcified specimen. Likewise, non-collagen proteins varied from 12.87% to 1.52% dry weight, respectively (Herring, 1964).

In bone deposition, precursors of the matrix molecules (e.g. procollagen) are secreted by the osteoblast and when attacked by osteoblastic extracellular enzymes (e.g. peptidase) will coalesce to form the bone matrix (Ham, 1974). This matrix, which initially is termed osteoid, will become progressively impregnated by hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) crystals as the matrix becomes calcified.

The mechanism of calcification in this matrix is very complex and not fully understood. According to Ham (1974), changes occur in both the matrix itself and in the osteoblastic induced

local environment prior to calcification. The blood and tissue fluids are normally saturated with Ca^{++} and PO_4 ions, but researchers have demonstrated further increases in these ions in the local environment of bone deposition, resulting in a precipitation of calcium phosphate salts. This is thought to be due to the alkaline phosphatase enzymes which are secreted abundantly by the osteoblasts, and result in the splitting of pre-existing organic phosphate compounds (e.g. hexosephosphate or glycerophosphate) that might be present in the locality, but are ordinarily not ionized. When these PO_4 ions are liberated under influence of the phosphatase, the local concentration of phosphate ions is increased to the point where calcium phosphate will precipitate into the matrix surrounding the cells.

Additionally, the organic matrix is thought to have a physical or chemical proclivity for becoming calcified under conditions where other tissues would remain uncalcified. This has been demonstrated by the fact that the matrix will calcify regardless of the presence of viable osteoblasts (Ham, 1975). Changes in composition, such as the mucopolysaccharides seen between the osteoid and calcified tissue have been suggested to have an effect in opening up spaces between the tropocollagen fibrils and to provide an active nucleus for the precipitation of the inorganic salts (Ham, 1974). In addition to changes in composition, the matrix also undergoes changes in physical properties with development. Ham (1975) demonstrated that as much as 10% of the collagen in

young bone matrix is water soluble at room temperature, but as the matrix matures the amount of soluble collagen decreases significantly in conjunction with an increase in collagen cross-linking.

On the other hand, bone resorption, which involves the removal of both mineral and organic intercellular substance, has been shown to be mediated by the osteoclast. The fact osteoclasts are found only in association with calcified tissue and never osteoid tissue, indicates that they act primarily by dissolving the mineral, and secondarily, by depolarizing the organic constituent. Ham (1974) suggests that the osteoclasts function by providing a sufficiently local acid environment in close association with the bone, which causes the bone salts to act as a buffer. In buffering an acid, the calcium phosphate becomes a much more soluble salt (e.g. $CaHPO_4$ is much more soluble than $Ca_3(PO_4)_2$) and is eroded away. Concomitant with the demineralization, enzymes secreted by the osteoclast (e.g. B glucuronidase) would degrade the organic matrix (Ham, 1974).

The control of bone deposition/resorption and, thereby, growth and remodeling are under a variety of local and systemic controls. One systemic control that has received a great deal of study has been the effects of hormones, particularly parathyroid hormone and calcitonin.

Parathyroid hormone has been shown to cause an increase in the number and activity of osteoclasts, as well as an increase in the level of blood calcium. This increase in serum calcium levels

has been attributed to a significant increase in osteoclastic bone resorption. Ham (1974) has suggested that parathyroid hormone (PTH) will induce the undifferentiated osteogenic cells present in the periosteum to become osteoclasts preferentially and, thereby, would account for the increased number present. Furthermore, the formation and function of bone forming cells is impaired, because parathyroid hormone will prevent the cells that are not directed into becoming osteoblasts from forming more new bone to compensate for that which is lost. Therefore, it seems that PTH affects all the cells of the bone cell series in one way or another, with the net effect being resorption.

Conversely, calcitonin, which is secreted in response to a rise in serum calcium, acts to lower these levels in a way that is more or less the reverse of the mechanism of PTH. It functions by diminishing resorptive processes and perhaps also by stimulating osteoblastic activity. Kalleo, Garant and Minkon (cited by Ham, 1974) have described an inactivation of osteoclasts in culture when calcitonin was present (e.g. disappearance of the ruffled border which is thought to be responsible for acid production). In addition, research by other investigators (Herring, 1964) has indicated that calcitonin actually increases the rate of new bone formation as well as decreasing the formation and activation of osteoclasts. Like PTH, calcitonin appears to affect all bone cells in one way or another, resulting in deposition (e.g. specific hormone receptors).

A basic understanding has been gained in the cause and effect of growth and remodeling, but when it comes to evaluating the determinants of osteoblastic/osteoclastic function and their subsequent activation/deactivation, knowledge is sparse. Which mechanisms control the biochemical machinery within the bone cell and thereby the direction and timing of bone metabolism is not yet understood. In the last 20 years there has been a flourishing of research in the area of local control mechanisms involved in growth and remodeling. With this research, many local factors have been alluded to as possible affectors and determinants of growth. Of these, the cyclic nucleotides, and most notably cyclic 3', 5'-adenosine monophosphate, have received a great deal of interest.

The intracellular concentration of these cyclic nucleotides has been shown to fluctuate concomitant with various cell processes (e.g. resorption). In addition, researchers (Chen, 1978; Davidovitch, 1980) have shown that various external stimuli known to produce a specific cellular change (e.g. PTH, calcitonin) also affect levels of these cyclic nucleotides.

Cyclic Nucleotides and Skeletal Remodeling

The skeleton is a complex mineralized connective tissue organ system that provides many functions, including maintenance of both mineral and acid-base homeostasis. Its growth and remodeling are governed by highly specialized cells (e.g. osteoclasts, osteoblasts and osteocytes), all of which are regulated by hormonal as well as non-hormonal factors. Although controversial, it is

currently held that these cells represent mitotically inactive derivatives of separate cell lines (Peck, et al., 1979).

Osteoblasts become osteocytes as they engulf themselves with newly formed bone. These osteocytes, however, will remain in communication with their neighbors and with surface osteoblasts by way of cytoplasmic processes that traverse bony cannaliculi. Thus, these vast interconnections provide a network having enormous surface area that is thought to act in the rapid transfer of ions and as buffer between bone and the extracellular fluids. In addition to this mediation of ion transfer, osteocytes are also capable of both bone deposition and resorption (e.g. osteocytic osteolysis) in response to external regulatory factors.

Hormones, locally acting chemical mediators, and mechanical forces, have all been shown to affect the various biochemical activities as well as the number of these specialized cells and, thereby, resorption or deposition (Peck et al., 1973).

Direct stimulators of bone resorption are known to include parathyroid hormone, prostaglandins, the biologically active metabolites of Vitamin D, and osteoclast activating factor, whereas calcitonin, insulin and growth hormone (e.g. somatomedin mediated) have been demonstrated to stimulate osteoblastic activity (Peck and Klahr, 1979). Coinciding with these events, changes in the levels of the cyclic nucleotides have also been noted. Evidence accumulated thus far has provided a strong indication that the cyclic nucleotides act as mediators (e.g. second messengers) in

the external stimulation of biochemical processes, and therefore, control bone remodeling.

Most of the evidence points to cAMP as the second messenger in bone resorption. Although evidence is sometimes conflicting, most stimulators of bone resorption prove to also increase cAMP levels. Time-course studies reveal that the rise in cAMP precedes the resorptive response in a cause-effect relationship (Peck and Klahr, 1979). These effects, however, must be examined in relation to cell type. Bone is a heterogeneous tissue consisting of both osteoclasts and osteoblasts, each with a different function. Stimulators of resorption such as parathyroid hormone have been shown to activate adenyl cyclase in both cell types (Jilka and Cohn, 1981). This concomitant rise in cAMP was then hypothesized to place the cells into a "resorption mode" by activation of the osteoclasts and inhibition of the osteoblasts. These effects cannot be solely isolated to cAMP, however, without possible consideration of the effects of Ca^{++} and cGMP along with other probable factors.

History of the Cyclic Nucleotides

With the flourishing of research in local control mechanisms, cyclic AMP, and to a lesser extent cyclic GMP, have been implicated as second messengers in a variety of important cellular mechanisms (Bliziotes et al., 1982; Draper et al., 1980).

Cyclic 3', 5'-adenosine monophosphate was first discovered by Sutherland and Rall (1960) in connection with their study of adrenergic induced glucose release in hepatic cells. This mole-

Figure 1.-- Schematic of cyclic nucleotide metabolism. Top- Adenosine Triphosphate is converted into cyclic 3'5' adenosine monophosphate by an adenyl cyclase enzyme. Subsquent degradation of this cAMP involves a phosphodiesterase -catalized hydrolysis of the cyclic phosphate ring yielding 5' AMP. Bottom- A similar metabolism as described for cyclic AMP has been demonstrated for cyclic 3'5'guanosine monophosphate

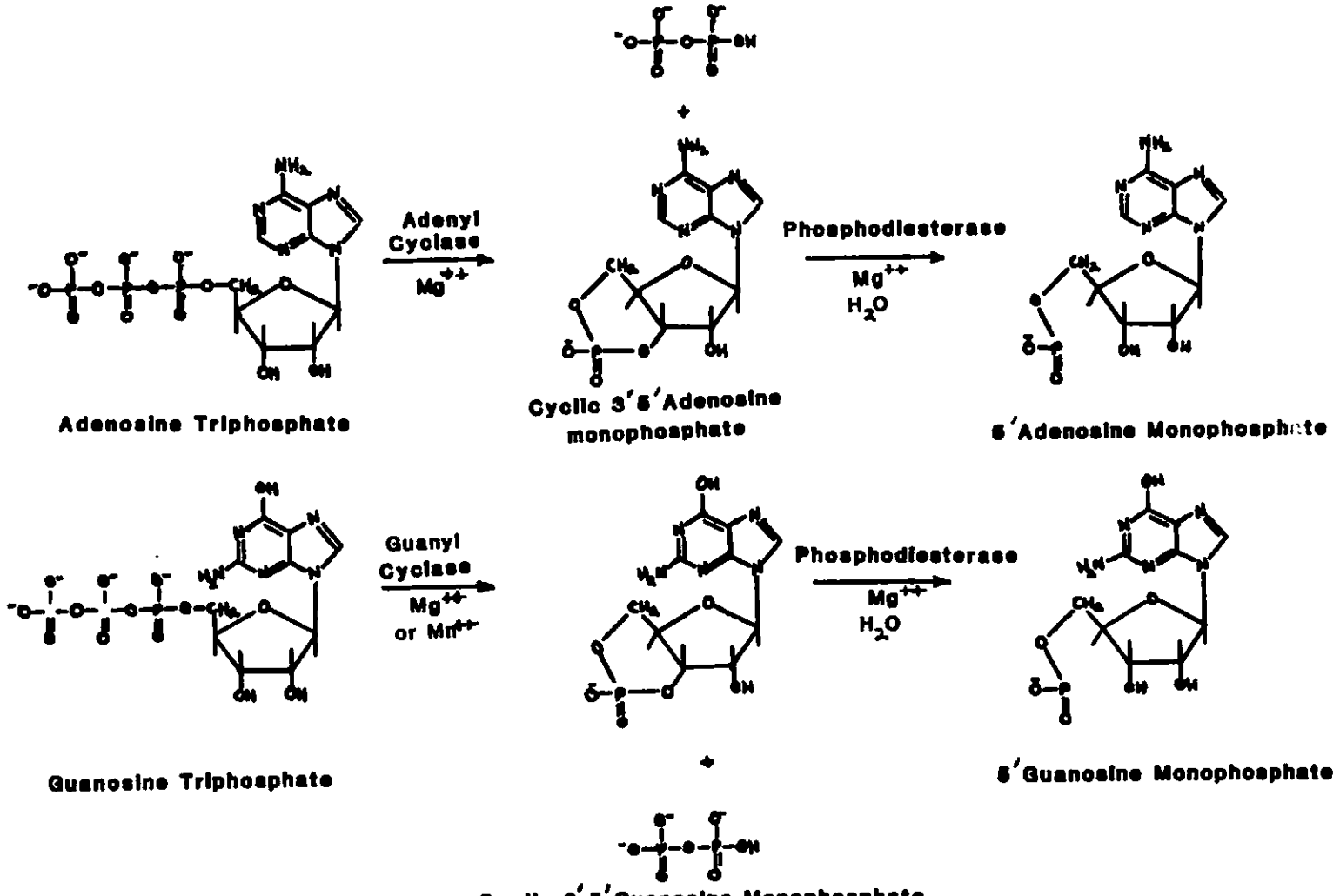
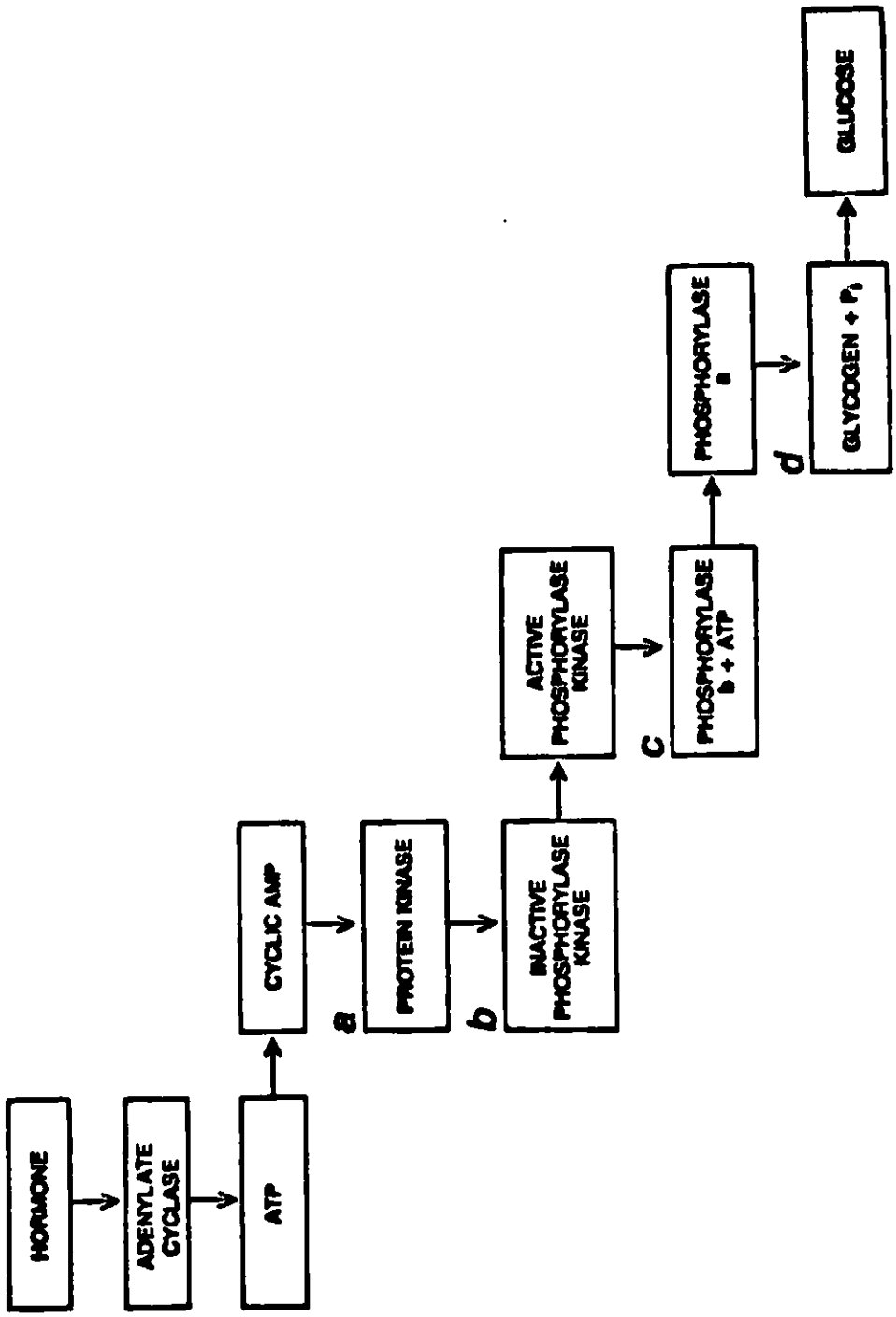

Adenyl Cyclase
Mg^{++}
Phosphodiesterase
Mg^{++}
H_2O
Adenosine Triphosphate
Cyclic 3'5'Adenosine monophosphate
5'Adenosine Monophosphate
Guanyl Cyclase
Mg^{++} or Mn^{++}
Phosphodiesterase
Mg^{++}
H_2O
Guanosine Triphosphate
5'Guanosine Monophosphate
Cyclic 3'5'Guanosine Monophosphate

Figure 1b.-- Example pathway of cyclic AMP action. This schematic represents the initial sequence in a cAMP mediated-hormone induced transformation of glycogen into glucose in muscle tissue. First (a) the cAMP that has been formed from ATP in the cell cytoplasm activates a protein kinase. The kinase in turn activates a second kinase (b) that is capable of transforming phosphorylase b into phosphorphorylase a. When this takes place (c), the transformed phosphorylase then starts the sequence (d) that converts glycogen into glucose (Pastan, 1972).


HORMONE
ADENYLATE CYCLASE
ATP
CYCLIC AMP
a
PROTEIN KINASE
b
INACTIVE PHOSPHORYLASE KINASE
ACTIVE PHOSPHORYLASE KINASE
c
PHOSPHORYLASE b + ATP
PHOSPHORYLASE a
d
GLYCOGEN + Pi
GLUCOSE

cule, although 1,000 times less abundant than ATP, has been found in virtually every cell, both normal and patholigical. The "second messenger" mechanism which was proposed by Sutherland, et al. (1960) involved an adenylate cyclase enzyme which was incorporated within the cell membrane (Figure 1b). This enzyme, normally low in activity, became activated by local perturbations in the membrane caused by external stimuli (e.g. first messengers). Once activated, this enzyme then converted intracellular ATP into cyclic AMP. The cAMP, thus formed, diffused through the cytoplasm and attached to the regulatory subunit of a protein kinase enzyme. Once bound to this enzyme, it would cause dissociation of the regulatory subunit and subsequent activation of the remaining catalytic portion of the kinase enzyme. This protein kinase enzyme would then further activate a second phosphorylase kinase enzyme which is thought to have a broad spectrum (Miyamoto, et al., 1969) and catalyze the phosphorylation of a variety of molecules involved in cellular metabolism, thereby creating a cascade effect in response to the first messenger (e.g. hormone, mechanical stress, etc.) (Lehninger, 1975).

A similar mechanism of action has been worked out for cyclic GMP. The natural occurrence of cGMP was first recognized by Ashman and co-workers in 1963 from rat urine (cited by Goldberg, et al., 1973). A clue to its biological role, however, did not become evident until almost 7 years later when it was noted that acetycholine-induced cardiac depression could be associated with a rapid tissue cGMP accumulation. This cholinergic action on the

heart was opposite to that promoted by epinephrine, which coincidently elevated myocardial cAMP concentrations. At present, cyclic AMP and cyclic GMP appear to be the only naturally occurring 3', 5'-cyclic nucleotides (Goldberg, et al., 1973). Like cAMP, cGMP has been found in virtually every tissue examined, although the concentration varies over a wide range. It has generally been shown that tissue levels range between 10^{-8} and 10^{-7} moles/kg and are usually found at 1/10 to 1/100 of the levels of cAMP (Goldberg et al., 1973).

The mechanism of action by which cyclic GMP initiates its biological effects is very similar to that proposed for cyclic AMP. It is synthesized by both membrane-bound and cytoplasmic (e.g. soluble fraction) guanylate cyclase, and once formed will activate a cGMP-dependent protein kinase (separate regulatory/catalytic subunits have not, however, been demonstrated in the cGMP specific protein kinase). Levels of these protein kinases vary widely with the tissue and cross over has been noted at high levels of either cyclic nucleotide (Goldberg, et al., 1973).

Although support for Goldberg's (1973) "dualism" hypothesis in which cAMP and cGMP were associated with promoting antagonistic biological events has been discredited by recent investigations, evidence still indicates that cGMP and cAMP are not induced by the same affectors, and that steady state levels of the two cyclic nucleotides are probably regulated by different biological factors. However, an apparent discrepancy may appear at high cGMP concen-

trations, mild cAMP-like effects have been noted in several tissues (Braun, et al., 1969). A possible explanation for this may be found in the probable feedback inhibition that normally maintains the cyclic nucleotides within certain physiological limits.

Since cAMP and cGMP are such powerful regulators of cell function, the cell must be able to control their concentration levels precisely. This can be done either by regulating synthesis or nucleotide elimination. Elimination can take two forms. Some of the cyclic nucleotides are pumped out of the cell (Pastan, 1972) where they no longer exert an effect. A majority, however, are degraded by one or more phosphodiesterase enzymes. The activity of these enzymes seems to fluctuate with the level of their respective cyclic nucleotides, thus assuring a rapid return of the second messenger to basal levels upon dissociation of the first messenger from its membrane receptor (Rodan, et al., 1975).

In addition to the metabolic effects within the cell, the levels of cGMP and cAMP have been observed to affect the expression of genetic information, although by which method is still unclear (Pastan, 1972). It has been shown in bacterial studies and in certain disease states, that in non-replicating cells, which are actively secreting cellular products (e.g. enzymes), the ratio of cAMP to cGMP is high. Conversely in rapidly proliferating cells, as seen in psoreasis, which is an epidermal disorder involving greatly expanded cell division, there is a low cAMP to cGMP ratio (Lehninger, 1975).

Pastan (1972) has provided evidence that cells in cultures will not undergo virus-induced malignant transformation when grown in the presence of cyclic AMP. Likewise, cells which have previously undergone transformation will tend to return toward normal in many aspects when placed in the presence of cAMP (Pastan, 1972). Evidence has also indicated that cyclic GMP may have some effect in stimulation of DNA synthesis.

Mechanical Effects on Cyclic Nucleotides

Throughout history, orthodontic treatment has been based on the principle that teeth will move and bones will remodel as a result of applied forces. These forces act by creating resorptive/depository changes within the bone. Due to the proven effect of bone seeking hormones, such as parathyroid hormone, in affecting similar bone remodeling through probable cAMP/cGMP mediated pathways, these cyclic nucleotides have also received a great deal of attention as possible mediators of the mechanical effect on remodeling.

Davidovitch, et al. (1975) demonstrated that cyclic nucleotide levels do, in fact, change as a result of mechanical stresses. They found by examination of the alveolar bone surrounding a tooth that had previously undergone orthodontic movement, that cAMP levels initially decreased, then increased 3-4 fold at 1-2 days. It was felt that the initial drop was probably due to pressure-induced necrosis, but that the following increase was fairly indicative of a cAMP mediated second messenger function.

Similar results were found by Roden, et al. (1975), in which a compressive force of 60 gm/cc^2, applied to the epiphysis of chick embryo tibiae, resulted in a decrease in both cAMP and cGMP, with the drop in cGMP being larger than that in cAMP. This effect was more pronounced in the absence of theophylline, a potent phosphodiesterase inhibitor, indicating that phosphodiesterase enzymes were responsible for the effect. It has been shown that Ca^{++} will fluctuate along with the cyclic nucleotide levels, thus implicating a possible Ca^{++} dependent phosphodiesterase (Peck, et al., 1981). Further research by Roden (1975), using the tibiae epiphysis, implied a possible cell specific cAMP/cGMP mechanism in bone cells under mechanical stimuli. To study this effect, Roden separated the epiphysis into a distal segment rich in proliferating cells, a middle section of enlarging, non-dividing cells and a proximal segment containing bone forming cells. This separation unmasked differences among the cells obtained from the three tissue segments. When hydrostatically stressed in culture, the cells from the distal segment demonstrated an increase in cGMP, whereas in the middle segment both cAMP and cGMP were elevated and in cells from the proximal segment the cGMP concentration dropped.

It has been shown rather conclusively, by these and other investigators (Somjen, et al., 1980) that mechanical stress is converted to biochemical effects via a cyclic nucleotide mediated pathway. How this biochemical effect is actually initiated is controversial. However, the presence of a membrane receptor,

sensitive to mechanical deformation, is doubtful. An interesting hypothesis has been worked out by Harrell, et al. (1977). According to this hypothesis, the cyclic nucleotide mechanism is mediated through denovo prostaglandin synthesis. Mechanical tension acting upon the cell is hypothesized to cause a distortion in the membrane. These distortions will lend to conformational changes exposing phospholipids to the action of phospholipase. Free arachadonic acid would then be made available for prostaglandin synthetase. The prostaglandins formed would then elicit increases in cAMP by inhibition of the phosphodiesterase enzyme. Prostaglandin would also cause changes in Ca^{++} membrane permeability. As the cAMP levels rise, a concomitant rise in Ca^{++} would inhibit adenyl cyclase and activate the Ca^{++} dependent phosphodiesterase enzyme (Harrell, et al., 1977). These two combined would result in a drop in cAMP levels. This hypothesis is supported by the fact that prostaglandin (PGE_2) elicits the same response. In addition, indomethacin, a potent inhibitor of prostaglandin synthesis, has been shown to inhibit the cAMP response of mechanical stimuli (Harrell, et al., 1977).

Somjen, et al. (1980), in a similar study, also found physical force to be prostaglandin-influenced, with cyclic nucleotides secondary. In addition, Somjen found that thymidine incorporation into DNA was significantly increased in response to physical forces but that this effect was likewise inhibited by indomethacin. Therefore, it is possible that prostaglandins mediate some effect

of mechanical stress upon DNA synthesis.

Other external stimuli beside mechanical stress have also been shown to affect increases in cyclic nucleotides and thereby bone remodeling. One of the more notable, electric current, has received a considerable amount of investigation. Davidovitch, et al. (1980a, 1980b) has shown that 15 microamperes of d.c. current, when applied to alveolar bone of a cat, can effect increases in both cAMP and cGMP in the surrounding bone, along with concomitant bone apposition near the cathode, and bone resorption near the anode. In addition, Davidovitch found that the levels of cAMP and cGMP fluctuated, and not always in concert with one another. A possible mechanism for the observed effect has been suggested to involve an electrically induced alteration of membrane potential allowing for the influx of ions, including Ca^{++}.

Other studies by Davidovitch (1980a, b) where electric current has been combined with mechanical stress have indicated an additive effect between the two stimuli. According to Davidovitch, this observed effect may result from either a more intense reaction within the cells, or more likely a wider activation of bone cells. Either way, Davidovitch provided evidence that bone remodeling occurs at a faster rate when the two stimuli are combined.

Along these same lines, Burrows, et al. (1978) showed a similar additive effect when mechanical stress in the form of orthodontic tooth movement was performed in conjunction with val-

ium administration, a drug which reportedly acts as a phosphodiesterase inhibitor. In this study, Burrows presented evidence that a significant increase in tooth movement occurred with a phosphodiesterase-inhibited cAMP increase. This added support for the premise that cAMP did, in fact, mediate at least some of the effects of mechanical stress on bone.

As with the hormonal effects, cAMP probably doesn't represent the entire picture. One must also include the less studied factors such as Ca^{++} and cGMP in order to grasp a fuller understanding (Peck, et al., 1981).

Hormonal Regulation of Skeletal cAMP Metabolism

Investigations involving both intact bones and isolated cell cultures have alluded to many effectors of increased cAMP levels, either through increased synthesis, decreased breakdown, or both. Some of the better studied affectors of synthesis include: parathyroid hormone (PTH), Prostaglandins (E and F series), calcitonin, epinephrine, osteoclast activating factor (OAF), calcium, mechanical and electrical stimulation. While at the same time, various phosphodiesterase inhibitors such as theophylline, and methylisobutylxanthine have been used to amplify the nucleotide changes by these various stimulators of skeletal cyclic AMP formation. In addition, thyroid hormones (L-Thyroxine and L-Triiodothyronine; Marcus, 1975) and glucocortocoids (Chen, and Feldman, 1978) have also been shown to inhibit skeletal phosphodiesterase activity.

Parathyroid Hormone

Parathyroid hormone, which is secreted by the parathyroid gland in response to plasma hypocalcemia, is generally accepted as a major physiological stimulator of bone resorption. This hormone represents one of the first cyclic AMP dependent mechanisms worked out in skeletal tissue. Evidence accumulated thus far indicates that parathyroid hormone, at least at high concentrations, is capable of increasing cyclic AMP production in osteoblasts, osteoclasts and osteocytes (Smith and Johnson, 1975; Peck, et al., 1978). Its direct effects, however, are to enhance osteoclastic activity, osteocytic osteolysis and to inhibit bone formation (Jilka and Cohn, 1981). These effects may become modified by such factors as nucleotide concentration, duration or on local environmental pH.

Jaffe (1933), presented evidence that chronic exposure of animals, particularly the young, to parathyroid hormone can be associated with activation of osteoblasts and increased bone formation. Furthermore, Herrmann-Erlee, et al. (1976) have reported an increased osteoid formation in cultured embryonic bones treated with low concentrations of parathyroid hormone. Likewise, low doses of synthetic human parathyroid hormone (hPTH 1-34) have also been shown to accelerate bone formation in patients with postmenopausal osteoporosis (Peck, and Klahr, 1979).

Martin, et al. (1980) have shown a significant increase in cAMP production with an increase in surrounding hydrogen ion concentration (e.g. low pH) as is seen in metabolic acidosis. Pre-

sumably the increased H+ ions are in some way altering the normal cellular ion balance, thereby potentiating the effects of the parathyroid hormone.

Generally, in isolated bone cell systems, which respond more rapidly to parathyroid hormones than intact bone, PTH will cause cyclic AMP to rise within several minutes, peak to as much as 30-40 fold with a phosphodiesterase inhibitor within 30 minutes, and then gradually decline (Peck and Klahr, 1979). Structure-function studies have revealed close similarities between the capacity of parathyroid hormone to augment cyclic AMP formation and the capacity to elicit biochemical responses.

At first, it was thought that a rise in cyclic AMP coincided directly with an increase in bone resorption. This hypothesis appears valid for the most part, but there is evidence that increased cAMP levels will elicit entirely different and sometimes opposing effects in different cell types. Bone is a heterogeneous tissue made up of several types of cells. Studies on isolated bone cell populations (Wong and Cohn, 1975; Luber, et al., 1976; Jilka and Cohn, 1981), enriched with either osteoblast-like cells or osteoclast-like cells, exposed to PTH, showed cell type specific biochemical responses concomitant with cyclic AMP rise. Evidence indicates that PTH increased cAMP levels in both populations, thus moving them into a "resorptive mode" (e.g. activation of osteoclasts and inhibition of osteoblasts). The net overall function was the same, but resulted through opposing cyclic AMP mechanisms.

Draper, et al. (1980) showed evidence of a differential responsiveness to parathyroid hormone as bone ages. Although cyclic AMP levels remain fairly constant, the bone responsiveness appears to decrease with age.

Prostaglandins

Prostaglandins, both of the E and F series, have been found to stimulate osteoclastic bone resorption directly by a calcitonin inhibited process, and at high concentrations will suppress bone formation (Peck and Klahr, 1979). In addition, prostaglandins (particularly E_2) are potent stimulators of skeletal cyclic AMP (Marcus and Orner, 1977). Aspirin, an inhibitor of prostaglandin synthesis, was found in one in vitro study (Powles, et al., 1972) to block parathyroid hormone-induced bone resorption, thus creating a possibility that prostaglandins mediate the effects of PTH. This assumption proved false, however, when indomethacin, another potent inhibitor of protaglandin synthesis, failed to blunt the cyclic effect of parathyroid hormone at a physiological concentration (e.g. inhibition was seen at high concentrations) (Marcus, et al., 1976). Although disproven in the case of parathyroid hormone, prostaglandins are still thought to have a mediating function in the effects of other cyclic AMP stimulators such as the osteoclastic activating factor and mechanical stress (Luben et al., 1979).

Marcus, et al. (1976) have shown that the effects of prostaglandins and parathyroid hormone are additive and that they

probably induce their cellular effect through different receptors. Preliminary evidence also suggests that prostaglandins, unlike parathyroid hormone, increase cAMP preferentially in the osteoclast-like cells rather than the osteoblast-like cells, an observation which coincides with the fact that prostaglandins are only mild inhibitors of bone formation (Peck and Klahr, 1979).

Calcium

Calcium is known to have many vital functions in a variety of tissues (Peck and Klahr, 1979). The extracellular concentration of Ca^{++} has been shown to determine, at least in part, the magnitude of cAMP response to various stimulation of adenyl cyclase activity in bone. Peck, et al. (1981) provided evidence that cyclic AMP responses to both parathyroid hormone and prostaglandin E_2, but not the basal cAMP levels were augmented as extracellular Ca^{++} was increased. Peck also determined that verapamil, an inhibitor of calcium cellular penetration, blunted these responses. In fact, research has indicated that parathyroid hormone is inactive in the absence of extracellular calcium (Peck and Klahr, 1979).

A methoxy derivative of verapamil, D600, that blocks voltage sensitive entry of Ca^{++} into the cells has also been shown to inhibit the prostaglandin-effected stimulation of bone resorption, but not its ability to increase cAMP levels in the bone cells (Peck et al., 1981). Whether Ca^{++} mediates the effects of parathyroid hormone upon cAMP by direct stimulation of adenyl cyclase has never been shown. Increasing evidence, however, now points to

Ca^{++} as not only another possible second messenger in itself, but also as a modulator in cyclic nucleotide metabolism (Peck and Klahr, 1979).

Since the finding by Chase and Aurbach (cited by Marcus, et al., 1979), that parathyroid hormone resulted in an increased cellular accumulation of cAMP, there has been a general assumption that at least some of the effects of PTH on bone are mediated by cyclic AMP. Recent investigations have been somewhat contradictory, with other possible routes for mediation of parathyroid hormone action proposed: a more notable example being the calcium uptake by the bone cells (Peck and Klahr, 1979). This hypothesis of Ca^{++} as a modulator of bone resorption may be further strengthened when one looks at studies involving osteoblast activating factor. This lymphokine, which is liberated from lymphocytes, has the ability to stimulate osteoclast mediated bone resorption.

In a study by Dziak, et al. (1980), the osteoclastic activating factor was shown to increase calcium uptake and concomitant bone resorption in fetal rat cells without affecting cyclic AMP levels. Dziak proposed that the osteoclastic activating factor achieved its effect by altering membrane permeability to Ca^{++} and thus proposed that cAMP may not be the primary or even a necessary mediator of bone resorption.

A contradiction was found in a similar investigation by Luben, et al. (1979) with osteoclastic activating factor (OAF) in mouse cranial bones which indicated that OAF did, in fact, elevate

cAMP. Luben did suggest, however, that "cAMP accumulation and resorption could be disassociated under some conditions," but that generally osteoclastic activating factor exerts its effect by means of cyclic AMP mechanisms.

A calcium dependent regulator (calmodulin), which stimulates phosphodiesterase enzyme has been found in tissues other than bone by a number of investigators. In these investigations, a low molecular weight, acidic, calcium dependent regulator protein (CDR) was found which, when complexed with calcium, could activate a variety of enzymes (Wang, et al., 1973; Lin, et al., 1974). This CDR molecule was shown to be an activator of both cyclic AMP phosphodiesterase (Wong, et al., 1973) and also adenyl cyclase (Brostrom, et al., 1976).

The importance of Ca^{++} cannot be disputed, but the second messenger function of Ca^{++} has never conclusively been shown. Because of the evidence favoring cAMP as a second messenger, it seems most likely that the Ca^{++} interacts within the cAMP regulatory system in governing cell response under physiological conditions (Peck, et al., 1981).

Calcitonin

Calcitonin, which is a peptide elaborated by the thyroid parafollecular cells, was shown to be a direct inhibitor of osteoclast-mediated bone resorption. Investigations have demonstrated that this hormone will antagonize the stimulatory effects of parathyroid hormone, prostaglandins and vitamin D metabolites, while

not directly influencing bone formation (Aliapoulios, et al., 1966).

Calcitonin appears to increase cAMP levels preferentially in populations enriched with osteoclast-like cells. Several studies, however, indicate that osteoblasts are calcitonin resistant (Jilka and Cohn, 1981). Like parathyroid hormone, calcitonin has the ability to activate adenyl cyclase and thereby increase cyclic AMP. Peak responses are for the most part far lower than those produced by either PTH or prostaglandin (Peck and Klahr, 1978). A slight contradiction might be seen in the osteoclast cyclic AMP responses of parathyroid hormone and calcitonin. It has been suggested that this discrepancy may be due to a compartmentalization of the cAMP or a concentration effect (Peck and Klahr, 1978).

Vitamin D and Steroid Hormones

The physiologically active form of vitamin D, 1, 25 $(OH)_2D_3$, has been found to mimic many of the effects of parathyroid hormone. Raisz, et al. (1978) have shown that, like parathyroid hormone, vitamin D_3 will stimulate bone resorption directly and at the same time inhibit bone formation. However, this metabolite will differ in its reduced capacity to increase skeletal cAMP formation (Peck and Klahr, 1978). Moreover, the metabolites of vitamin D will not influence the effect of calcitonin on skeletal cyclic AMP levels.

Several reports have suggested that vitamin D may, in fact, augment the effects of parathyroid hormone (Peck, et al., 1981) upon producing cyclic AMP. Crowell, et al. (1981) showed that 1,

25 $(OH)_2D_3$ in hypocalcemic animals increased serum Ca^{++} levels and also augmented the parathyroid hormone response, while this metabolite had a negative effect on the PTH response in hypercalcemic animals. Crowell hypothesized that vitamin D_3 somehow was responsible for the generation of bone cells responsive to parathyroid hormone and that these cells were lacking in vitamin D deficient bones. Whereas, the reduced response in the hypercalcemic animals was due to a vitamin D desentization of cell membrane receptors to PTH. Another plausible explanation may involve Ca^{++} mediation of the parathyroid hormone response as previously described (Peck and Klahr, 1979).

Vitamin D_3 has also been shown to augment the effects of somatostatin (Silve, et al., 1981). This tetradecapeptide has been characterized as a growth hormone release-inhibiting factor in the hypothalamus. Silve has shown that somatostatin will induce a Ca^{++} dependent stimulation of cAMP and that interaction with vitamin D_3 will enhance this effect.

Estrogen, testosterone, progesterone and glucocorticoids have all been shown to inhibit parathyroid hormone mediated bone resorption in vitro (Peck and Klahr, 1979). Contradictory to this finding, Chen and Feldman (1978), presented evidence that the glucocorticoids acting through specific membrane receptors potentiated PTH mediated cAMP response. Chen proposed that this effect was due to glucocorticoid inhibition of cAMP phosphodiesterase. In another study by Chen (1978), he also presents evi-

dence that several of the glucocorticoids have a direct inhibitory effect on bone RNA and protein synthesis.

Cyclic GMP

Cyclic 3', 5'-guanosine monophosphate has been shown to serve as a second messenger in a variety of tissues, but interest in this area has been greatly overshadowed by cyclid AMP. Unfortunately, studies with cGMP in skeletal tissue are limited, and most of what is known about this cyclic nucleotide has come from studies of soft tissue. Generally speaking, affectors of cGMP produce cellular effects which are somewhat opposed to those of the cAMP affectors, although no strict cAMP/cGMP relationship has been demonstrated.

Goldberg, et al. (1973) have found that the administration of epinephrine or glucagon to rats had no effect upon hepatic cGMP, but greatly elevated cAMP levels, thereby strengthening the premise that the two cyclic nucleotides were probably regulated by different biological factors.

Acetycholine has long been known to affect cGMP levels. Studies by Goldberg, et al. (1973) have shown that acetycholine perfusion of rat hearts produced an elevation of myocardial cGMP coincident with cardiac depression, while at the same time a slight drop in cAMP was noted. The addition of atropine, an anticholinergic drug, was found to further potentiate both cGMP accumulation and cardiac depression. Similar results have also been

noted in studies with other tissues including neural tissue (Goldberg, et al., 1973).

The administration of oxytocin, serotonin and prostaglandin F_2 have been shown by Goldberg, et al. (1973) to promote cGMP accumulation in rat uterus with no subsequent cAMP change. In a similar study, Schultz, et al. (1972) showed that histamine also caused an increase in cGMP concentrations in intestinal smooth muscle concomitant with intestinal mobility. Compounds which enhance cGMP generation have been shown to inhibit intestinal mobility (Goldberg, et al., 1973).

Glucocorticoids have been shown to have both negative and positive inhibitory effects on cGMP levels in various tissues (Goldberg, et al., 1973). These apparent cell specific reactions to glucocorticoids have been postulated to be due to a direct effect of guanylate cyclase.

The process of cell division has not yet been proven to be under hormonal control, but a number of recent studies have implicated cGMP as partial affectors of cell division (Goldberg, et al., 1973). This stems from the fact that mitogenic agents such as concanavalin A, which are used to induce lymphocyte proliferation, will also increase cGMP levels 5 to 50 fold with little alteration of cAMP (Hadden, et al., 1972). It has been postulated from this evidence that cyclic GMP may represent a membrane-to-nucleus signal. Preliminary evidence has shown that cGMP at concentrations of 10^{-9}M can stimulate both RNA synthesis and histone acetylation

in isolated lymphocyte nucleus', two events which occur early in the process of cell division (Goldberg, et al., 1973).

In skeletal tissues, the strong antagonistic cAMP/cGMP relationship is not as evident. Research has shown that humoral agents and mechanical factors can influence skeletal cGMP. Roden, et al. (1976) showed acute decreases in cGMP content of chick embryo tibial epiphysis in response to a physiological compressive force. Roden suggested that increased phosphodiesterase activity may be responsible for this effect since cAMP levels also fell and theophylline blunted both of these effects.

In studies conducted on thyroparathyroidectomized rats, Goldberg (1973) found that infusion of calcitonin produced an immediate elevation in urinary excretion of cGMP. In similar experiments conducted with parathyroid hormone infusion, an increase in cGMP was likewise seen, but did not occur until 3-5 hours following infusion and seemed closely related to elevations in urinary calcium. From this evidence, it was suggested that calcitonin produced a direct stimulation of skeletal cGMP, whereas the delayed increase seen with PTH was thought to be due to a secondary action of Ca^{++}. This is consistent with observations that Ca^{++} stimulates cyclic GMP levels and is required for the maintenance of endogenous tissue cGMP levels, as well as the responsiveness of tissues to hormones mediated through this nucleotide (Peck and Klahr, 1979).

A contradiction to this was presented by Davidovitch, et al. (1977) in which parathyroid extract, when administered to

cats, caused an increase in total bound alveolar bone cyclic GMP. Through immunohistochemical techniques, Davidovitch was able to localize this cGMP increase to the osteoblasts, osteocytes and periodontal cells, but not the osteoclasts. In subsequent studies by Davidovitch (1980a, b, c), he showed similar results when cat alveolar bone was subjected to microcurrents and/or mechanical stress. Along with this Davidovitch also found fluctuations in cAMP/cGMP with time. Although the fluctuations were roughly reciprocal a direct antagonism was not evident.

Phosphodiesterase Inhibition

Phosphodiesterase, which degrades cyclic nucleotides, serves a vital function in the biochemical regulation of cell metabolism. This enzyme was first found to destroy cyclic AMP in the cell, and prior to the discovery that cyclic GMP was a naturally occuring compound, it was also noted by various investigators that cGMP could serve as substrate for this phosphodiesterase enzyme, but at only one-third the rate of reaction (Peck and Klahr, 1979).

Since the earlier investigations with the enzyme, evidence has accumulated indicating that different phosphodiesterases exist which are specific or more selective for either cAMP or cGMP. This was demonstrated in a study by Brooker, et al. (1968), which showed that cGMP did not affect the rate of cAMP hydrolysis. From this evidence, it was reasoned that if a single nucleotide were responsible for the hydrolysis of both nucleotides, cGMP would have

been expected to act as a competitive inhibitor of cAMP degradation. Likewise, Butcher and Sutherland (1962) found that imidazole, a compound known to produce activation of cAMP phosphodiesterase, competitively inhibited cGMP hydrolysis without altering the rate of cAMP degradation.

Similar studies by Goldberg, et al. (1973) revealed that cAMP could inhibit the hydrolysis of cGMP competitively. This then provided evidence for the existence of two species of enzyme, one highly specific for cAMP that did not degrade cGMP, and another more selective for cGMP but which could also degrade cAMP. Beavo, et al. (1970) found and partially purified phosphodiesterase from beef heart tissue that hydrolized both nucleotides, but degraded cGMP preferentially. Further characterization of the phosphodiesterase enzyme indicate that a high molecular weight protein (ca400,000) was active in degradation of both, but preferentially cGMP, while a low molecular weight (ca200,000) protein seem to degrade cAMP exclusively (Lin, et al., 1974).

Early investigation by Marcus (cited by Goldberg, et al., 1973) identified particular and soluble cAMP phosphodiesterases functioning in bone, each of which is kinetically separatable into high Km and low Km forms exhibiting pH, cation and temperature requirements that resemble those of phosphodiesterase from other tissues.

Another intriguing regulatory influence was uncovered by Kakiuchi and co-workers (cited by Goldberg, et al., 1973). They

found that a fraction of the phosphodiesterase activity from rat cerebral cortex, when supplemented with a non-dialzable, heat stable, phosphodiesterase activating factor (PAF) (e.g. calmodulin) could be stimulated by Ca^{++} in such a way that the enzyme becomes more specific for cGMP. A heat stable calcium dependent protein was likewise described in research by Levin and Weiss (1978). They separated the various phosphodiesterase enzymes, isolated from rat brain by acryl gel electrophoresis, and determined the presence of four isozyme peaks, each demonstrating different characteristics. Out of the four isozyme peaks, one isozyme was not significantly affected by any phosphodiesterase inhibitor tried, and only one could be activated to any significant degree by the calcium dependent regulator protein. Theophylline was shown to inhibit both inactive/active forms of this enzyme.

Many inhibitors of the phosphodiesterase enzyme have been found. Research into the affectors of this enzyme by Levin and Weiss (1979) suggest that these inhibitors can influence the isozymes of phosphodiesterase by several distinct mechanism, namely by: (a) competitive inhibition with the cyclic nucleotide at its catalytic site; (b) action on the substrate site non-competitively; (c) action at an allosteric site to affect enzyme-substrate affinity; or (d) action on endogenous cofactors of the enzyme (e.g. Ca^{++}).

Studies by Norman and Drummond (1979) have shown that neuroleptic drugs (e.g. major psychosedatives and anti-psycotics) which are proven inhibitors of phosphodiesterase activity will

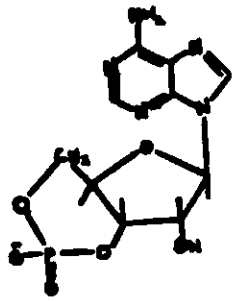
bind to the calcium dependent protein molecule affecting its ability to activate the phosphodiesterase enzyme. It has also been shown that this same calcium dependent protein may activate adenyl cyclase. Thus, it has been suggested that these drugs effect nucleotide levels both by decreasing synthesis and increasing degradation.

On the other hand, theophylline, which is a xanthine with a structure similar to the purines of the cyclic nucleotides, has been hypotehsized to affect its action by competitive inhibition at the catalylatic site (Levin and Weiss, 1979).

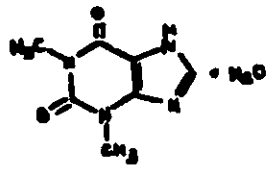
The administration of theophylline by Levin and Weiss (1978) to parathyroidectomized rats caused an elevation in serum calcium similar to that resulting from the administration of a moderate dose of parathyroid hormone. They further showed that this effect of theophylline was mediated by cAMP by virtue of the fact that isoproterenol, which also enhances cAMP snythesis, produced similar elevations. In another study, Wells and Lloyd (1968) showed that theophylline inhibited the fall in serum Ca^{++} elicited by the administration of thyrocalcitonin, but did not affect normal basal serum Ca^{++} levels in intact rats. From this it can be suggested that theophylline acts primarily on those isozymes that undergo activation/deactivation in accordance with cyclic nucleotide eliciting stimuli.

Evidence from a study by Jilka and Cohn (1981) conducted on the phosphodiesterase activity in isolated bone cell popula-

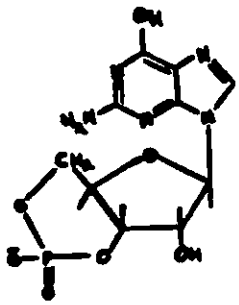
Figure 2.— Comparison of Molecular structures between the cyclic nucleotides and theophylline. This schematic demonstrates the similarities between the purine rings of cAMP/cGMP and theophylline and may account for the competitive inhibition seen with the phosphodiesterase enzyme.



CYCLIC 3'5' ADENOSINE MONOPHOSPHATE



THEOPHYLLINE



CYCLIC 3'5' GUANOSINE MONOPHOSPHATE

and cell type. Theophylline appears to affect those phosphodiesterase isozymes that are affected by stimuli directed at changing cyclic nucleotide levels and not at the isozymes which function to maintain the basal level of nucleotides.

Craniofacial Morphogeneses in the Rat

In a growth study on rats, Cleall, et al. (1968) examined the growth of various bones between birth and 66 days of age (e.g. early adulthood). From results using vital dyes, he found that most of the normal growth enlargement in the rat's skull occurred in the nasal and maxillary region (e.g. face) with only moderate growth occurring in the neurocranial region.

The frontal bone was found to increase in thickness by equal extents of bone deposition on both the inner and outer tables at a fairly low, uniform rate. As this occurred, the contained cancellous space expanded by resorption and remodeling (e.g. cortical drift). The frontoparietal suture was noted to grow at a more moderate rate as compared to the frontonasal suture.

In the parietal bones, increases likewise occurred on both the inner and outer tables, with the inner being slightly greater. An important observation indicated that while some incremental growth occurred along the full length of the bone on the outer table, the greater extent occurred at the lateral margins resulting in a flattening of the bone with continued growth. Likewise, the frontoparietal suture and the interparietal-parietal suture also grew at a relatively moderate rate.

tions enriched with either osteoclastic or osteoblastic cells, indicate that the phosphodiesterase activity in osteoclasts is twice that in osteoblasts, both inhibited by methylxanthine. This difference in activity may account for the higher cAMP levels found in parathyroid hormone stimulated osteoblasts.

In summary, it can be seen that multiple forms of phosphodiesterase enzyme exist, and that they vary with tissue and cell type. Theophylline appears to affect those phosphodiesterase isozymes that are affected by stimuli directed at changing cyclic nucleotide levels and not at the isozymes which function to maintain the basal level of nucleotides.

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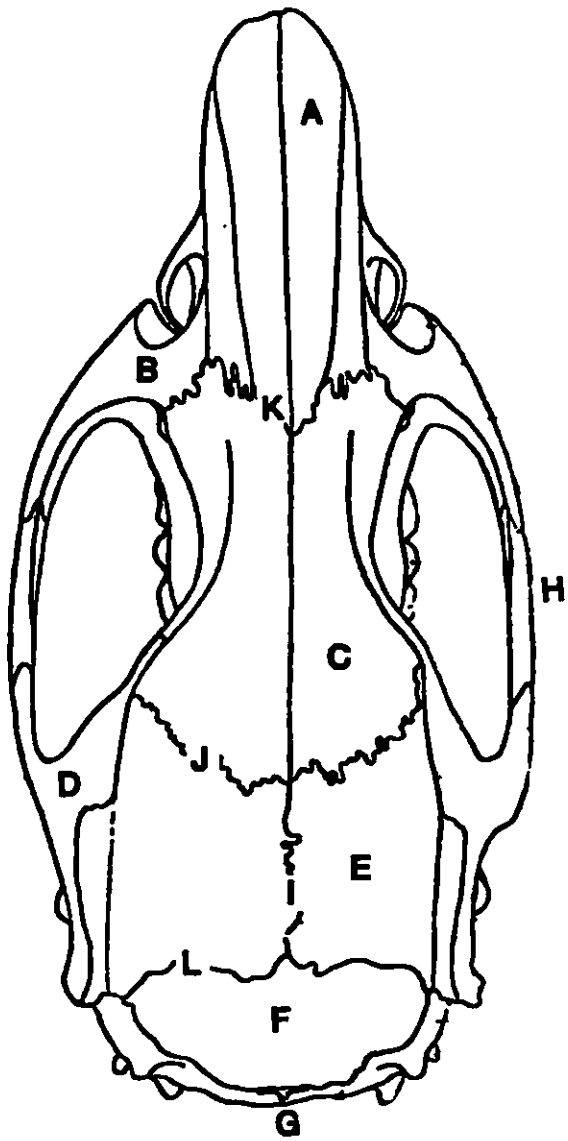
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Figure 3.-- Cranial Anatomy of the Rat: Dorsal view.

A - Nasal Bone
B - Maxillary Bone
C - Frontal Bone
D - Squamosal Bone
E - Parietal Bone
F - Interparietal Bone
G - Occipital Bone
H - Zygomatic Bone
I - Sagittal Suture
J - Frontoparietal Suture
K - Nasofrontal Suture
L - Interparietal Suture


A
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STATEMENT OF THESIS

Bone tissue investigations by Davidovitch (1980a, 1980b), Roden (1975), Burrows (1978) and others demonstrated that external stimuli such as electric current, mechanical force and various chemical mediators (e.g. hormones, prostaglandins, etc.) have a significant effect on intracellular cyclic nucleotide concentrations. Furthermore, Davidovitch and Burrows provided strong evidence indicating that the levels of cyclic nucleotides play an important role in alveolar bone remodeling in response to the application of orthodontic forces.

The effects of an orthopedic force (e.g. Headgear), when applied to the bones of the craniofacial complex upon cyclic nucleotide levels and subsequent sutural growth and remodeling changes, are still unclear.

The objective of the present investigation is to evaluate the effects that externally applied forces have upon the cyclic nucleotide levels of the involved bones, and how these levels relate to growth and remodeling changes that occur at the sutures. To study this effect an expansile force was applied across the sagittal suture in young growing rats and responding cyclic nucleotide levels measured. In addition, effects of orthopedic expansion upon cyclic nucleotide concentration were further potentiated by the use of theophylline, a potent phosphodiesterase

inhibitor, in an attempt to extend these growth and remodeling processes. Levels of cyclic AMP and cyclic GMP in both the experimental and control animals were quanitated by a radioimmune assay procedure specific for each particular cyclic nucleotide. Resultant growth and remodeling changes that occurred in the sutural areas were examined directly by implant measurement and evaluated histologically in attempts to relate the levels of cyclic nucleotides to a corresponding bone change.

METHODS AND MATERIALS

Animals

Twenty two female Sprague Dawley rats were utilized for this study. The animals were approximately 28 days old at the beginning of the experimental period. They were fed on a diet of Purina Rat Chow (Ralston Purina Co., St. Louis, MO) and tap water ad libitum. Initial weights at the beginning of the experimental period ranged from 63 to 93 g (Table I).

The rat was chosen as the experimental model in this research for several reasons. It was chosen not only because of size, cost and availability, but more importantly due to the fact that it undergoes a large amount of rapid early postnatal cranial growth and development. During the time between birth and approximately 66 days of age, the general body growth curve is relatively straight and steep indicating rapid growth (Cleall, 1968). The experimental period, thus, was selected to occur between the ages of 28 days and 49 days of life in order to optimize any experimentally induced growth changes.

The rats were received at approximately 21 days of age. They were then allowed to become acclimated to their new environment during one week in order to overcome any trauma inflicted during transport. At the end of this period, it was assumed that the animals were sufficiently recovered to physically with-

TABLE I

INITIAL WEIGHTS
OF ANIMALS (grams)

GROUP	ANIMAL	EXPERIMENT VARIABLE	INITIAL WEIGHT (gm)
CONTROL	1	CONTROL	72.0
	2		70.5
	3		73.0
	4		76.0
	5		81.0
T GROUP	1	THEOPHYLLINE	67.5
	2		68.0
	3		66.0
	4		75.0
	5		63.0
S GROUP	1	SPRING	69.5
	2		81.0
	3		74.0
	4		70.5
	5		79.0
	6		81.5
T + S GROUP	1	THEOPHYLLINE SPRING	63.0
	2		73.0
	3		70.0
	4		81.5
	5		76.0
	6		93.0

stand the necessary surgery.

Experimental Design

The animal sample was divided into four groups (Table I). The first two groups (e.g. control and T group) contained five animals each while the last two groups (e.g. S and T + S), due to increased risk, each contained six animals. At the time of surgery, three stainless steel implants were placed in a triangular pattern in the cranium of all animals. One implant was placed in each of the two parietal bones and one in the frontal bone. Interimplant distances were measured to the nearest 0.1 mm with a Bowley gauge both pre- and post-experimentally. This arrangement provided a method by which to measure lateral and anteroposterior expansion across both the sagital and the frontoparietal sutures. As the rat is a long snouted animal, it is logical to expect a considerable amount of growth to take place in the anteroposterior direction (Cleall, 1968). In addition to the implants, animals in the S and T + S groups also received a stainless steel expansion spring that was designed to deliver a known and constant amount of force across the sagittal suture. This force was expected to act as a lateral growth stimulant in both of these groups.

Following the surgery, one animal from each group was chosen randomly and injected intraperitonally with the vital dye Calcein Green. The purpose of the dye was to label the calcification front at day zero and allow measurement of the amount of new appositional growth that occurred in each group during the exper-

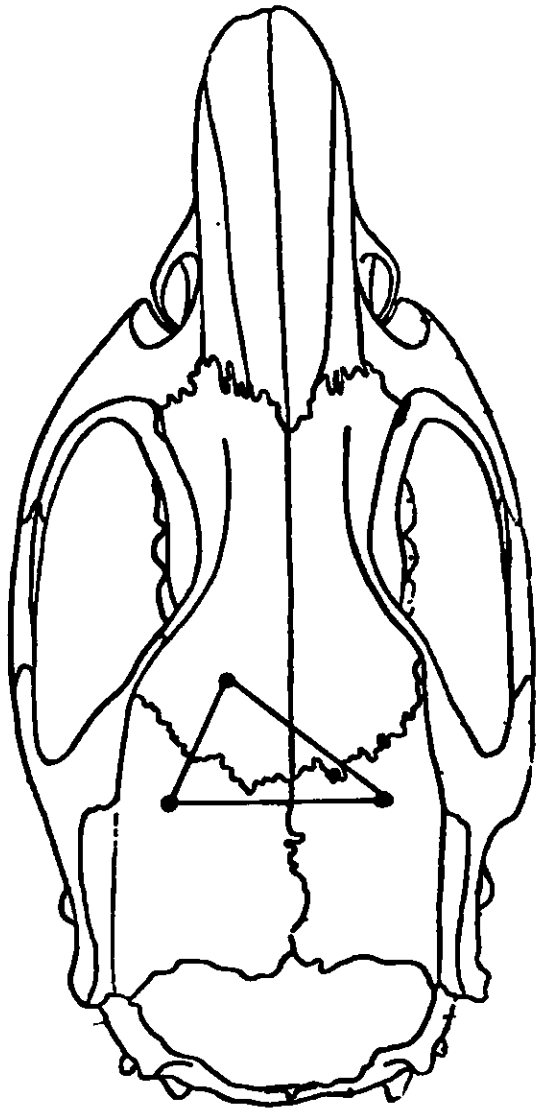
mental period.

The animals were allowed to recover completely in individual cages to avoid possible adverse interferences from other members of their respective groups. Once the animals had completely recovered from the effects of anesthesia, they were then placed together with their group. The animals for groups S and T + S, respectively, were chosen at random from all those animals which had received the expansion spring. This was done in order to overcome any possible discrepancies between the surgery performed on the first animal and the surgery performed on the last as surgical technique improved with experience.

Following surgery, a period of two days was allowed to provide the animals time to overcome the insults of surgery, vital dyes or any post-operative infection which might occur. After this time, beginning on Day 3 of the experimental period, the animals in groups T and T + S were started on a daily drug regiment. This involved "force feeding" a 1 mg/100 g body weight dose of Theophylline liquid (Theolair^{R}, Riker Laboratories, Inc., Northridge, CA) to the animals four times a day. This regiment was repeated daily up until the experimental period ended.

All animals were weighed approximately every three days during the experimental period. The reason for this was twofold. First, it allowed a method to measure physical development of the various animals, and secondly, it allowed for modifications in the drug dosage as the animals grew.

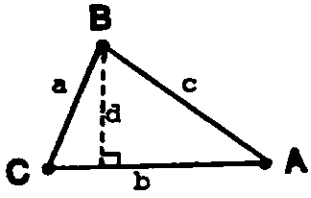
Figure 4.-- Schematic of the implant triangle. Cranial implant markers placed in the frontal bone and in each of the parietal bones were used to estimate calvaria growth change over the experimental period. The anteroposterior dimension and total area were determined trigonometrically using the indicated formulas.



$\alpha = \angle BAC$

$\beta = \angle ABC$

$\Gamma = \angle BCA$



a = measure $\overline{BC}$

b = measure $\overline{AC}$

c = measure $\overline{AB}$

LAW OF COSINE = $\cos\alpha = \frac{b^2 + c^2 - a^2}{2bc}$

ALTITUDE(d) = $c \sin\alpha$

Area = $\frac{1}{2}bc \sin\alpha$

At the end of the 21 day experimental period, all animals were sacrificed. The parietal bones and a portion of the frontal bone was removed from each of the 22 animals. The bone samples from the animal in each group that had received the vital dye following the initial surgery, were separated out and placed in 70% alcohol. These samples were then utilized in the histological evaluation. The bone samples from the remaining 17 animals were frozen in liquid nitrogen, pulverized and evaluated for cAMP, cGMP and protein content by the appropriate methods.

Appliance Construction

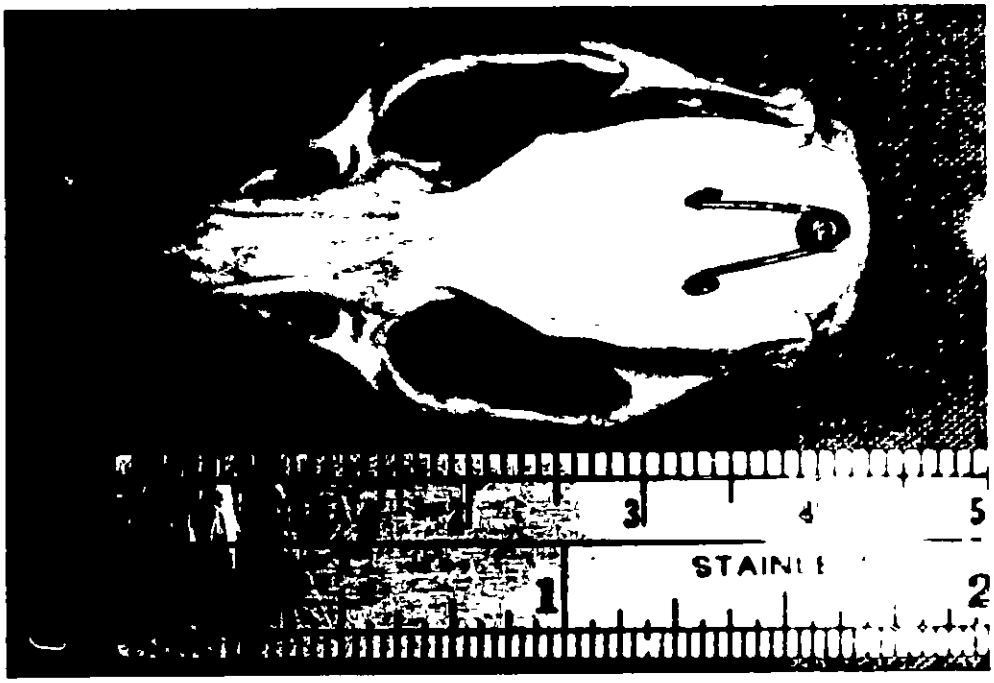
The expansion springs were a modification of the springs described by G. Hinrichsen and E. Storey (1968). The basic appliance was fabricated from 0.014 round 18:8 Austenitic stainless steel wire (Unitek Corporation, Monrovia, CA). It consisted of two arms, each 7 mm in length, separated by a helical loop (Plate I, II). This helix was constructed of four loops of wire formed around the 0.050 barrel of a Tweed loop forming plier (Unitek Corporation).

In order to prevent the arms of the spring from slipping into the bone, a helix was placed at the end of each arm. In addition, these helices also provided a convenient handle by which to manipulate the spring into place during surgery. Following their fabrication, the springs were heat treated in a Rocky Mountain Welder to relieve any internal stress created during construc-

Plate I.
Lateral View of Expansion Spring in Place on Dry Skull.

Plate II.
Dorsal View of Expansion Spring in Place on Dry Skull.




3
4
5
STAINL
1
2

tion, and thereby assuring tolerance stability.

All springs were designed to deliver the same force over the same distance. Spring dimensions were kept as constant as possible and the tension was calibrated with a precision force gauge (Haldex, Halmstad, Sweden). The appliance was designed and adjusted to deliver 60 gms of force at an arm separation of 5 mm. This was adjusted at the time of spring construction and checked immediately prior to surgical insertion.

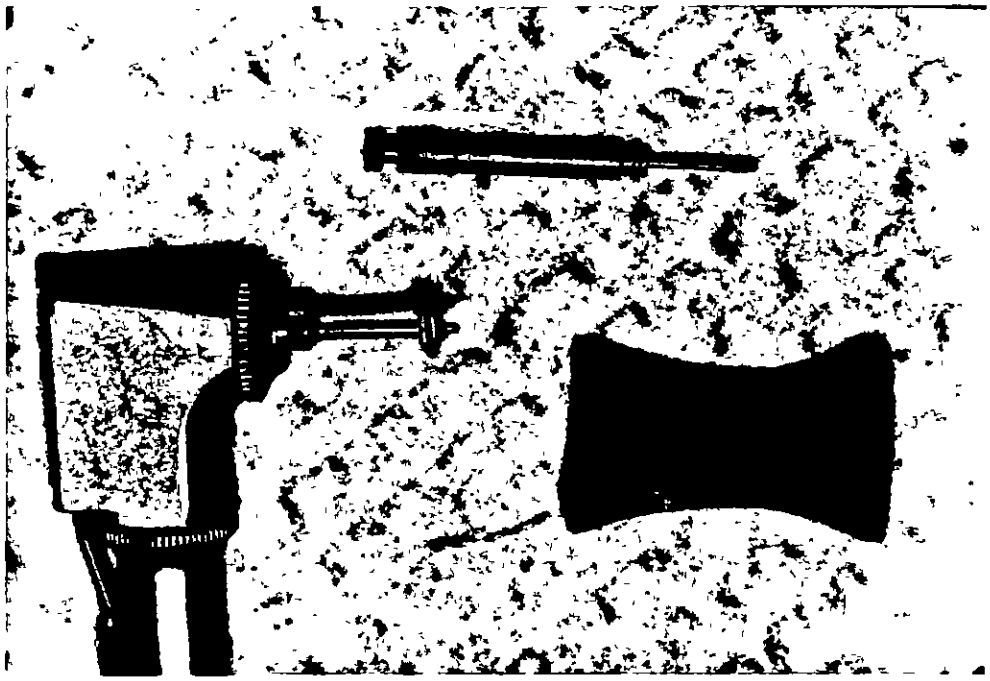
To assure congruent placement of the appliance in each animal, an acrylic template (Orthoacryl,^{Tm} Stratford-Cookson Co., College Park, GA) was constructed (Plate III) with two holes, exactly 5 mm apart, and with the location of the sagittal suture marked. The distance of 5 mm was used to avoid penetration into the midsagittal sinus. During surgery, this template was lined up with the suture and the holes drilled with the assurance that each hole would be 2.5 mm from the suture and exactly 5 mm apart. Retention of the appliance was obtained by bending the end of each arm below the helix out at about a 60^{o} angle. Migration of the spring through the bone does not occur to any significant degree (Hinrichsen and Storey, 1968), with the only macroscopic change being a slight thickening of the bone and connective tissue at the site of entry of the spring arms into the bone.

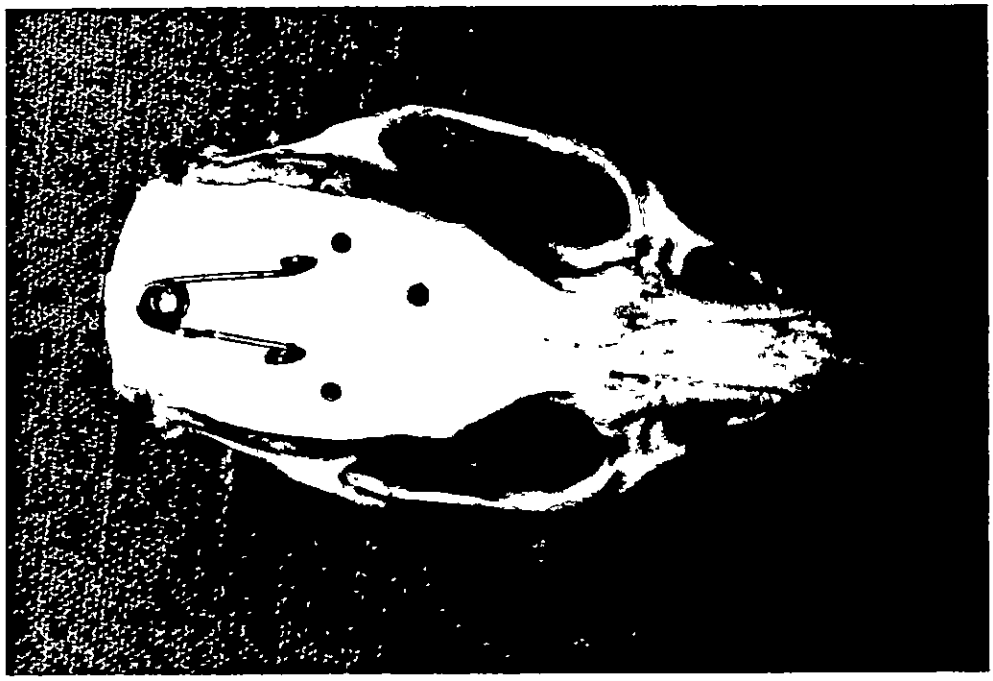
Anesthesia

Anesthesia prior to surgery was induced by intramuscular

Plate III.
View of the TMS Drill, Implant Marker and Acrylic Template used in placing the implants.

plate IV.
Dorsal View Showing the position of the Implant markers on the Dry Skull.





injection of 0.2 ml of a solution containing 10% Acepromazine Maleate[1] (Ayerst Laboratories Inc., New York, NY) and 90% ketamine hydrochloride (Ketaset[R], Bristol Labs, Syracuse, NY). The acepromazine was used in combination with ketaset to tranquilize the animal. During surgery, local infiltration injections of 2% xylocaine hydrochloride with Epinephrine 1:100,000 (Astra Pharmaceutical Products, Inc., Worcester, MA) were given adjacent to the area of incision.

Surgical Procedure

Following the induction of anesthesia, the animals were weighed and the weight recorded. The area on top of the head, from the middle of the neck to right in front of the eyes, was shaved. Copious amounts of Povdone-iodine (Betadine[R], The Purdue Frederick Co., Yonker, NY) was then applied to the area of the incision and blotted dry with a sterile 2 x 2 gauze. The incision was made with a pair of sharp surgical scissors, in the midline, from just behind the ears to a line between the eyes. The tissue was then dissected back from the midline with a scapula and held with a hemostat on each side of the incision. The periostrum was scraped away in the areas in which the holes were to be

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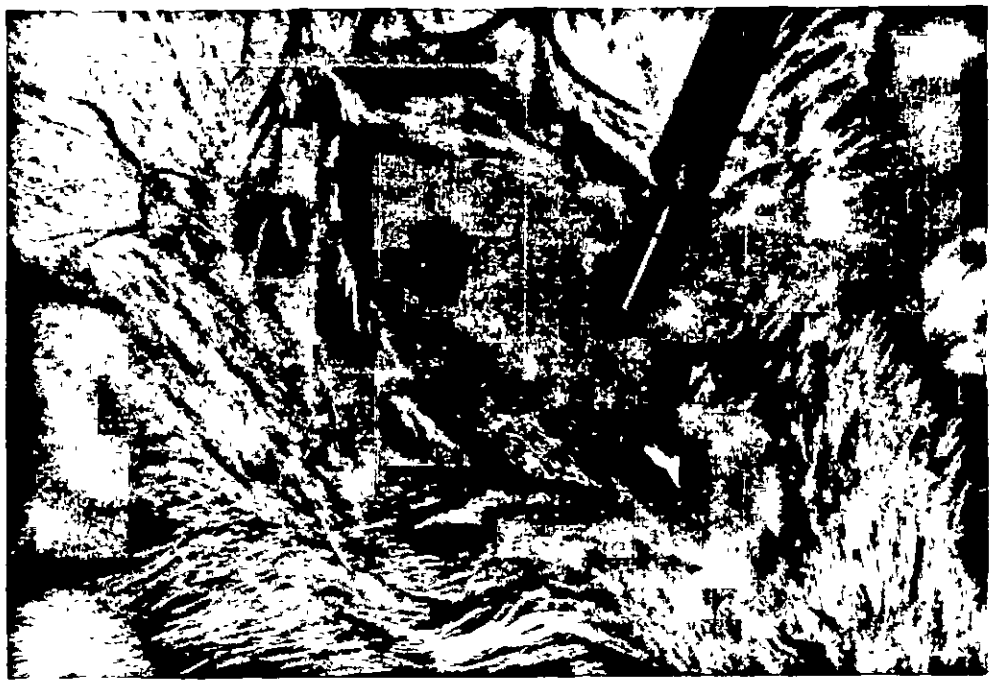
[1]Phenothiazines have been shown to inhibit the activation of phosphodiesterase in nerve tissue by Levin and Weiss (1976). Possible interference in the final results, however, are highly unlikely due to its single low does administration at the beginning of the experimental period.

drilled to prevent fouling of the drill. Once the area was clear of periostreum, holes were drilled into the cranium with a 0.020 in. TMS drill (Threadmate®, Henry Schein, Inc., Port Washington, NY), in a slow speed dental handpiece. Penetration was restricted to 1 mm by a plastic shim.

In those animals which were to receive the expansion spring, the acrylic template was placed on the skull with the holes in the middle of the parietal bones and the template lined up with the suture. The exact site for the spring holes was marked with a 1/4 round bar revolving at slow speed in the dental handpiece. The template was removed and the spring holes made with the TMS drill. In all animals, three holes were drilled for the placement of the implant markers. One hole was drilled in each of the two parietal bones and one in the frontal bone. In those animals which were to receive the spring, the implant holes in the parietal bone were placed anterolateral to the spring hole to avoid possible interference with the spring. Once made, distance measurements between the implant holes were taken to the nearest 0.1 mm with a Bowley gauge (Beerendonk Speitz, OIS Orthodontic, Wilmington, DE) and recorded. Implants, which consisted of 0.021 in. TMS pins (Minim Link®, Henry Schein Inc., Port Washington, NY) were then screwed into each of the three holes by hand and the excess cut off with a pair of heavy ligature cutting pliers. The reason for using TMS pins over the typical titanium implants was twofold. First, because they could be screwed in, they provided

Plate V.
Surgical View of Implant Placement Prior to Cutting Off Excess.

Plate VI.
Surgical View of Expansion Spring in Place on the Skull.





excellent retention in very thin bone, and secondly due to the fragility of the rat skull at this early age, it lessened the chance of fracture. Once the pins had been placed, springs were inserted in the animals from the experimental groups S and T + S. The springs were placed with the helical portion located between the pinnae and the arms projecting anteriorly (Plate IV). This was done to lessen possible appliance interference by the animal. One arm was placed in the bone and the other arm was rotated and compressed with a pair of hemostats and inserted in its hole. Due to the natural retention created in the appliance, it then snapped firmly into place against the skull.

The area was lightly dusted with an antibiotic (Neosul II,[R] Carson Chemical Inc., New Castle, IN) to prevent post-operative infection and sutured closed with 4-0 silk suture (Ethicon Inc., Henry Schein, Port Washington, NY). Once the incision was completely closed, the area was swabbed with Betadine again to reduce the possibility of infection. The animal was permitted to recover completely in an individual cage prior to placement back with its group mates.

Vital Dye

In order to make a histological evaluation of the experimental effects upon bone development, one animal was chosen randomly from each group. This animal received a 0.2 mg intraperitoneal injection of the vital dye Calcein Green (Decalf,[R] Amino methyl

Fluorsein; ICN Pharmaceuticals, Inc., Life Science Group, Cleveland, OH) immediately following surgery. This animal was then treated in the same manner as the rest of the members in its group until sacrifice.

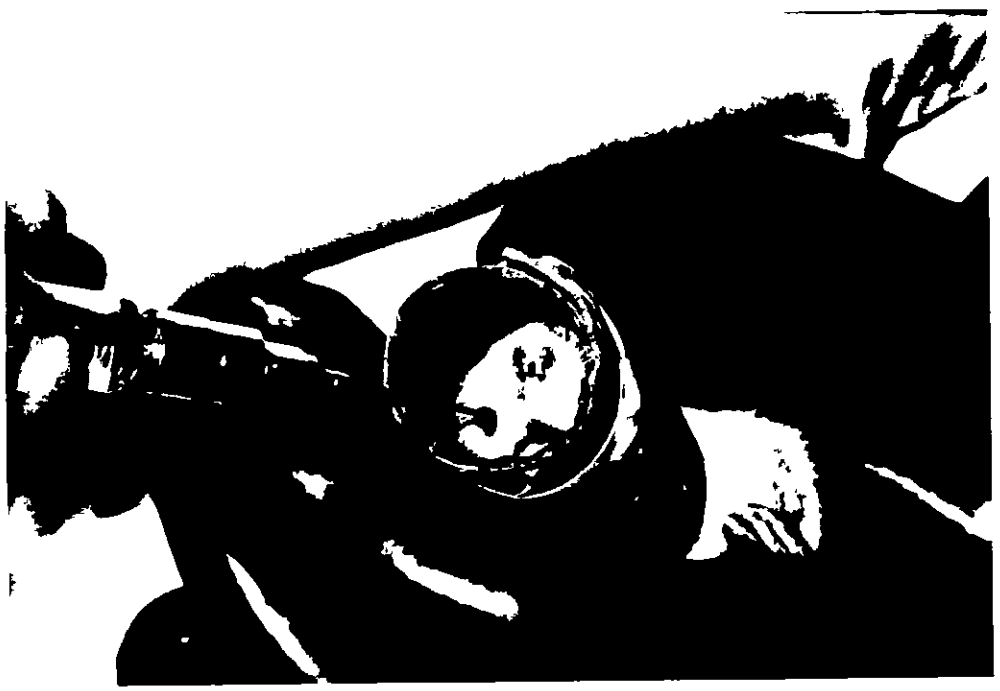
Drug Administration

Theophylline was the drug of choice for this experiment, due to its known potent inhibition of the phosphodiesterase enzymes responsible for the breakdown of the cyclic nucleotides, particularly cAMP and cGMP. It was administered to the animals in liquid form because of ease of handling and also due to the fact that the drug itself, apart from the sweetened liquid, is extremely bitter tasting making force feeding more difficult.

The drug was fed to the animals utilizing a 5 ml glass luer-lock syringe (Interchangable, Union Broach Co., Inc.) with a modified 21 gauge blunt needle bent at a 45^{o} angle.

The animal's head was stabilized during feeding in a cardboard tube harness (Plate VII). Perforation of the animal's esophagus was avoided by the application of a small polished bead of solder at the end of the needle. The theophylline liquid, which comes in a stock concentration of 80 mg/15 ml was diluted to the appropriate concentration with distilled water. This concentration varied as the mean weight of the animals changed. Feeding, itself, consisted of administration of between 0.5-0.8 ml of diluted theophylline liquid over a period of 30-60 sec., with the actual amount depend-

Plate VII.
Force Feeding of the Experimental Animal



ing upon body weight of the individual animal. This dose, which amounted to 10 mg/kg, was repeated four times a day and the dosage adjusted as the animal's body weight increased. At approximately 14 days into the experimental period, however, a noticeable variation became evident in the body weights between the animals receiving the drug and those that did not. At this time, it was felt that the drug was affecting the animals' feeding habits and the administration of the drug reduced to 3 times a day without changing the dose. In addition, administration of syrup without theophylline was begun in the non-drug experimental groups.

Sacrifice and Preparation of Bone Samples

At the end of the twenty one day experimental period, all the animals were sacrificed utilizing a guillotine. Immediately following decapitation, the head was immersed for approximately 10 seconds in an ice bath to bring the temperature down. The skin covering the cranium was then quickly removed by making an 'L' shaped cut extending from the right ear to the right eye and then across the midline to the left eye. The scalp was reflected back to expose the top of the skull. The calveria was sectioned using a separating disc (Dedeco^{R} Dental Development and Mfg. Corp., Brooklyn, NY) in a slow speed dental handpiece. Cuts were made behind the orbits and just posterior to the lambdoid suture. These cuts were then connected by parallel cuts along the insertions of the temporalis muscle on both sides of the skull. The rectangular segment of calvaria was then teased off and immediately

immersed in liquid nitrogen to freeze the sample. This whole procedure was kept under two minutes in duration to preserve as much of the integrity of the sample as possible.

Once frozen, the sample was taken out of the liquid nitrogen and the interimplant distances measured with a Bowley gauge. At the same time, the expansion spring, if still in place, was removed and remaining fibrous tissue scraped away with a scapula. Care was taken during these procedures to keep the sample frozen by periodic immersion in the liquid nitrogen. The sample was then pulverized for approximately three minutes in a small liquid nitrogen-cooled mortar and pestile and the powdered bone, which was kept frozen by the continual addition of liquid nitrogen, placed in a pre-cooled 13 mm x 100 mm Pyrex test tube (#9820, Corning Glass Works, Corning, NY). The sample was lyophilized for 12 hours (Virtis, Uni-Trap Model 10-100, Vistis Co., Gardiner, NY), tightly sealed and stored at -20^{o} C until utilization in the assay procedure.

The procedure for those animals that were to be utilized for the histological evaluation was similar to the above described, except that upon sample removal, freezing was omitted. Instead, after the implant measurements were made and the fibrous tissue removed, the sample was immersed in 70% ethyl alcohol and stored at 5-8^{o} C.

Extraction and Assay of Cyclic Nucleotides

The lyophilized bone samples were weighed to the nearest 0.0001 gm using a microbalance and transferred to Nalgene^{R} polycarboxylate centrifuge tubes (Nalge Co., Rochester, NY). The extraction of cyclic nucleotides was performed at 0-4^{o} C by a method similar to that of Oleinick, Daniel, and Brewer (1981).

The samples were homogenized in enough cold 1M perchloric acid (e.g. 1.0-2.5 ml) to make an approximate sample concentration of 50 mg/ml (W/V). A previous pilot study had shown a relatively straight line relationship for extraction concentrations between 10-100 mg/ml, thus assuring complete extraction at this concentration (Figure 5, Table II). The samples were then centrifuged at 20,000 g for 15 minutes in the cold. Following decantation of the supernate, the acid-insoluble pellet was stored at 5-8^{o} C until the material could be assayed for protein content by the method of Lowry (1951). The perchloric acid supernate was then neutralized with a saturated potassium carbonate solution and the precipitated potassium perchlorate collected by a second centrifugation for 15 minutes at 20,000 g. Extraction efficiency of this procedure was checked by the addition of tritiated recovery nucleotides to bone extracts immediately following homogenization with perchloric acid. Upon evaluation of the final supernate, cAMP and cGMP recoveries of 97.7% and 98.6%, respectively, were realized following counting in a scintilation spectrometer (Intertechnique^{R} SL30) (Table III).

The resultant supernate was stored at 5 - 8^{o} and assayed directly for cAMP and cGMP by a radioimmunoassay procedure. A high degree of sensitivity was obtained in this assay by utilizing the acetylation procedure employed in the appropriate kits purchased from the New England Nuclear Corp. (kit #NEX-132 and NEX-133, New England Nuclear, Boston, MA).

The basic principle of radioimmunoassay depends upon a competition between radioactive and non-radioactive antigen for a fixed number of antibody binding sites. Thereby, increasing amounts of non-radioactive antigen (e.g. standard or unknown) in relation to a known amount of radioactive antigen when allowed to react with a constant amount of antibody, will decrease the amount of radioactive antigen bound to the antibody (Figure 6). This relationship can be expressed in the form of a standard curve and the unknown amounts of unlabeled antigen determined by interpolation. The kits, which utilize radioactive I^{125} for the antigen markers (e.g. succinyl tyrosine (^{125}I) methyl-ester derivative of cAMP), were adopted from the procedures of Steiner et al. (1974).

Research by Steiner and others have demonstrated that cyclic nucleotides substituted at the 2' - 0 - position had a higher offinity for the antibody and thus displaced the (^{125}I) labeled derivative better than the unsubstituted cyclic nucleotide. This substitution, which resulted in a 100 fold increase in the sensitivity of the assay, was achieved in this study by sample acetylation with acetic anhydrid (e.g. 2' - 0 ' acetyl cAMP).

Figure 5.-- Cyclic nucleotide extraction efficiency. A graphical representation of data found in Table II on the concentration(pmole/mg.bone) of cAMP/cGMP extracted at sample concentrations ranging between 10 and 100mg.bone/ ml.1M perchloric acid.

CYCLIC NUCLEOTIDE EXTRACTION EFFICIENCY

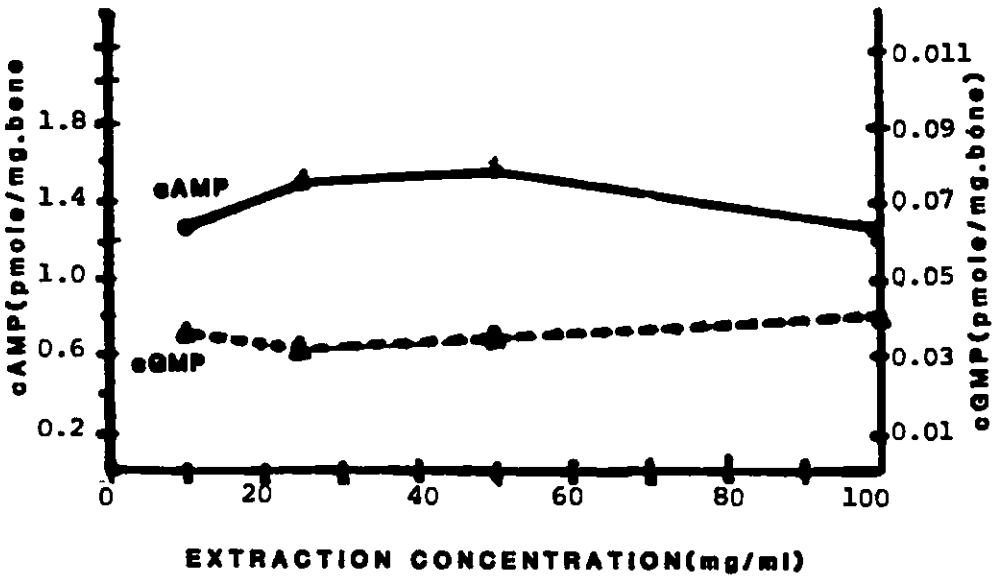


TABLE II

ANALYSIS OF NUCLEOTIDE EXTRACTION EFFICIENCY
(pmole/mg. bone)

SAMPLE	EXTRACTION CONCENTRATION (mg/ml)	cAMP (pmole/mg)	cGMP (pmole/mg)
1	100	0.133	0.0420
2	50	0.154	0.0368
3	25	0.150	0.0318
4	10	0.130	0.0383

TABLE II, represents cyclic nucleotide extractions at varying concentrations of 1M perchloric acid. All samples were obtained from the calvaria of the same animal.

Figure 6.— Schematic of radioimmunoassay mechanics. The principle of this assay technique depends upon competition between radioactive and non-radioactive antigen for a fixed number of antibody binding sites.

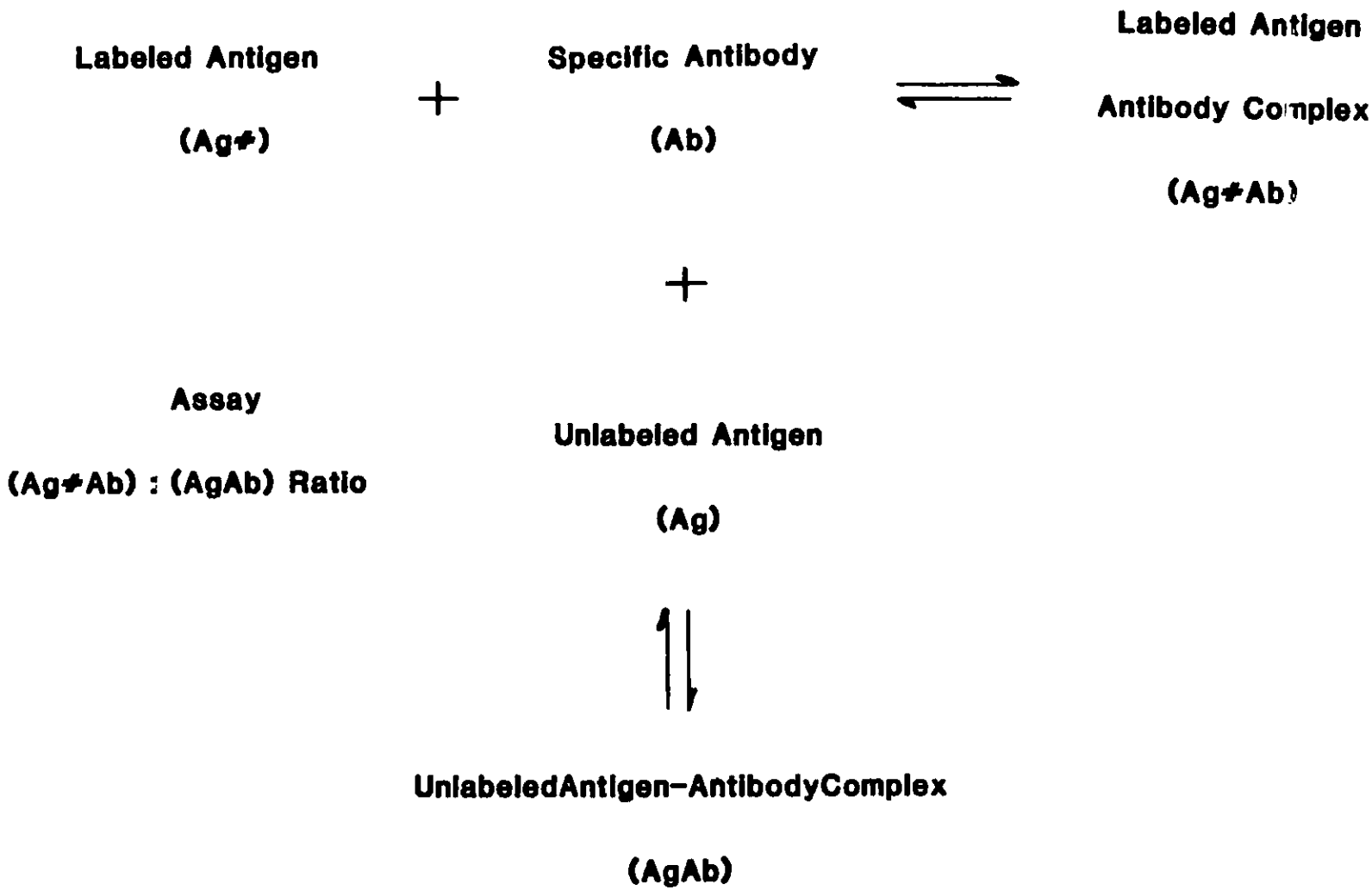

Labeled Antigen
(Ag*)
Specific Antibody
(Ab)
Labeled Antigen
Antibody Complex
(Ag*Ab)
Assay
(Ag*Ab) : (AgAb) Ratio
Unlabeled Antigen
(Ag)
UnlabeledAntigen–AntibodyComplex
(AgAb)

Figure 7.-- Standard curve for assay of cAMP. Values of cAMP for unknown samples were interpolated from a standard curve determined by assaying known quanities of cAMP and plotting concentration(pmole) vs. % binding of the labeled antigen to the antibody. Due to the rapid decay rate of the I^{125} label, a standard curve was determined with each sequence of unknown samples

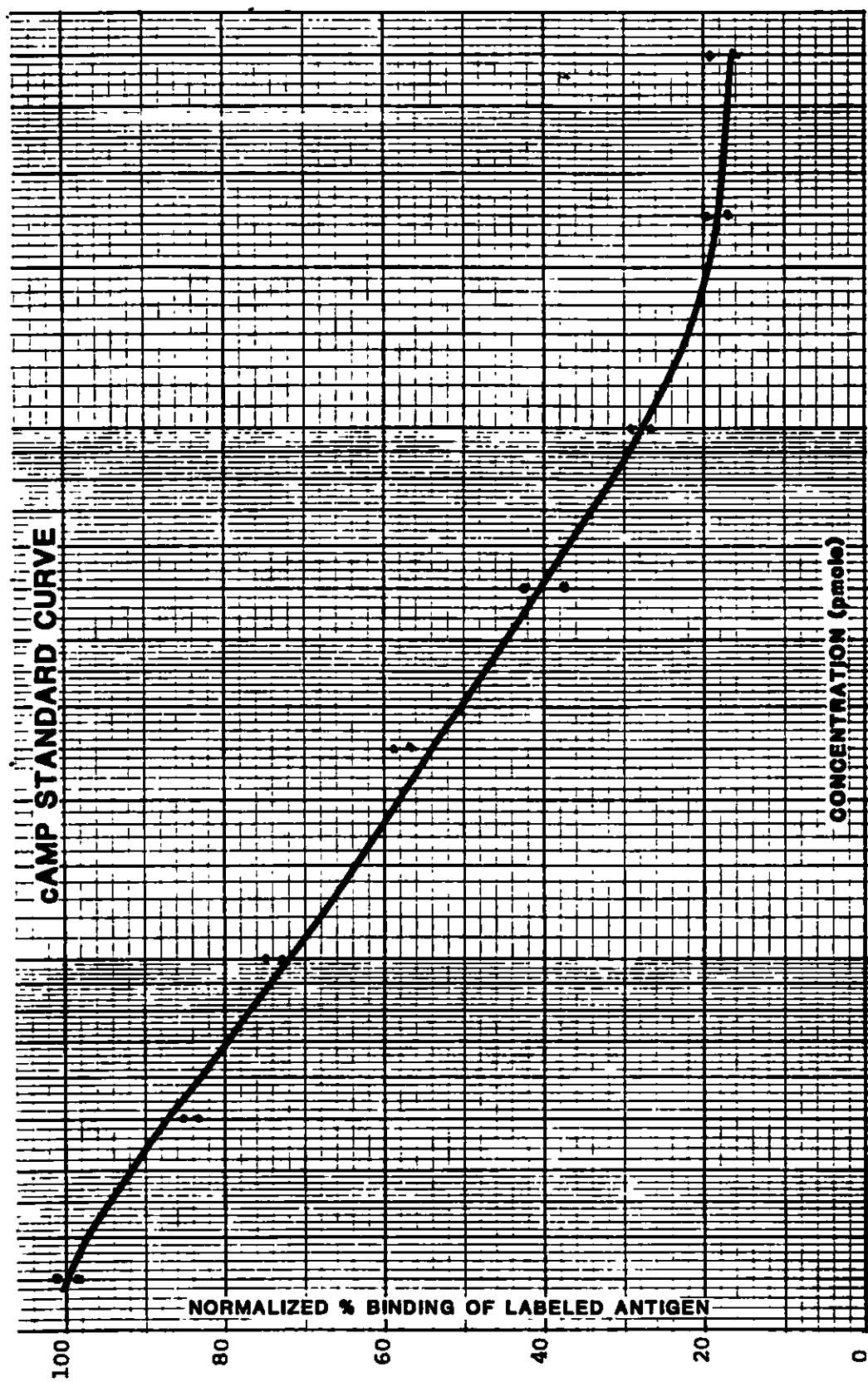
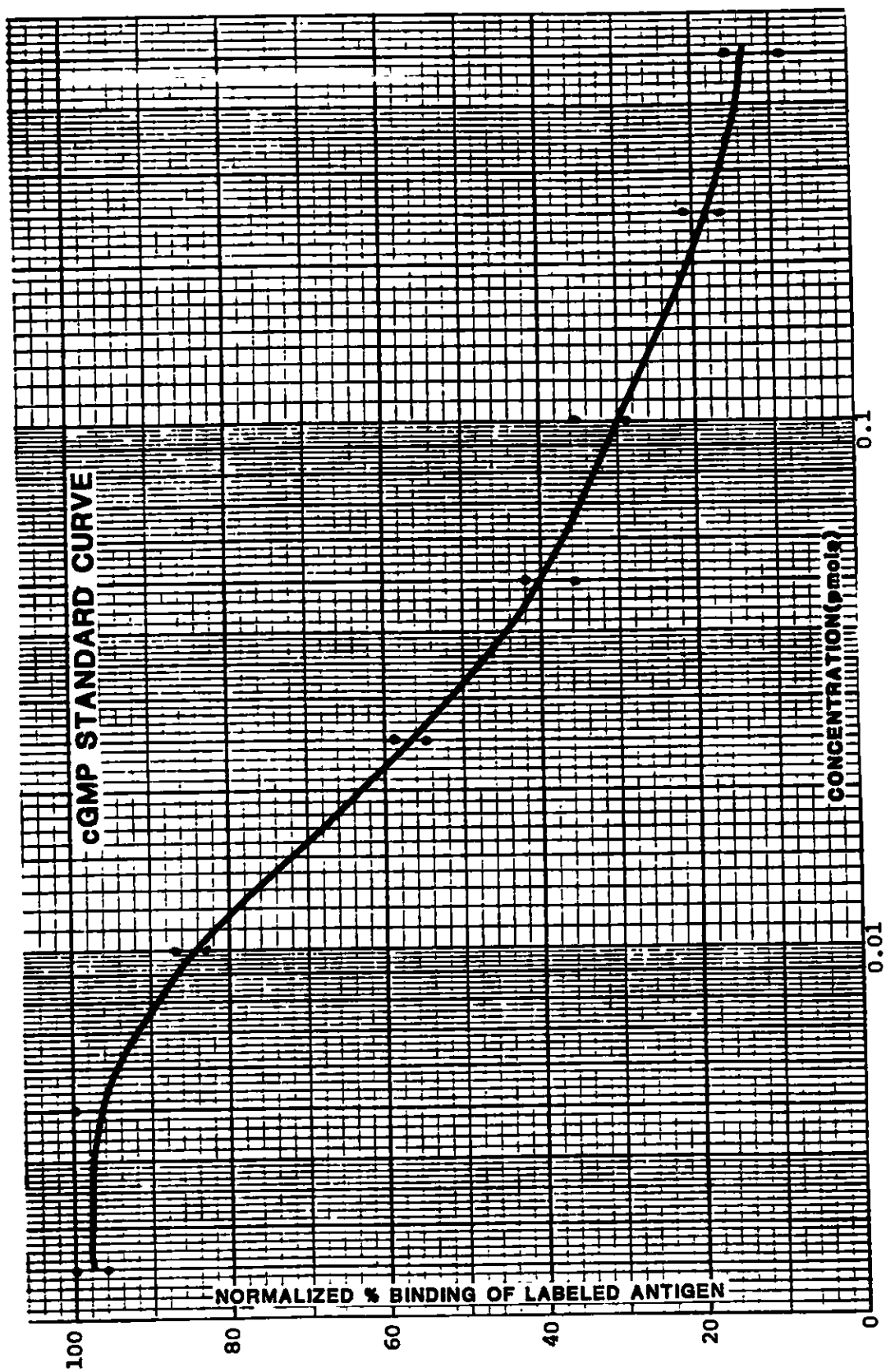

cAMP STANDARD CURVE
NORMALIZED % BINDING OF LABELED ANTIGEN
100
80
60
40
20
0
CONCENTRATION (pmole)
0.1
0.01

Figure 8.--Standard curve for assay of cGMP. Values of cGMP for unknown samples were interpolated from a standard curve determined by assaying known quanities of cGMP. Unknown samples were diluted in such a way that the majority of the values fell in the middle one-third of the curve where the slope was the steepest and thus the most accurate.


cGMP STANDARD CURVE
NORMALIZED % BINDING OF LABELED ANTIGEN
100
80
60
40
20
0
0.01
0.1
CONCENTRATION(pmole)

Separation of bound from free antigen is achieved by the use of a pre-reacted primary and secondary antibody complex. The primary antibody, which was included in the kit, was prepared in rabbits against a succinyl cAMP - albumin conjugate, while the second antibody was prepared in sheep against rabbit-gobulin. After incubation for 22-24 hours at 4^{O} C buffer was added, the tubes centrifuged at 2000 g for 20 minutes, the supernatent carefully discarded and the precipitate counted in a gamma counter (Liquimat^{R}, Picker Nuclear). In these evaluations, 200 ul of the final extract (50 mg/ml) for cGMP and 20 ul for the cAMP proved sufficient to provide measurable assay activity and allow for experimental fluctuations.

The possibility of extraneous activity from material other than the desired cyclic nucleotides was checked by reacting samples with a 0.1 mg/ml phosphodiesterase enzyme preparation (0.21 units activity/mg; Sigma Chemical Co., St. Louis, MO) in a 1:2 sample to enzyme ratio and processing. In those samples so treated, a 91-100% destruction of activity was observed indicating a high assay integrity (Table IV). In practice, cross-reactivity of either cAMP or cGMP antiserum with other nucleotides was low enough as not to interfere with assay results (see kit brochure).

Protein Assay

The protein content of the bone samples was measured utilizing a variation of the Folin Protein assay developed by Lowry et al. (1951). The determination of protein with the Folin Phenol rea-

TABLE III. represents activity obtained from $[H^3]$ recovery. Comparison between initial activity (standard) and the amount of activity recovered (recovery) following nucleotide extraction permits evaluation of extraction efficiency. This average value was then factored into the nucleotide concentrations obtained from the unknown bone samples.

TABLE IV. represents an evaluation of extraneous assay activity from sources other than the specific cyclic nucleotide. In this procedure, a 100ul aliquot of (1) known standard (2) unknown sample and (3) perchloric acid(e.g. extraction medium) were reacted with 200ul of 0.1mg/ml phosphodiesterase enzyme preparation to eliminate assayable cyclic nucleotides and permit evaluation of non-nucleotide interferences. Comparison between the reacted and unreacted samples thereby demonstrates the high specificity of the RIA.

TABLE III

ANALYSIS OF NUCLEOTIDE RECOVERY EFFICIENCY
([H^3] Counts/min.)

CYCLIC NUCLEOTIDE	SAMPLE	ACTIVITY (CPM)	AVERAGE (CPM)	% RECOVERY
cAMP	STANDARD	3745	3467.2	97.7
	STANDARD	3642		
	RECOVERY	3343	3388	
	RECOVERY	3433		
cGMP	STANDARD	5087	4690.8	98.6
	STANDARD	4937		
	RECOVERY	4574	4625	
	RECOVERY	4676		

TABLE IV

PHOSPHODIESTERASE DESTRUCTION
OF CYCLIC NUCLEOTIDE ACTIVITY

SAMPLE	cAMP pmol/mg.bn	% DESTRUCTION	cGMP pmol/mg.bn	% DESTRUCTION
STANDARD	0.5	96.7	0.5	97.4
ENZYME RXN	0.165		0.013	
SAMPLE(S_2)	0.393	95.5	0.067	100
ENZYM RXN.	0.0177		++++	
PCA BLANK	0.0165	--	0.0125	

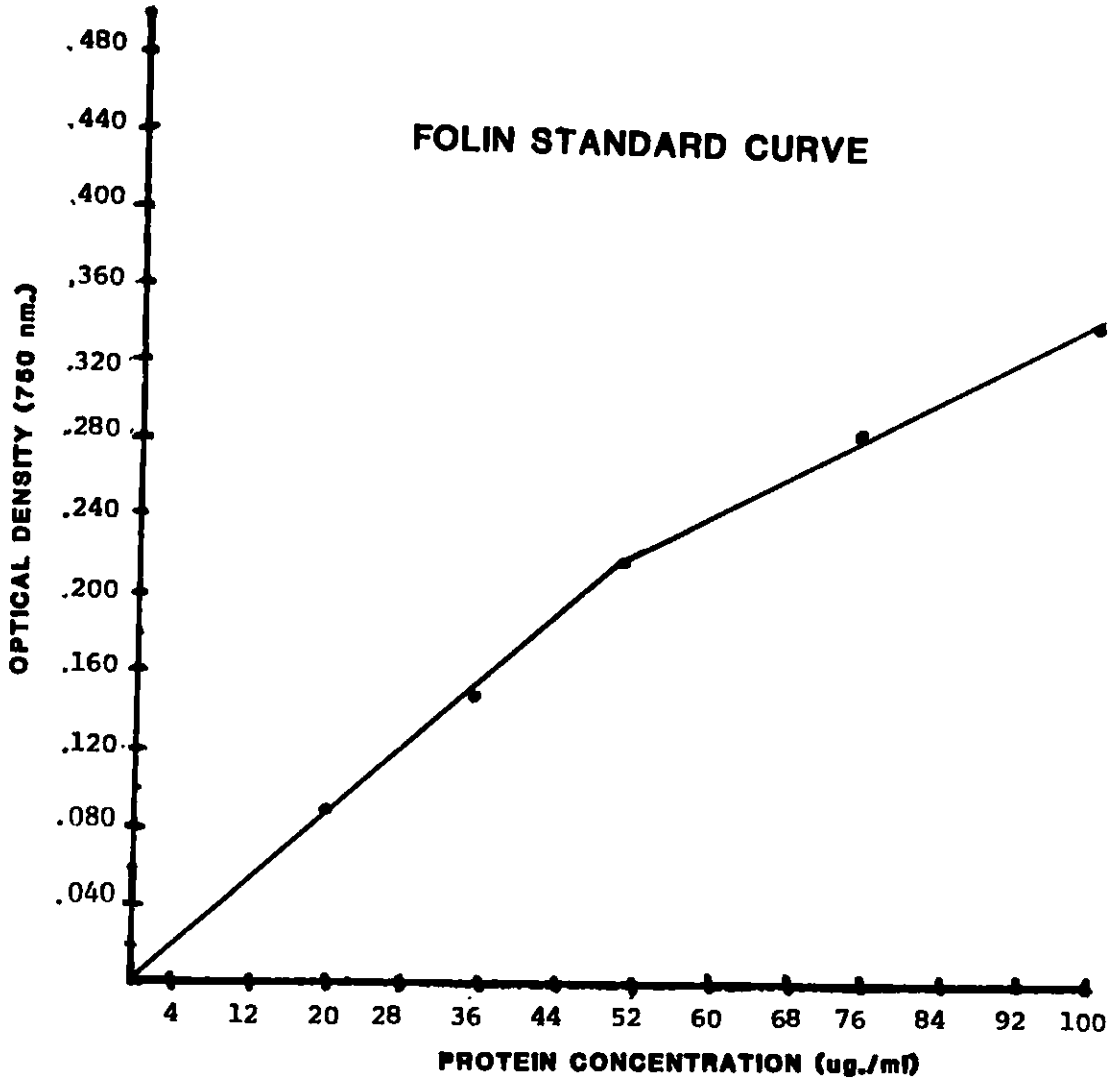
gent (Fischer Scientific Co., Warrensville, OH) is based on the principle that in alkaline solution a complex will form between protein and copper. This complex, when reacted with a phosphomolybetic-phosphotungstic reagent (e.g. Folin reagent) will reduce it forming a colored compound that can be measured photometrically.

This assay, which is primarily sensitive to the amino acid tyrosine, was chosen because of its insensitivity to collagen (e.g. 4 tyrosine/1000 amino acid residues in Type I collagen). In addition, this determination provided a sensitive standardized method by which to evaluate cell protein. Protein to be assayed was extracted from the acid-insoluble pellet from the perchloric acid extraction previously described, using 5.0 ml of 0.4 N Sodium hydroxide per 50 mg sample for 48 hours in the cold with periodic agitation to suspend the bone material. Due to the high collagen contect of bone (e.g. 23 to 31% total dry weight) the extraction procedure was performed with a weak base in the cold in order to keep extraction of the collagen fraction to a minimum.[2] At the end of the 48 hour period, the samples were centrifuged at 20,000 g for 10 minutes. The supernate was then decanted and the base insoluble pellet extracted a second time with another 5.0 ml of 0.4N NaOH to assure complete extraction of all assayable protein.

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[2]To eliminate further the possibility of collagen assay interference, differential NaCl precipitation as described by Ehrlich (1979) was performed on random protein extraction samples and the results compared. No significant differences were found between the precipitated samples and those assayed directly indicating the absence of significant extracted collagen.

Figure 9.-- Bovine serum albumin(Cohn fraction V) standard curve for Folin non-collagen protein determination was constructed by making various dilutions of a 500ug./ml. standard BSA solution. Due to the linearity between 0 and 50ug., unknown samples were diluted so that the majority of their values fell in this range.


FOLIN STANDARD CURVE
OPTICAL DENSITY (750 nm.)
.480
.440
.400
.360
.320
.280
.240
.200
.160
.120
.080
.040
4
12
20
28
36
44
52
60
68
76
84
92
100
PROTEIN CONCENTRATION (ug./ml)

The extracted supernate was diluted 3-5 times with 0.4 N NaOH and a 50 ul aliquote assayed against a bovine serum albumin (Cohn Fraction V; Fisher Scientific, Warrensville, OH) protein standard, prepared by dilution of a 500 ug/ml protein solution (Figure 9). Due to the linearity of the Folin assay between 0 to 50 ug, all samples were diluted to within this range prior to assay.

Following a two hour development time, both samples and standards were read for absorbance at 750 nm in a Beckman Model 25 Spectophotometer (Beckman Instruments Inc., Irvine, CA). Protein content of the unknown samples was then determined utilizing the following formula:

$$y \text{ protein} = \frac{(\text{O.D. unknown} - \text{O.D. Blank})\ (\text{ug Protein Standard})}{(\text{O.D. Standard} - \text{O.D. Blank})}$$

Histological Evaluation

In preparation for embedding, undecalcified cranial specimens which had been stored in 70% ethyl alcohol since decapitation, were dehydrated through a routine series of progressive alcohol baths (e.g. 70%, 95% and 100% ethanol). Once dehydrated, the specimens were cleared twice in xylene and imbedded in Bioplastic,[R] (Ward's Natural Science Establishment, Inc., Monterey, CA).

Sagittal 90 - 150 u undecalcified sections were cut utilizing a thin 200 u diamond disc in a high speed Precision[R] (Precision Corp., Racine, WI) microtome. These sections were dehydrated through a series of alcohol washes and cleared in xylene. They were then mounted on glass slides using a UV inert mountant (Gurr[R]

Biomedical Specialties Inc., Santa Monica, CA) and examined by fluorescence microscopy under blue light (450-490 nm) at 125X magnification. The specimens were evaluated for gross differences in the relationship of the vital dye lines. Amounts of bone growth were measured with an eyepiece micrometer and areas of appositional bone deposition were noted with particular attention to growth around the sutural area.

RESULTS

General Response of Animals to Experimental Procedures

Visual examination of the animals approximately 3 to 15 days following surgery revealed that the area of incision, in some of the experimental groups with spring implantation (e.g. S and T + S groups), showed signs of mild post-operative infection. As a possible cause, it was observed that the animals, particularly among those with the implanted springs, were interfering with the surgical wounds of their groupmates, even though the springs had been placed under the scalp between the pinnae to minimize such damage by the individual animals themselves. Fortunately, post-operative infection proved only a minor problem, and all areas healed without apparent complications with exception of Animal 1 in the T + S group. The post-operative infection in this animal proved to be a problem and could not be resolved, despite isolation and repeated application of Betadine. The expansion spring was eventually sequestered on Day 16 of the experimental period, and the tissues then healed without further interceptive treatment.

Animal health and physical development, as well as theophylline dosage, were approximated by continually monitoring the weight gain in the animals of the various groups (Figure 10). Toward the end of the second week, a significant differential in weight gain per day was beginning to become evident between the

control (5.96 gm/day) and the experimental animals (Figures 10 and 11). This effect was of concern, particularly in the group which received both the spring and theophylline (e.g. T + S). A possible explanation for this result involves the external variables that relate to the various groups. A moderate and almost uniformly reduced growth rate was observed in the two experimental groups (e.g. T and S) which had only one experimental variable; either a spring or drug but not both. However, in the experimental group, which had two variables (e.g, both spring and drug), a synergistic effect of the two variables could be seen in the greater reduction in growth rate realized (Figure 11). These variables could conceivably act as insults and affect the animals' appetite and thereby their growth rate. The expansion appliance, which exerts a lateral force on the skull, could easily have been a source of discomfort to the animals and in turn have affected their feeding habits. It may be suggested that the stretching action generated on the periosteum by the spring could have made the entire cranial complex sensitive, particularly to the force generated by the muscles of mastication (e.g. the temporalsis muscle force vector is similar to that of the spring).

Theophylline, on the other hand, could also stress the animal in several ways. First, theophylline is known to have a diuretic effect, and since the animals were maintained on high levels of this drug, it is plausible that they may have been kept in a somewhat dehydrated state. A second and probably more significant

Figure 10.-- Summary of average group weight increase over the experimental period. Animals were weighed approximately every three days to guage physical development and to re-adjust theophylline dosage. Due to an apparent discrepency in weight gain which became evident in the second week, theophyline dose interval was reduced from 4 to 3 times/day on Day 14 (indicated by the arrow). Groups are denoted by experimental variable; T=theophylline, S= expansion spring.

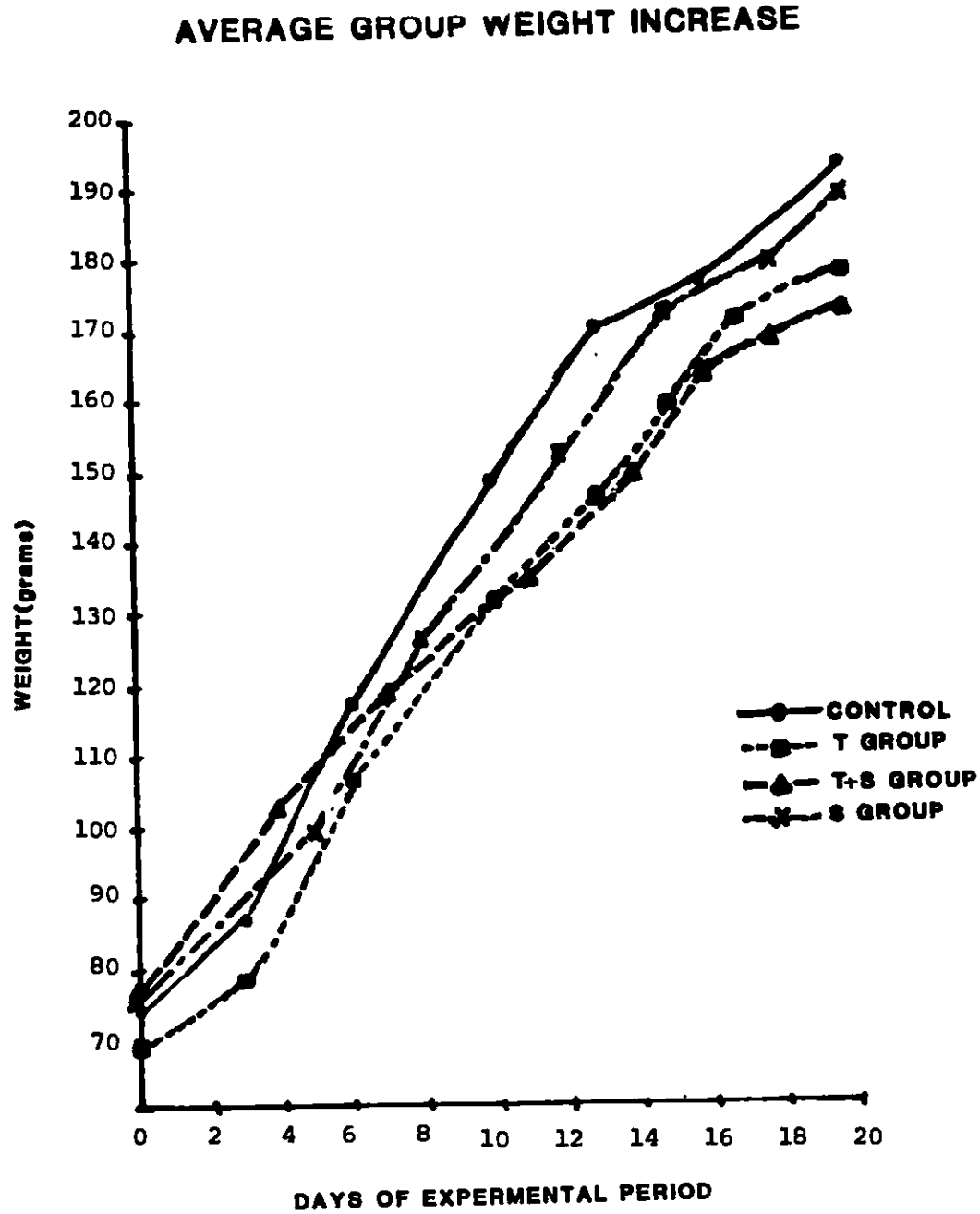
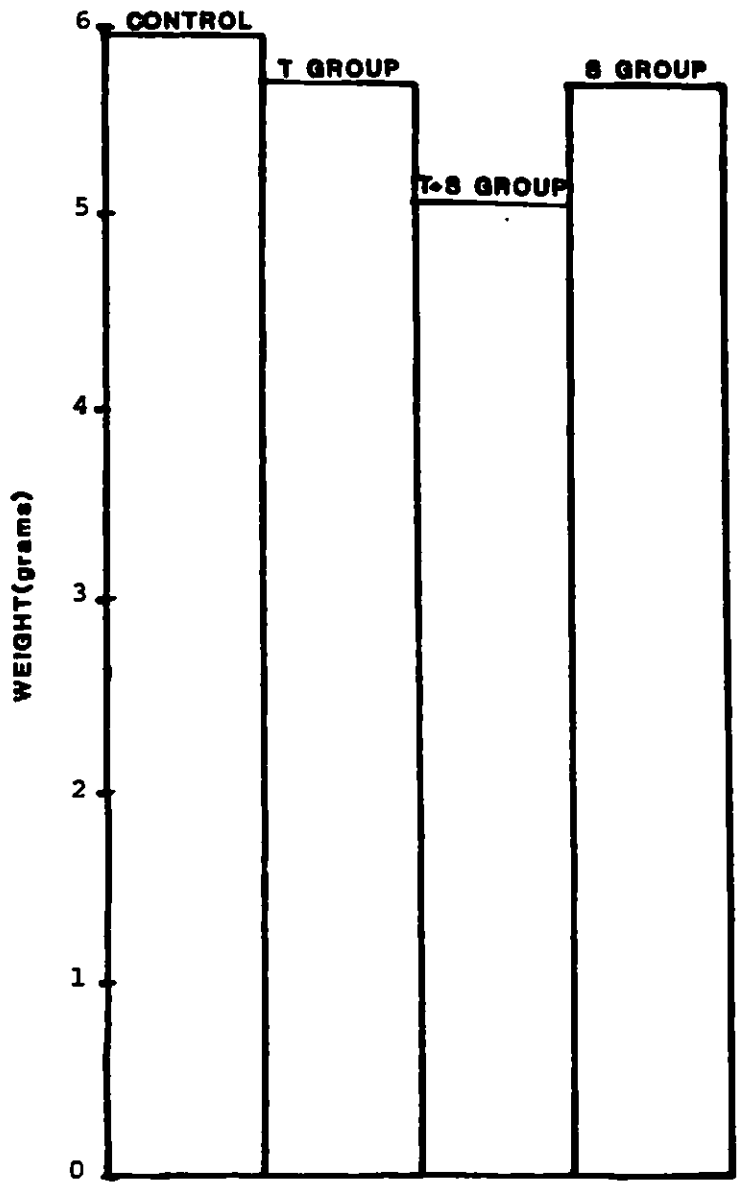

AVERAGE GROUP WEIGHT INCREASE
WEIGHT(grams)
200
190
180
170
160
150
140
130
120
110
100
90
80
70
0
2
4
6
8
10
12
14
16
18
20
DAYS OF EXPERMENTAL PERIOD
CONTROL
T GROUP
T+S GROUP
S GROUP

Figure 11.-- Histogram of Average Daily Weight Gain
This diagram summarizes the weight increases, by group, depicted graphically in Figure 10.
(T= theophylline, S = expansion spring)

AVERAGE DAILY GAIN



effect of theophylline is that even moderate doses of this drug can cause restlessness and agitation, both of which could affect appetite. Either way, a modification was made in the drug regiment at Day 14 of the experimental period, in which the dosing frequency was reduced from 4 to 3 times per day. This change resulted in a slight increase in daily growth rate of both theophylline variable experimental groups (T and T + S) more toward that of the controls.

The experimental period passed with few other complications. Although drug administration proved to be a troublesome problem initially, force feeding improved as the animals began to accept the drug more readily. Of the 22 animals, only one death was noted at Day 10 in an animal from the T group, and this was thought to be the result of a punctured esophagus incurred in drug administration.

Gross examination of the skull at the time of sacrifice indicated that in many of the experimental animals with the spring, there were varying degrees of fibrosis around the spring continuous with both the scalp and bone, possibly related to the post-operative infection present initially. The extent of such fibrosis, however, did not appear severe enough in any of the animals to affect expansion of the spring. Some epiphytic bone proliferation occurred around the spring attachment points to the bone as an attempt by the tissues to reinforce this area. In addition, due to the increased bony surface accretion, the morphology was

significantly more irregular than in those groups without the springs.

Analysis of Sutural Expansion

The results of sutural expansion as depicted by direct implant measurement will be presented in three parts: (1) average lateral expansion; (2) average anteroposterior increase; and (3) average change in the surface area defined by the implants. A summary of the changes in inter-implant measurements of the experimental period are summarized in Table V.

The average lateral expansion was determined by direct measurement across the sagittal suture, between the implants in the parietal bones (Figure 4). The average change in this lateral distance for each group between the pre- and post-experimental periods is graphically depicted in Figure 12. Results from this measurement indicate a moderate increase in the transverse cranial dimension of the skull in the control animals (0.54 $\pm$ 0.23 mm) with only a slightly greater increase being noted in the animals from the T group (0.90 $\pm$ 0.34 mm), which had received only theophylline. As expected, however, the transverse dimensional change in the S and T + S groups, in which the springs had been implanted, was proportionally larger in relation to the controls. The results, indicative of the active expansile force present, demonstrate a greater than five-fold increase in this dimension of both the S and T + S groups (2.83 $\pm$ 0.41 mm and 2.73 $\pm$ 0.76 mm, respectively), with

TABLE V

SUMMARY OF SUTURAL EXPANSION

GROUP	LATERAL (mm)		ANTEROPOSTERIOR (mm)		AREA (mm^2)	
	$\bar{X}$	SD	$\bar{X}$	SD	$\bar{X}$	SD
CONTROL	0.54	0.23	0.71	0.29	3.33	1.52
T GROUP	0.90	0.34	0.84	0.28	5.69	1.72
S GROUP	2.83	0.41	0.60	0.37	9.50	4.39
T+S GROUP	2.73	0.76	1.38	0.78	13.61	4.03

TABLE V. represents the average and standard deviation($\pm$) of sutural expansion in each of the experimental groups.(T = theophylline, S = expansion spring).

TABLE VI

ANALYTICAL STASTICS - Student t-test

Sutural Expansion (mm^2)

MEASUREMENT	n_1	n_2	$\bar{X}_1$	$\bar{X}_2$	S_1^2	S_2^2	t	α
CONTROL TO T GROUP	5	4	3.33	5.69	9.24	8.88	1.146	0.25
CONTROL TO S GROUP	5	6	3.33	9.50	9.24	96.36	1.340	0.10
CONTROL TO T+S GROUP	5	6	3.33	13.61	9.24	81.20	2.671	0.025
T GROUP TO S GROUP	4	6	5.69	9.50	8.88	96.39	0.824	0.25
T GROUP TO T+S GROUP	4	6	5.69	13.61	8.88	81.20	2.022	0.05
S GROUP TO T+S GROUP	6	6	9.50	13.61	96.39	81.20	0.748	0.25

α = Level of significance

the S group predominating slightly. Levels of significance (e.g. student t distribution) for expansile changes can be found in Table VI.

In an attempt to determine any gross dimensional compensations concomitant with the above described transverse expansion, changes in anteroposterior dimensions were determined trigonometrically from changes in pre- and post-experimental implant measures (Figure 4). These results, summarized in Table V, appear as the shaded bars in Figure 12.

Examination of these results indicate a slightly larger anteroposterior increase (0.71 $\pm$ 0.29 mm) in relation to the transverse change seen in the control animals. The anteroposterior change in the T group was somewhat larger (0.84 $\pm$ 0.28 mm) than the control group, but was smaller in relation to the concomitant transverse change noted in this same group. Animals from the S group demonstrated a moderately reduced anteroposterior dimensional increase (0.60 $\pm$ 0.37 mm) in relation to the control, but as expected, a large differential was noted between the anteroposterior and transverse dimensions. Conversely, a large change in the A-P cranial dimension was noted in the T + S group (1.38 $\pm$ 0.78 mm) in relation to the control. In fact, this anteroposterior change amounted to almost 1/2 of the change seen in the transverse dimension of that same group.

An attempt was also made to combine the transverse and anteroposterior dimensional changes into a third measure of the

Figure 12.-- Histogram of lateral and anteroposterior calvarial expansion. This diagram depicts graphically data found in Table V for both lateral and anteroposterior growth changes which occurred over the experimental period. (T = theophylline, S = expansion spring)

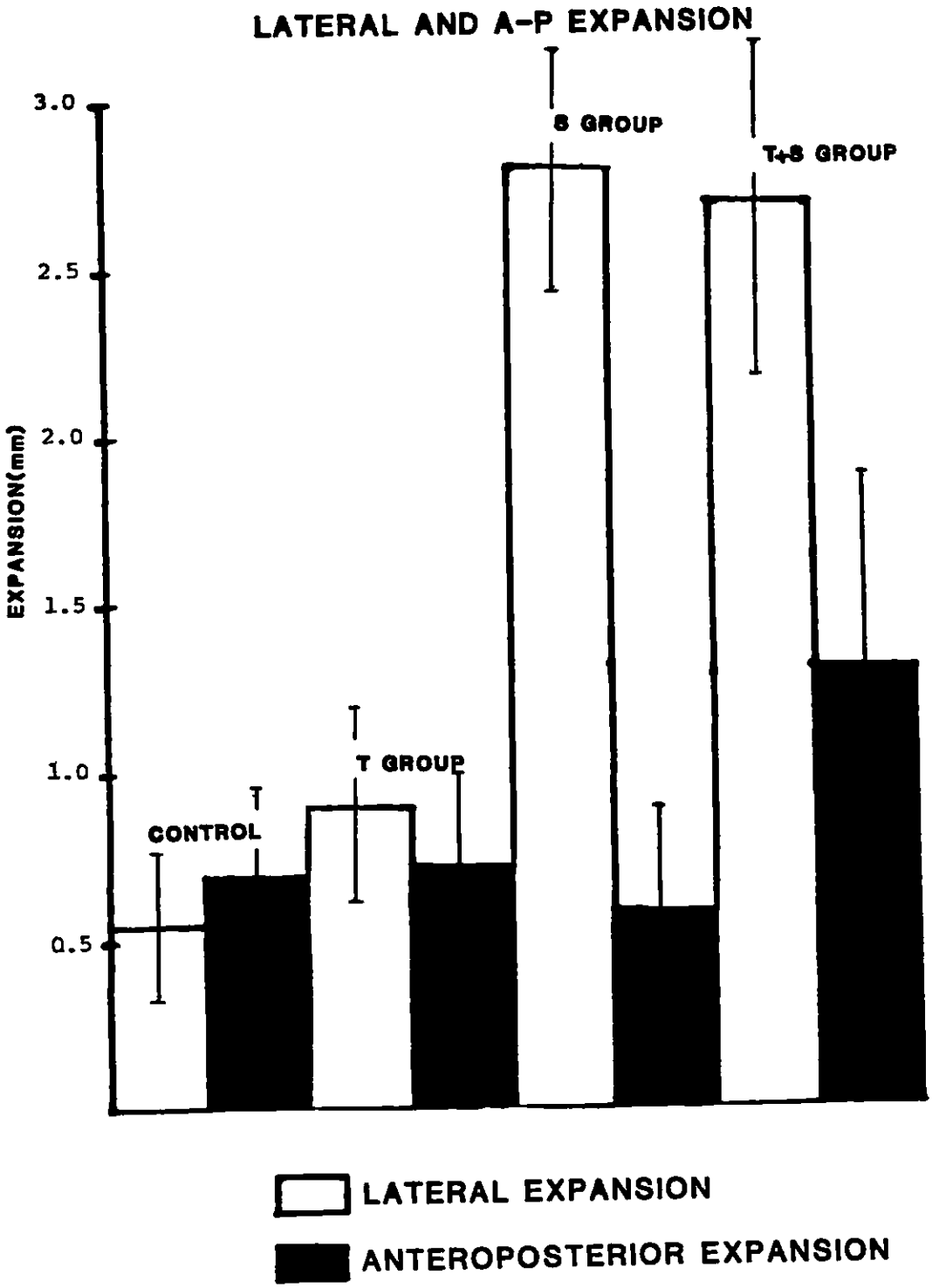
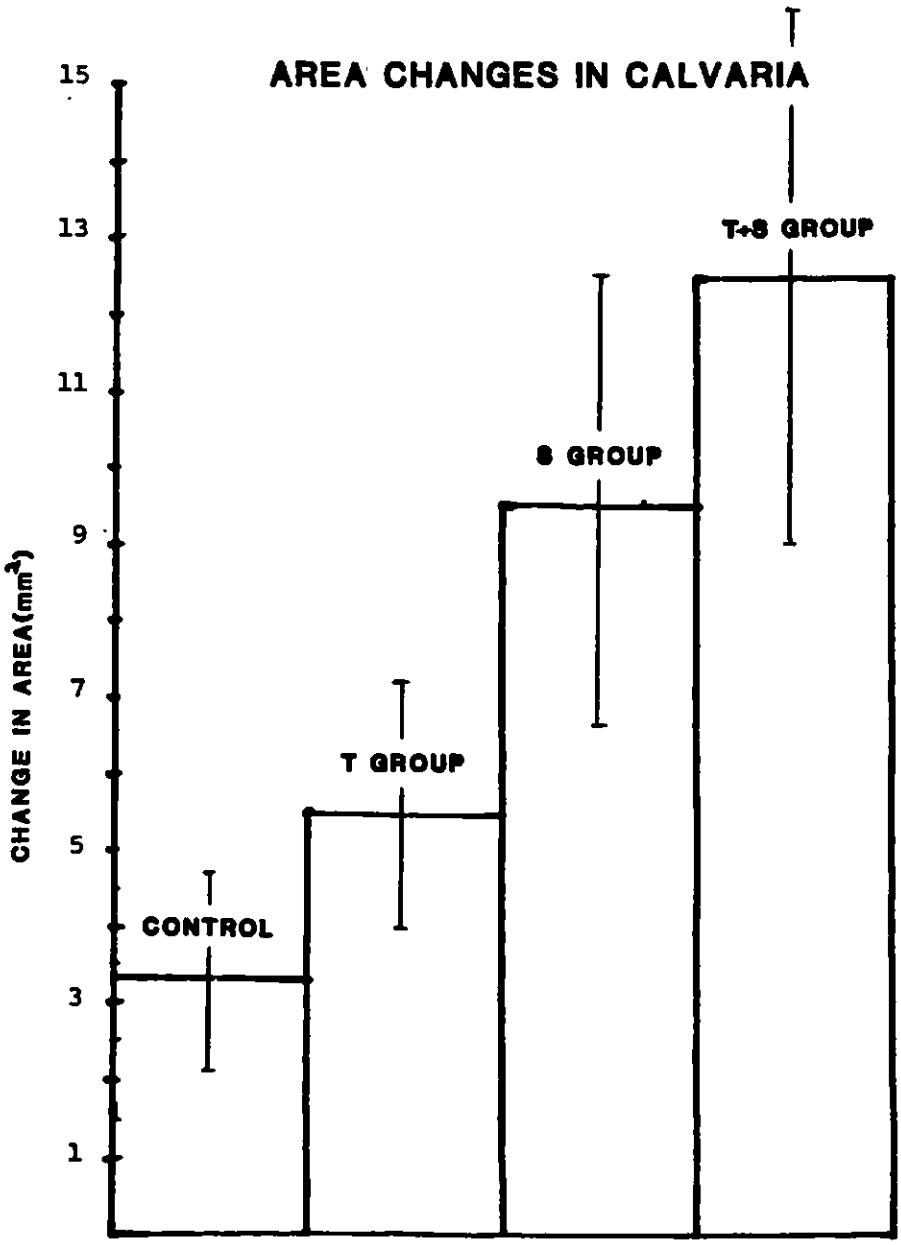

LATERAL AND A-P EXPANSION
3.0
2.5
2.0
1.5
1.0
0.5
EXPANSION(mm)
CONTROL
T GROUP
S GROUP
T+S GROUP
LATERAL EXPANSION
ANTEROPOSTERIOR EXPANSION

Figure 13.-- Histogram of area changes in the calvaria This diagram depicts graphically the data found in Table V for changes in area of the implant triangle over the experimental period.(T= theophylline, S = expansion spring)


AREA CHANGES IN CALVARIA
CHANGE IN AREA(mm²)
15
13
11
9
7
5
3
1
CONTROL
T GROUP
S GROUP
T+S GROUP

surface area of the implant triangle (Figure 13). It was thought that this measure, although relatively small, would better depict the overall dimensional changes which occurred in the calvaria during the experimental period than either the transverse or anteroposterior measurement. Area was determined trigonometrically from interimplant measurements and is summarized in Table V with average area measurements graphically depicted in Figure 13. Overall evaluation showed an almost step-like increase in surface area, in which mechanical stress proved greater than the drug effect, and an additive effect was noted when these two variables were combined. Results indicate that a moderate increase in area occurred in the animals from the T group (5.69 ± 1.72 mm^2) compared to the control (3.33 ± 1.52 mm^2). A much greater increase in area was demonstrated in the S and T + S groups with spring implantation (9.50 mm^2 and 13.61 mm^2, respectively). This was greatest in the T + S group in which both variables were combined.

Cyclic Nucleotide Analysis

Analysis of cyclic nucleotides involved the quanitation of both cAMP and cGMP levels utilizing the radioimmunoassay procedure of Steiner, et al. (1972). These values, expressed in picamole (pmole) units, were compared in terms of both dry bone weight (mg) and protein weight (mg) of the sample from which the nucleotides were extracted. Due to the heterogenous nature of bone tissue and possible differences in the cellular elements (e.g.

osteoclasts/osteoblasts, hemopoietic cells, etc.) present in the various samples, a non-collagen protein determination was done. Since the cyclic nucleotides are primarily intracellular compounds, this determination of cellular protein (e.g. other than collagen) provided a method by which the cyclic nucleotide concentrations of the various samples could be standardized. Comparison of cyclic nucleotide concentrations and protein weights to total dry bone weight is presented here only as confirmation for information already established by the protein content.

Non-collagen protein content, as determined by the Folin protein assay, was compared to total dry bone weight in all groups. The results of this comparison, summarized in Table V, demonstrate that the protein content of the bone samples from the various groups was rather uniform. The protein content varied over a range of only 1% from 21.4% to 22.4% of the total weight, and even though a trend might be construed, all values were within one standard deviation, making any difference of little significance.

Data for the cyclic nucleotide concentration in relation to protein weight are summarized in Table VII. For comparison, this data is also graphically represented in Figure 15. Evaluation of these results demonstrates that the levels of cyclic AMP increased to a varying degree in all three of the experimental groups in relation to the control animals (0.77 ± 0.1 pmole/mg). These effects appeared to be variable-dependent in that the mechanical stress generated by the spring had a slightly greater effect

TABLE VII

SUMMARY OF CYCLIC NUCLEOTIDE ANALYSIS

GROUP	cAMP pmol/mg.bone		cGMP pmol/mg.bone		cAMP pmol/mg.prot.		cGMP pmol/mg.prot.		RATIO cAMP/cGMP		mg.PROTEIN/mg. BONE	
	$\bar{X}$	SD	$\bar{X}$	SD(10^{-3})	$\bar{X}$	SD	$\bar{X}$	SD(10^{-2})	$\bar{X}$	SD	$\bar{X}$	SD
CONTROL	0.164	0.014	0.016	5.49	0.77	0.10	0.076	2.09	10.82	3.89	0.214	0.012
T GROUP	0.302	0.107	0.0087	1.55	1.36	0.456	0.039	0.60	34.18	6.95	0.221	0.006
S GROUP	0.334	0.044	0.0087	2.26	1.51	0.124	0.040	1.03	40.04	11.67	0.221	0.033
T+S GROUP	0.537	0.046	0.0092	1.97	2.42	0.374	0.042	1.17	59.90	12.28	0.224	0.036

TABLE VII. Summarizes the average cyclic nucleotide levels determined for each group. These results appear in terms of both dry bone weight and non-collagen protein weight with the relationship between these two standards likewise demonstrated.

Figure 14.-- Histogram of cyclic nucleotide concentration: pmoles/mg.bone. This diagram depicts graphically data found in Table VII for cAMP/cGMP concentrations in relation to total dry weight of bone.(T= theophylline, S = expansion spring)

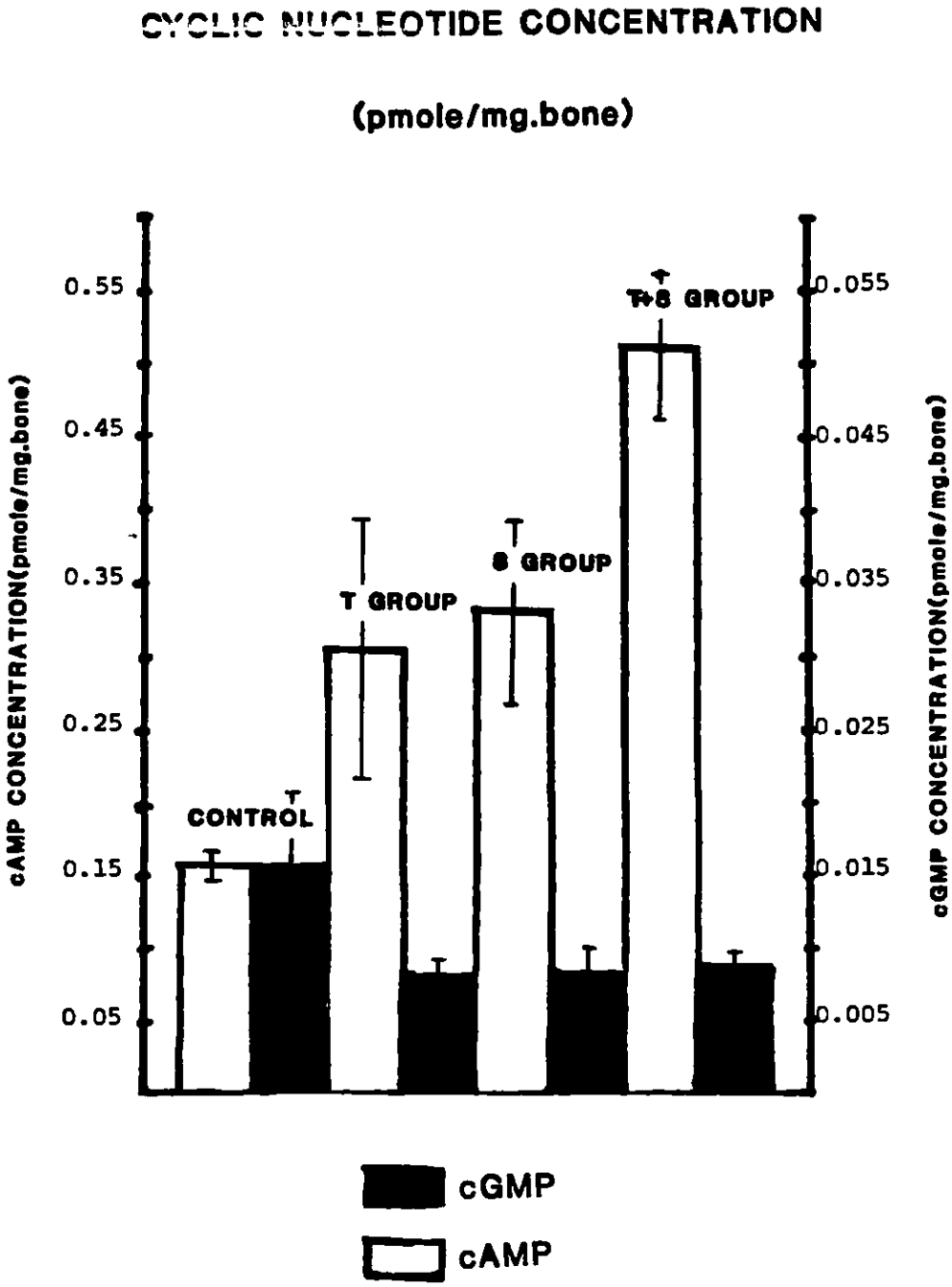

CYCLIC NUCLEOTIDE CONCENTRATION
(pmole/mg.bone)
cAMP CONCENTRATION(pmole/mg.bone)
cGMP CONCENTRATION(pmole/mg.bone)
0.55
0.45
0.35
0.25
0.15
0.05
0.055
0.045
0.035
0.025
0.015
0.005
CONTROL
T GROUP
S GROUP
T+S GROUP
cGMP
cAMP

Figure 15.--Histogram of cyclic nucleotide concentration: pmoles/mg.protein. This diagram depicts graphically data found in Table VII for cAMP/cGMP concentrations in relation to mg. protein.(T = theophylline, S = expansion spring)

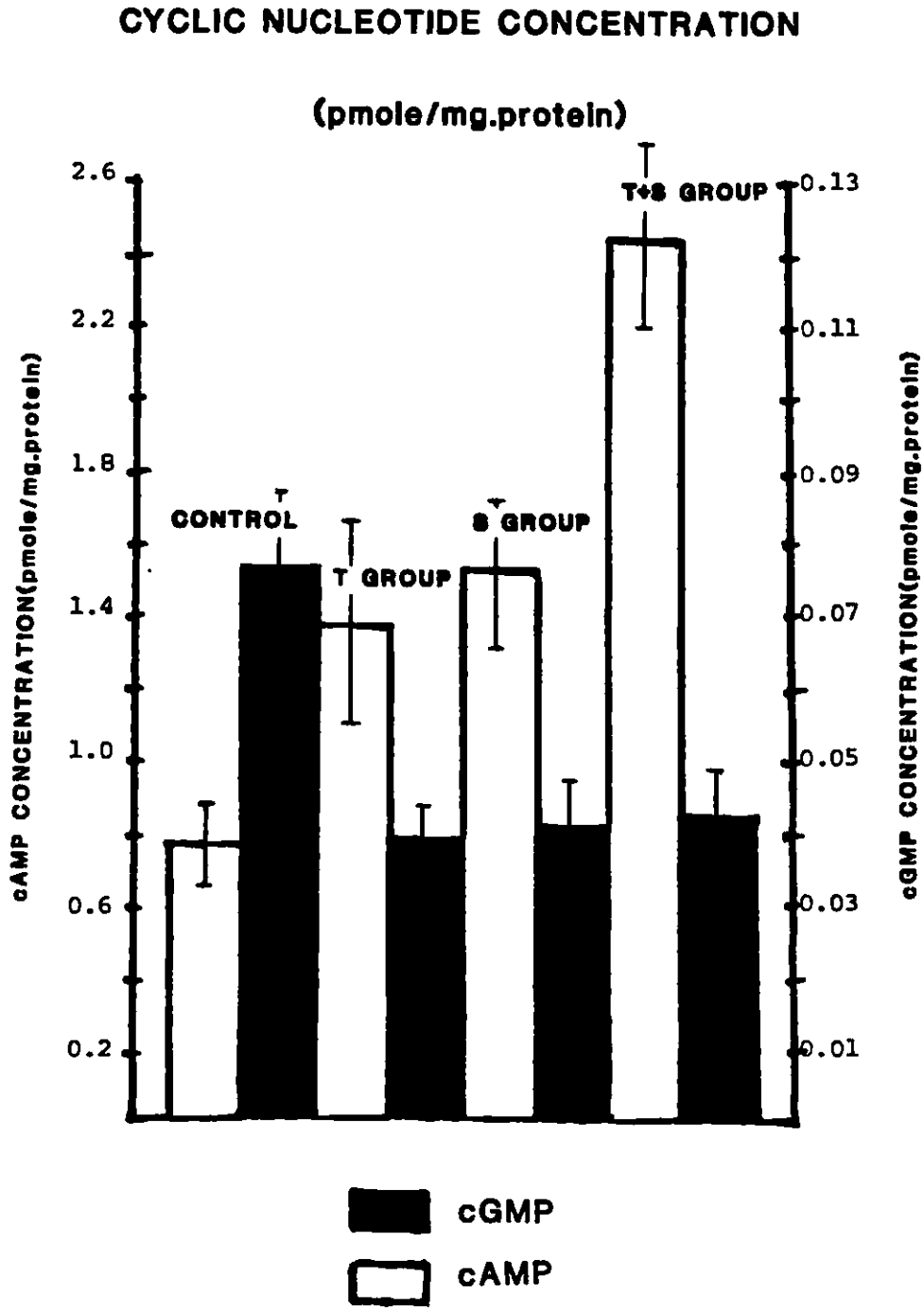

CYCLIC NUCLEOTIDE CONCENTRATION
(pmole/mg.protein)
cAMP CONCENTRATION(pmole/mg.protein)
cGMP CONCENTRATION(pmole/mg.protein)
2.6
2.2
1.8
1.4
1.0
0.6
0.2
0.13
0.11
0.09
0.07
0.05
0.03
0.01
CONTROL
T GROUP
S GROUP
T+S GROUP
cGMP
cAMP

TABLE VIII

ANALYTICAL STATISTICS- Student t-test

Cyclic AMP (pmole/mg.protein)

MEASUREMENT	n_1	n_2	$\overline{X}_1$	$\overline{X}_2$	S_1^2	S_2^2	t	α
CONTROL TO T GROUP	4	3	0.77	1.36	0.03	0.415	5.485	.005
CONTROL TO S GROUP	4	5	0.77	1.51	0.03	0.062	5.035	.005
CONTROL TO T+S GROUP	4	5	0.77	2.42	0.03	0.559	4.265	.005
T GROUP TO S GROUP	3	5	1.36	1.51	0.415	0.062	0.485	NS
T GROUP TO T+S GROUP	3	5	1.36	2.42	0.415	0.559	2.029	.05
S GROUP TO T+S GROUP	5	5	1.51	2.42	0.062	0.559	2.582	.05

α = Level of sigificance

TABLE IX

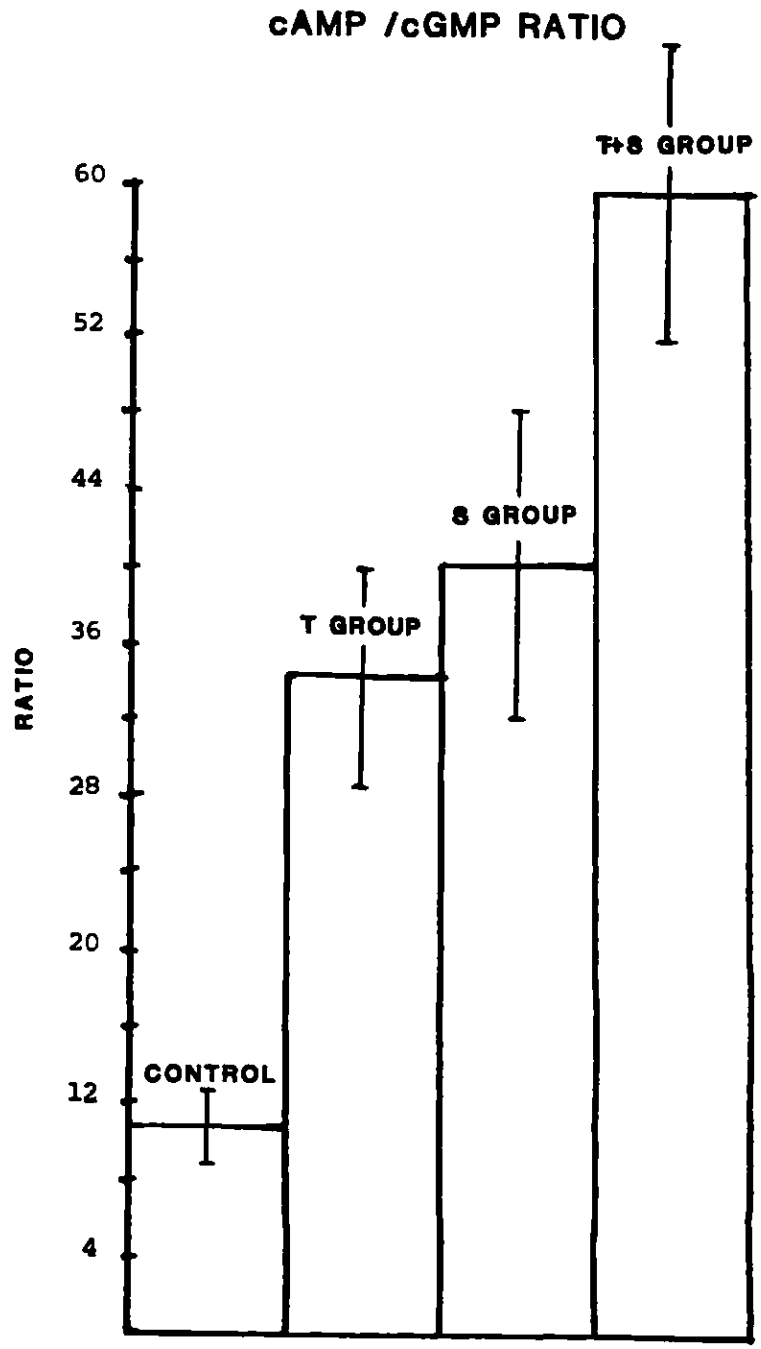
ANALYTICAL STATISTICS - Student t-test

Cyclic GMP (pmole/mg.protein)

MEASUREMENT	n_1	n_2	$\bar{X}_1$	$\bar{X}_2$	$S_1^2(10^4)$	$S_2^2(10^4)$	t	α
CONTROL TO T GROUP	4	3	0.076	0.039	13.1	0.72	1.697	.10
CONTROL TO S GROUP	4	5	0.076	0.040	13.1	4.244	1.893	.10
CONTROL TO T+S GROUP	4	5	0.076	0.042	13.1	5.47	1.714	.10
T GROUP TO S GROUP	3	5	0.039	0.040	0.72	4.24	0.0782	NS
T GROUP TO T+S GROUP	3	5	0.039	0.42	0.72	5.47	0.208	NS
S GROUP TO T+S GROUP	5	5	0.040	0.042	4.24	5.47	0.1434	NS

α = Level of significance

Figure 16.--Histogram of cyclic nucleotide ratio. This diagram depicts graphically data found in Table VII for cAMP/cGMP ratio. (T= theophylline, S = expansion spring)


cAMP /cGMP RATIO
RATIO
60
52
44
36
28
20
12
4
CONTROL
T GROUP
S GROUP
T+S GROUP

(1.51 ± 0.124 pmole/mg) upon cAMP levels than the phosphodiesterase inhibitor, theophylline (1.36 ± 0.456 pmole/mg), and also that a significant additive effect (t = 2.581 and 2.029, respectively) occurred when both variables were comibned (2.42 ± 0.374 pmole/mg).

A similar positive variable-dependent cyclic nucleotide response was not seen, however, in the coinciding cyclic GMP data (Table VII). Instead, examination of the results for cGMP, represented by the shaded bars in Figure 15, indicate that the levels in the experimental animals (e.g. T, S, and T + S groups) dropped approximately 50% and then remained fairly constant (0.0087 to 0.0092 pmoles/mg) relative to the control animals (0.0164 pmoles/mg), regardless of the nature or number of variables involved (e.g. negative variable response). Furthermore, due to the uniform protein content of the various bone samples, evaluation of the data relating cyclic nucleotide concentration to bone weight reinforces recognition of these aforementioned trends for both cAMP and cGMP.

In comparison, the ratio of cyclic GMP to cyclic AMP generally varies from 1/10 to 1/100 depending upon the tissue and the conditions. The nucleotide ratios of the various groups are summarized in Table VII. Evaluation of this data, which is depicted graphically in Figure 16, indicates that the ratio of cGMP to cAMP in the control is approximately 1/10. However, this ratio increased drastically in the experimental groups due to the previously demonstrated decrease in cGMP and subsequent rise in cAMP. Examination of the relationship among the three experimen-

tal groups demonstrates a variable-dependent response much like that seen in the cAMP response. This is not surprising since cGMP levels changed very little, and the ratio largely reflects the changes in cAMP concentrations previously noted.

Sutural Expansion Vs. Nucleotide Concentration

An attempt was made to relate the concentration of either cAMP or cGMP with concomitant expansion. In order to achieve this, data on nucleotide levels were evaluated in two ways: (1) in relation to increased lateral expansion; and (2) in relation to change in surface area.

The relationship between lateral expansion and nucleotide concentrations was examined by comparing the individual levels of cAMP or cGMP in all 17 animals assayed with coinciding changes in lateral skull dimensions, regardless of animal group. This data (Table X) was then plotted graphically (Figure 17) with respect to lateral expansion (x) vs. nucleotide concentration (y) in an attempt to determine a trend for the pmole levels of both cAMP and cGMP relative to the extent of expansion.

Evaluation of this data indicates that as expansion increases, levels of cAMP rise gradually until reaching a peak at about 2.0 to 2.5 mm of sutural expansion. Beyond this point the levels of cAMP gradually decrease. Conversely, in evaluating the relationship of cGMP with lateral expansion (Figure 17), the levels of cGMP decreased rather rapidly with increases in expansion

until a plateau was reached at approximately 1.0 to 1.5 mm of expansion. From this point the concentration remained at low levels through the remaining expansion range.

Association between surface area and cyclic nucleotide levels was evaluated with the same method as described for the lateral expansion relationship. Examination of this data (Figure 18), demonstrates an effect similar to that seen previously with respect to lateral expansion. Even though some points were scattered, a rough trend was apparent and indicated that cAMP gradually increased to a plateau at about 8 mm^2 of surface area and then tapered off gently as the change in area increased. In comparison, the data for cGMP demonstrated a gradual decrease as area increased. This slope reached a plateau and remained fairly level as changes in area increased.

It must be kept in mind when examining these relationships that they represent only a static portion of a kinetic process which took 21 days to develop. They are in no way intended to represent the condensation of this kinetic process. In other words, these values represent only static relationships at Day 21 and cannot be taken as representative of events which occur at any other time during the experimental period. The fact that the various samples are probably at slightly different stages in their cyclic nucleotide kinetics may account for the variability seen in Figures 17 and 18.

Figure 17.-- Correlation of lateral expansion and cyclic nucleotide concentration. This diagram plots the lateral expansion(mm) in each animal regardless of group against both cAMP and cGMP concentrations(pmole). The solid line represents cGMP and the broken line represents the cAMP correlation. ■ = individual cAMP level, ▲ = individual cGMP level, ◉ = group cAMP average, and ◭ = group cGMP average.

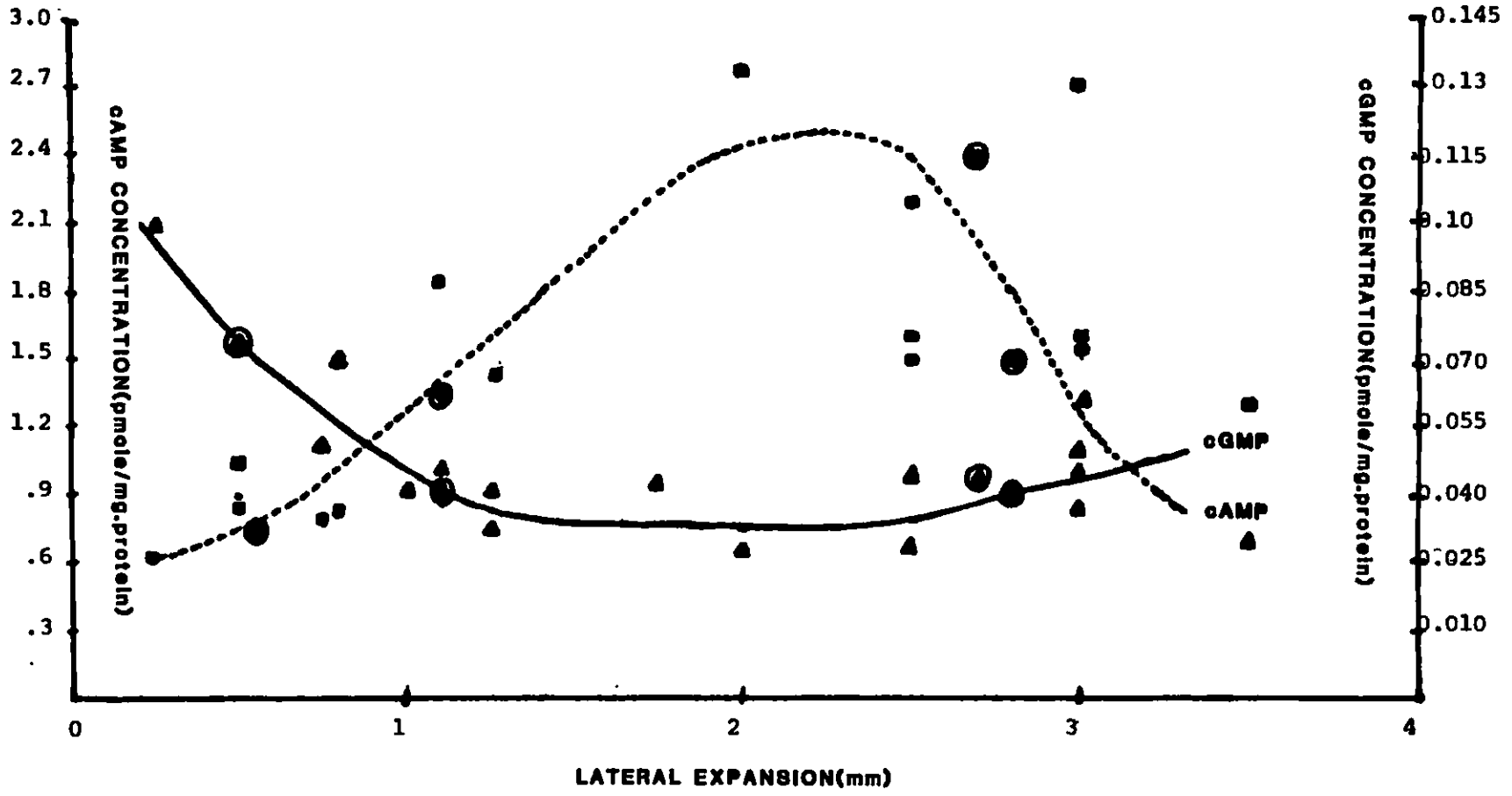
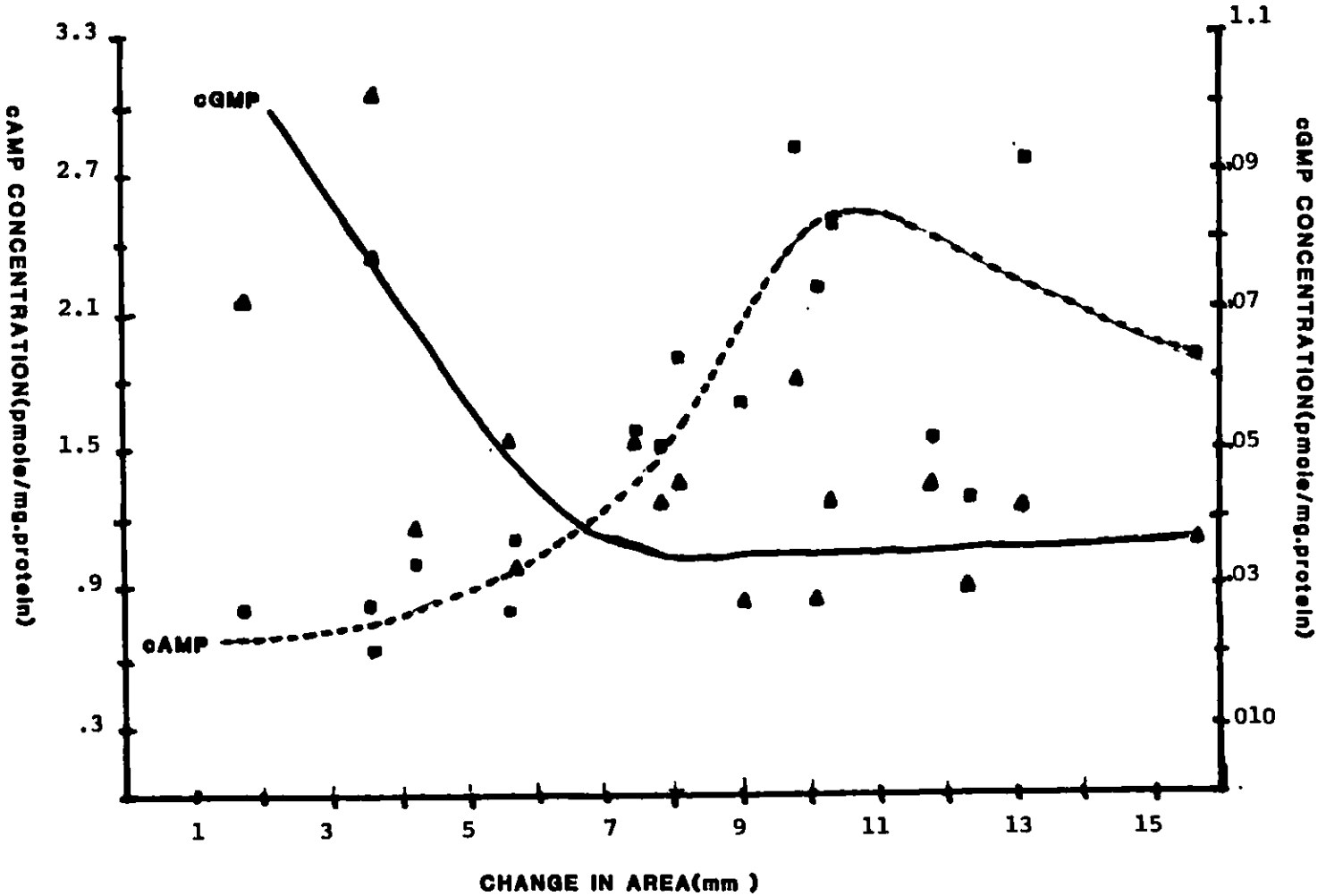

LATERAL EXPANSION vs. NUCLEOTIDE CONCENTRATION
cAMP CONCENTRATION(pmole/mg.protein)
cGMP CONCENTRATION(pmole/mg.protein)
3.0
2.7
2.4
2.1
1.8
1.5
1.2
.9
.6
.3
0.145
0.13
0.115
0.10
0.085
0.070
0.055
0.040
0.025
0.010
0
1
2
3
4
cGMP
cAMP
LATERAL EXPANSION(mm)

Figure 18.-- Correlation of area and cyclic nucleotide concentration. This diagram plots the change in area of the implant triangle(mm^2) in each animal regardless of group against both cAMP and cGMP concentration(pmole). The solid line represents cGMP and the broken line represents the cAMP correlation. ■ = individual cAMP concentration, ▲ = individual cGMP concentration.


AREA vs. CYCLIC NUCLEOTIDE CONCENTRATION
cGMP
cAMP
3.3
2.7
2.1
1.5
.9
.3
1.1
09
07
05
03
010
1
3
5
7
9
11
13
15
CHANGE IN AREA(mm)
cAMP CONCENTRATION(pmole/mg.protein)
cGMP CONCENTRATION(pmole/mg.protein)

Histological Evaluation

Histological examination utilized thin, undecalcified ground sections prepared from a representative animal of each group. These sections were viewed under blue light at 125X magnification and evaluated on the basis of: (1) microscopic appearance; (2) amount of new appositional growth; (3) evidence of remodeling changes; and (4) evaluation of the suture.

In evaluating the general appearance of sections from the various groups, it was noted that in those animals in which springs had been implanted, the bone was generally thicker and appeared more irregular and cancellous, presumably as a result of compensation for the increased force. This was especially evident in the animal from the T + S group. Bone sections from this animal were very atypical relative to the other groups. The bone was much thicker (e.g. 630 u) and composed of a fine cancellous, nonlamellar bone tissue. This "woven bone," which is indicative of rapid bone growth, was not seen in any of the other animals, even in the S group. This may suggest the occurrence of a secondary infection or other pathological process in addition to the experimental variables. Unfortunately this animal was sole representative of its group and comparisons could not be made.

Sections through the implant holes indicate a thickening of the bone in the vicinity of the hole. This build-up, which occurred mainly on the endocranial surface, was seen in sections from animals with and without springs. Presumably, the implants,

which probably protruded inward, acted as an irritant to the endosteal tissue, thereby stimulating localized bone opposition. Gross examination of sections from animals in the control and T groups revealed a more typical uniform histological pattern. Samples were somewhat thinner (e.g. approximately 300 u) with a well defined inner/outer cortical plate and diploe.

In the control animals a continuous vital dye line with a fairly uniform apposition of bone (e.g. 80 u) occurred on the endocranial surface.[3] Conversely, a slightly greater amount of apposition occurred on the ectocranial surface (90-100 u) and appeared greatest at the lateral edges (120 u). This provided a flattening of the external contour in relation to the curvature of the internal vital dye line (e.g. the initial bony contour). A similar pattern was found in sections from the T group animal, and no discernable difference between the groups were evident.

Evaluation of the animal from the S group indicated a relatively flat bone contour compared to the control. The vital dye line, which marked the original calcification front, was discontinuous (resorbed) in the middle one-third of the section, indicating a greater amount of new bone remodeling in

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[3]Measurements are approximate and were made from the vital dye line to the outside surface using a calibrated microscope micrometer. Measurements were made only in areas in which the outside surface and dye lines were distinct. No attempt was made to differentiate the location of the calcification front or account for the amount of osteoid tissue in measurement.

the sutural area. Furthermore, a reversal in the rate of new bone apposition was noted relative to the control and T animals in whicn greater amounts of endocranial apposition (150 u) occurred in relation to ectocranial opposition (48 u).

Examination of consecutive sections gave the impression that the spring had resulted in a slight outward opening movement of the parietal bones causing them to pivot somewhat about their lateral edges. At the same time an inward, remodeling reaction in the area surrounding the sagittal suture maintained and even flattened the original bony contour.

A similar sequence apparently occured in the T + S animal, only to a greater extent. Vital dye lines in this animal were almost totally resorbed and were only found in the lateral edges of the bone section. As in the S group, a much greater extent of endocranial apposition (300 u) occurred in relation to ectocranial apposition (180 u). As suggested previously, this may relate to a remodeling reaction in response to outward parietal expansion.

Hinrichsen and Storey (1968) demonstrated an increase in sutural width concomitant with mechanically-induced lateral expansion. In the present investigation, however, no significant conclusions could be demonstrated concerning sutural width. This is not to suggest that differences were not present. The sections did not allow an accurate study of changes in sutural width. Largely, this was because well defined sections through the suture in this small sampling were insufficient to draw a conclusion. In

addition, due to the single injection of vital dye, it was impossible to define the present calcification front and thereby the amount of osteoid tissue present within the suture.

DISCUSSION

Many investigations have shown the general importance of the cyclic nucleotides in relaying the effect of external stimuli to their molecular targets within the cell (Goldberg, et al., 1973; Peck and Klahr, 1979). Evidence demonstrates, however, that this effect is both tissue and cell specific and that particular concentrations or ratio variances may have a diversity of actions for different cell types.

The purpose of this investigation was to examine the effects of varying concentrations of cyclic AMP and cyclic GMP on sutural growth and remodeling when subjected to an orthopedic force. Findings showed that an expansile force applied across a suture generated a significant increase in the cAMP concentration along with a subsequent decrease in cGMP in the area around the affected suture. The important role of cyclic nucleotides in sutural growth was further demonstrated by a phosphodiesterase-inhibited potentiation of cAMP and cGMP which, in the absence of an orthopedic force, also resulted in an effect similar, but of lower magnitude, to that demonstrated by the orthopedic appliance (e.g. expansion spring). Theophylline, which competitively inhibits cyclic nucleotide breakdown, was shown to increase cAMP and decrease cGMP with a slight resultant sutural expansion relative to the control. Although this difference is only significant

at the 0.25 level (student + distribution) and thereby only indicative of a trend, a greater level of significance may have been obtained had the sample size been larger. Furthermore, an additive expansile effect was obtained (Figures 11 and 12) when theophylline was used to potentiate the mechanically-induced change in cyclic nucleotide levels seen with the expansion spring, thus supporting the hypothesis that cAMP/cGMP play a mediating role in regulating the sutural effect of orthopedic forces.

Results from the cyclic nucleotide analysis (Figure 15) demonstrate that increases in cAMP occurred whenever either a mechanical force or phosphodiesterase inhibitor was administered separately, but that an additive increase occurred when the two variables were combined. This effect was similar to that demonstrated by Burrows (1978) in his study on diazepam potentiated alveolar bone remodeling responses to orthodontic forces. Diazepam (e.g. valium), which has been demonstrated to have moderate inhibitory effects upon phosphodiesterase activity in brain tissue (Levin and Weiss, 1975), resulted in a relatively greater amount of orthodontic movement when administered in conjunction with the mechanical force. Likewise in the present study, an additive increase in cAMP was reflected by a coinciding increase in the amount of expansion relative to that realized when either variable was examined separately.

This positive variable-dependent effect, however, was not seen in the cGMP levels. Davidovitch (1980), in his study on al-

veolar bone, demonstrated that a mechanically applied force on bone resulted in an increase in both cGMP and cAMP levels. This effect for cGMP was not observed, however, in the present study, although this may be due to either (1) the time factor involved (e.g. levels may have risen initially and thereafter fallen) or (2) the different methods of cyclic nucleotide quanitation. Davidovitch's immunohistochemical technique measures only semi-quanitatively the cyclic nucleotides which are bound to the kinase enzyme at the time of fixing. Conversely, the level of cGMP was found to decrease 50% regardless of the variable. Further, the additive effect that was seen with cAMP did not occur in cGMP. The level, once reduced, remained constant indicating a possible variable saturation of the cGMP response. The question arises as to why the levels of cGMP dropped, particularly in lieu of the presence of a phosphodiesterase inhibitor. Levin and Weiss (1979) demonstrated the presence of several phosphodiesterase isozymes in nerve tissue, only one of which was significantly affected by an xanthine inhibitor. Furthermore, elimination of cGMP has been shown to involve cellular excretion, as well as degradation (Goldberg, et al., 1973). Therefore, it is possible that either one of these mechanisms could account for the decrease in cGMP. However, a lack of responsiveness to the external stimuli (e.g. theophylline and/or the mechanical force) tends to suggest that an inhibition of guanylate cyclase occurred and may be the primary cause, even though the other two factors may also be exert-

ing an effect. A negative feedback mechanism between cAMP and cGMP has been demonstrated by White and Aurbach (cited by Goldbert, et al., 1973) in which high levels (0.4mM) of cAMP were shown to produce as much as 50% inhibition in guanylate cyclase activity. From this information, it is tempting to suggest a mechanism similar to that put forth by Jilka and Cohn (1981) for bone resorption, in that cGMP levels might function in some way with or without cAMP to switch the cells into an "active mode," and once activated the amount of cellular activity would become dependent upon the level of cAMP.

The non-collagen protein content of the various samples was used as an approximate estimate of cellular mass in order to standardize cyclic nucleotide measurements. A fairly uniform protein content was demonstrated in the present study and although no attempt was made to determine the type or number of cells present, it was felt that the changes in cyclic nucleotides observed were due primarily to intracellular cAMP/cGMP fluctuations rather than an increase in the total number of cells. The non-collagen content of the bone matrix, which can be expected to be between 1-13% of total dry weight (Ham, 1975), was demonstrated to be somewhat higher in this study (22%). This difference can be related to the fact that hemopoietic tissue contained within the cancellous layer, along with periosteal/endosteal tissue were also included in the determination.

In attempting to relate the actual concentrations of cAMP

and cGMP to millimeters of expansion (Figures 17 and 18), a rough trend develops in which concentrations of cAMP are shown to have a positive relationship with expansion up to a point. Beyond this peak, however, there is a gradual decline in slope. The apparent contradiction of this negative slope can best be explained by the fact that this investigation represents only a single time point in a kinetic function which operated over a 21 day development period. Data collected at this point are not meant to be representative of other points during the experimental period although trends are discernable. In a time-course study, Davidovitch (1980) showed that cyclic nucleotides fluctuate with time and that following a mechanical stimulus there is an initial rise in cAMP followed by a gradual decline. Furthermore, the design of the expansion spring was such that as the arms moved apart, the forces delivered decreased proportionally. Thus, it can be suggested that the negative slope is not a contradiction, but actually represents a decline in cAMP levels as the spring delivered force decreases. In other words, the points to the right of the peak (e.g. negative correlation) in Figure 17 merely represent concentrations of cAMP that have already reached their peak prior to Day 21 and are presently declining as the applied force decreases.

Evaluation of the sutural expansion analysis (Figure 12) indicates that lateral expansion reflects the variable dependent response previously described for cAMP levels (with the possible exception of the T + S group). This is similar to the correla-

tions between cAMP and the amount of orthodontically induced alveolar remodeling demonstrated by Davidovitch, et al. (1980) and Burrows, et al. (1978). An apparent contradiction, however, appears in the T + S group in that despite significantly increased cAMP levels relative to the other groups, its lateral expansion is, in fact, less than that seen in the S group. This discrepancy can be explained, however, when the anteroposterior dimensional changes of each group are taken into account (Figure 12). Enlow (1975) has pointed out that the internal dimensions of the neurocranium are determined primarily by the expanding brain. Assuming that brain mass is unaffected and its shape flexible within limits, an increase in lateral width would therefore necessarily result in a compensatory decrease in anteroposterior growth. An example of this can be demonstrated in a child in which one or more of the coronal or sagittal sutures fuse prematurely prior to completion of brain expansion. These prematurely closed sutures require compensation at the remaining sutures resulting in variable degrees of brachycephaly or dolichocephaly. Thus, anteroposterior dimensional changes, as determined in the present investigation, could be expected to compensate to some extent as the lateral dimensions increase. Such an effect was seen in the S group, but not in the T + S group (Figure 12). This discrepancy can be better understood once it has been pointed out that unless the lateral expansion force is exactly perpendicular to the suture, a skewing effect will occur in which some of the lateral force may be translated

into an anteroposterior vector. An increase in both dimensions relative to the control, as seen in the T group, however, demonstrates the non-specific potentiation of the phosphodiesterase inhibitor. By inhibiting this enzyme in a rapidly growing animal a potentiation of all cAMP/cGMP mediated stimuli (e.g. hormones) should be expected (Weiss and Levin, 1978).

Evaluation of the surface area of the implant triangle gives a better indication of increases in expansion because this measure did not depend solely on one vector but rather a combination of both anteroposterior and lateral changes. Measures assessing changes in the implant area (Figure 13) correspond well to the demonstrated variable-dependent response seen in the levels of cAMP. Therefore, a definite and positive relationship can be seen between expansion changes and levels of cAMP.

Histological evaluation of undecalcified sections of bone was carried out to access the remodeling changes that occurred with expansion. Due to the limited number of sections taken from each group, however, only a broad conclusion can be drawn. Evidence indicated that the expansion force resulted in an upward and outward movement of the parietal bones with a subsequent downward remodeling at the sagittal suture. Although actual cranial measurements were not taken in this investigation, it may be suggested that the animal's head, as a whole, did <u>not</u> become wider, but that the expansion force generated by the spring was primarily compensated for by an upward opening of the sagittal suture with very

little net change in the rest of the cranium. This can best be demonstrated when noting that the buttress of the zygomatic arch is immediately lateral to the parietal bones (Figure 3, Plate II) and can offer significant resistance to a lateral force.

The results of this investigation show that cyclic nucleotide levels, particularly that of cyclic AMP, may be important mediators of the sutural response of orthopedic forces. Even though general trends observed in this study indicate that sutural expansion can be positively related with the increase in levels of cAMP and a decrease in cGMP, a definitive test of the hypothesis regarding the relationship between cAMP/cGMP and bone growth will need the support of a time-course study in order to establish an overall kinetic picture of cyclic nucleotides with time. These changes in cyclic nucleotide levels are not as simplistic as they might seem from evidence presented in this investigation. Control of cellular processes is a very complex procedure which, in addition to cAMP and cGMP, is also thought to involve other mediators such as Ca^{++} (Peck, et al., 1981). Therefore, cellular events cannot be described solely in terms of the cyclic nucleotides, but must take into account the combination of interactions of all mediators.

SUMMARY AND CONCLUSIONS

Local control mechanisms that mediate the growth and remodeling in skeletal tissue of the craniofacial apparatus, and thereby their response to externally applied orthopedic forces, are complex and at present not fully understood. One control pathway, however, that has received growing attention in the last 20 years has been the action of the cyclic nucleotides (e.g. cAMP and cGMP). Reserach has shown that cAMP and, to a lesser extent, cGMP both have important mediating functions in the cellular response to environmental stimuli in a wide variety of tissues and cell types. In addition, the response of these cyclic nucleotides have been shown to be both tissue and cell specific. Results of previous studies demonstrated that cAMP and cGMP are important mediators in the alveolar remodeling response to orthodontically induced tooth movements. However, the specific nature of the effects that externally applied forces have on the growth and remodeling of bones in the craniofacial complex is still unclear.

The present study was performed to examine the effects of an orthopedic stimulus upon cyclic nucleotide levels of the involved bones, and how these levels relate to growth and remodeling changes that occur at the sutures. To study this effect an expansile force was applied across the sagittal suture in young growing rats and corresponding cyclic nucleotide levels were experimental-

ly altered by the use of theophylline, a phosphodiesterase inhibitor. The sample consisted of 22 female Sprague Dawley rats which were divided into four groups, with one group acting as a control. The remaining three experimental groups were as follows:

1. T group-animals were force fed theophylline (1 mg/100 g body weight), a potent phosphodiesterase inhibitor;
2. S group-animals received a surgically implanted expansion spring across the sagittal suture to act as a mechanical stimulus;
3. T + S group-animals received theophylline four times a day as well as the implanted spring.

Cranial implant markers were placed in the frontal bone and in each parietal bone in all animals to measure dimensional changes in regional surface area of the calvaria over the experimental period. In addition, one animal from each group was injected with a vital dye for subsequent histological evaluation.

At the end of a 21 day experimental period, the animals were decapitated and the area around the sagittal suture removed. The bone samples were analyzed and data collected in four categories:

1. Sutural expansion and increase in surface area of the calvaria were determined from cranial implant measurements in order to assess the sutural response to the different experimental variables;
2. Levels of cAMP and cGMP were individually quanitated by specific radioimmune assay procedures to evaluate any variable dependent changes in concentration;
3. Analysis of non-collagen protein content of the sample was conducted to provide a standard unit by which to compare the levels of cyclic nucleotide, and;
4. Histological observation to evaluate the remodeling changes that occurred at the suture during the experimental period.

Analysis of sutural expansion demonstrated that a greater amount of expansion can be obtained in response to a mechanical stimulus when the expansion force is combined with a phosphodiesterase inhibitor. In addition, it was shown that the phosphodiesterase inhibitor alone also elicited a small, non-specific (e.g. both lateral and anteroposterior) increase in calvarial growth relative to the control, indicating active growth stimuli other than the mechanical force (e.g. hormones).

Values for cyclic nucleotide concentrations indicated that levels of cAMP were increased in the presence of the phosphodiesterase inhibitor relative to the control. This increase was not as great, however, as present when the mechanical stimulus was applied. Also a significant additive effect was observed when both of these variables were combined. The effects related well with the coinciding expansion which occurred in these groups. Cyclic GMP, on the other hand, did not follow the same positive variable-dependent response as cAMP. Concentrations of this nucleotide decreased approximately 50% and remained constant regardless of the nature or number of variables involved.

Analysis of the non-protein content of the various bone samples indicated a fairly uniform protein content in a range between 21.4 and 22.4% of the total dry weight.

Histological examination indicates that, in addition to minor structural changes (e.g. bone thickening), the expansion force across the sagittal suture produced an outward-upward bone rotation at this suture with a subsequent increase in downward

remodeling, resulting in a slightly flattened cranial contour. The overall expansion effect on the head as a whole was thought to be minimal due to the buttressing effect of the zygomatic arch.

The most significant findings of the present study were:

1. Orthopedic forces have a definite effect on the intracellular cyclic nucleotide levels in the sutural areas of the involved bones, resulting in a significant rise in cAMP and a drop in the levels of cGMP;
2. Theophylline, a phosphodiesterase inhibitor, when used alone, resulted in a similar increase although of lower magnitude in cAMP levels in comparison to that seen with the mechanical stimuli with a significant additive effect when both variables were combined;
3. Concentrations of cGMP were found to decrease approximately 50% and remain stable relative to the control values regardless of the variable(s) involved;
4. A positive association was demonstrated between the amount of expansion observed and the level of cAMP present in the bone around the suture; and
5. Sutural expansion forces resulted in extensive remodeling changes primarily at the sagittal suture with very little expansile effects on the rest of the craniofacial complex as a whole.

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