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THE EFFECTS OF APPLIED TENSION AND PRESSURE UPON THE CONCENTRATIONS OF PROSTAGLANDIN E_2 AND CYCLIC 3' 5' ADENOSINE MONOPHOSPHATE IN THE MIDSAGITTAL SUTURE OF THE RAT

by

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Abstract

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The biochemical mediators in bone tissue responsible for the recognition of mechanical stress and its subsequent translation into a cellular response remain undefined. The role of prostaglandin E_2 in the local control process is receiving increased attention. cAMP has long been known as a "second messenger" of cell activation as it has been shown to be responsive to a host of stimulating agents. That these two substances interact during the local control process of bone remodeling is a postulation formulated, for the most part, from tissue culture research.

The present study was initiated to examine the effects of mechanical forces upon the levels of PGE_2 and cAMP *in situ*. Springs calibrated to deliver 20 g of tensile or compressive force were implanted across the midsagittal sutures of 21 day-old female Sprague Dawley rats. Osteotomies within the parietal bones were used in all animals to partially isolate the experimental sites and thus minimize the influence of adjacent cranial region.

In order to detect PGE_2 dependent changes in the cAMP concentration, indomethacin, a potent inhibitor of cyclooxygenase, was used in selected groups in a dosage of 1 mg/kg to suppress the PGE_2

response. A sample of 24 animals was used and divided equally (four animals each) into the following six groups: Tension; Tension + Indomethacin; Pressure ; Pressure + Indomethacin; Control; and Control + Indomethacin.

At the end of a five day experimental period, bone samples containing the midsagittal suture were removed under general anesthesia, immediately frozen in liquid nitrogen, and lyophilized. An extraction procedure that allowed recovery of PGE_2, cAMP, and cellular proteins from the same bone sample at an efficiency suitable for quantitative measurement was developed. Samples were extracted and individually quantitated for PGE_2 and cAMP content by specific radioimmunoassay procedures. These values were related to a measure of active bone tissue as determined by analysis of the non-collagen protein content for each sample.

This investigation demonstrated a simple surgical procedure that provided a good model of the bone remodeling response. Findings showed that PGE_2 levels were elevated equally in both tension and pressure groups over controls. Furthermore, subcutaneous administration of indomethacin decreased PGE_2 concentrations below controls even in stressed groups. Changes in cAMP levels were found to vary with tension and pressure. Applied tension caused an eight-fold increase above controls but pressure produced only a two-fold increase, suggesting different roles for cAMP in the interpretation of mechanical stress at the cellular level. Suppression of the PGE_2 response reduced cAMP levels by half with applied tension but had no

effect with applied pressure. PGE_2 thus partially regulates the cAMP response to tension, but other mechanisms beside PGE_2 production are contributing to increased cAMP levels in the tension group. However, the modest cAMP increase in response to pressure is not associated with PGE_2 synthesis.

DEDICATED

TO

Bronko and Martha, my parents, who have lovingly sacrificed throughout their lives to give me the opportunities they never had. My indebtedness is so great that my only form of repayment is to love my own children as my parents have loved me.

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INTRODUCTION

One of the most heavily studied yet poorly understood areas of orthodontic research is the cellular basis of local growth control. It is fairly well understood what happens but not how it happens. Recently, a great deal of effort has been focused upon the roles of prostaglandin E_2 (PGE_2) and cAMP in the local control of bone remodeling. It is toward these two mediators that this investigation is directed.

Rodan *et al.* (1975) were the first to implicate cAMP as a mediator of mechanical stress in cultured bone cells. Since then, a number of other studies have been done, particularly by Davidovitch (1975, 1976, 1984), showing elevated cAMP levels in bone cells surrounding orthodontically treated teeth. The first link between PGE_2 control of cAMP in bone cells was presented by Yu *et al.* (1976). PGE_2, like parathyroid hormone, was further shown to regulate cAMP levels by a direct stimulatory effect on adenyl cyclase (Marcus and Orner, 1977). Soon afterwards, Harell (1977), then Somjen (1980), found that mechanical stress applied to cultured bone cells caused an immediate increase of PGE_2 followed by elevated cAMP levels. This chain of events was linked together by Yoshizawa *et al.* (1983) who demonstrated specific PGE_2 receptors in rabbit alveolar bone. Unfortunately, the majority of evidence for PGE_2 anD cAMP as mediators of mechanical stress is derived from *in vitro* studies. Most investigations on endogenous PGE_2 or cAMP levels *in situ* have used

immunohistochemical techniques, which are indirect methods of quantitation. These methods can measure only those substances bound to enzyme systems at the time of fixing. Because of this, they are inadequate when used to determine tissue concentrations of a compound thought to be a local cell to cell messenger undergoing active secretion. The nature of the relationship between applied mechanical stress, prostaglandins, and cAMP *in situ* has not been conclusively shown.

A major difficulty encountered when investigating the processes of bone remodeling *in situ* is defining the tissue reactions to tension or pressure. A section of bone upon which tensile forces are applied can have a predominately resorptive surface. Likewise, the application of pressure does not guarantee a corresponding resorptive process. These contradictory events occur because the control process of bone remodeling is multifactoral. Each contributing factor, including stress, piezo effect, and local oxygen tension among others, influences the course of remodeling, sometimes in opposing directions. The manipulation of all local control mechanisms to attain a *pure* resorptive or depository response in an experimental animal is a difficult task.

With these biological limitations in mind, the present investigation attempts to establish a model of bone resorption and deposition *in situ*. A tensile or compressive force was placed across the midsagittal suture of growing Sprague Dawley rats. These sutures were isolated from the growth and structural influence of adjacent

sutures by means of osteotomies, designed to avoid free-floating bony segments. Cellular concentrations of PGE_2 and cAMP in response to applied tension and pressure were measured by direct radioimmunoassay of extracted suture samples. PGE_2 regulation of cAMP levels was assessed in tension and pressure with the use of indomethacin, a potent inhibitor of the cyclooxygenase enzyme. Sample values for PGE_2 and cAMP were expressed in terms of non-collagen protein content, a measure of active bone tissue, as determined by a variation of the Folin protein assay.

LITERATURE REVIEW

The basic principle underlying the field of orthodontics is that a physical force, whether applied orthodontically or orthopedically, will cause a clinically observable change in tooth or jaw position. The mechanism by which cells recognize physical stress is not fully understood. Early work by Sandstedt (1901), Oppenheim (1911), Reitan (1947), and others demonstrated that mechanical stress induced histologic changes in bone tissue. The nature of these changes remained descriptive, as the cause for cellular reorganization was elusive. A number of local "messengers" have been postulated, including electrical charges, cyclic nucleotides, prostaglandins and the divalent cation calcium (Ca^{+2}). These factors, if operable, must direct cellular response amidst an environment of hormonal influences, local tissue conditions, such as oxygen levels and pH, and availability of nutrients. It is therefore not unexpected that research in this area has been controversial.

Sutherland and Rall (1960) were the first to suggest that adenosine 3':5' cyclic monophosphate (cAMP) played a key role in the control of intracellular events. Following this initial work, a number of other investigators have since lent further support to the role of cAMP as a "second messenger". Studies with cultured bone cells (Marcus _et al._, 1977; Yu _et al._, 1976; and Hursch _et al._, 1978) have repeatedly produced results showing elevated intracellular levels of cAMP when such agents as parathyroid hormone

(PTH), calcitonin(CT), and prostaglandins (PG) were added to the medium.

Cyclic nucleotides, specifically cAMP and cGMP, were first implicated as a mediator of mechanical stress by Rodan _et al._ (1975). They exposed cultured tibia from chick embryos to physical stress. Nucleotide levels were either increased or decreased depending upon cell type (cells with proliferative potential showed increases in cGMP; hypertrophying cells showed increases in both cAMP and cGMP; and differentiated cells, chondrocytes and osteoblasts, showed decreases in cGMP). From these results, they postulated that cyclic nucleotides may be messengers in conveying physical stress into active bone remodeling. Furthermore, the nucleotide response appeared to be cell specific. Concurrently, Davidovitch and Shanfield (1975) studied cAMP changes in alveolar bone of orthodontically treated cats. They reported alterations in cAMP content of bone samples taken at both tension and compression sites. However, the increases in cAMP were modest, reaching levels only double that of controls.

In a follow-up study, Davidovitch and Montgomery (1976) used immunohistochemical techniques to localize cAMP containing cells in the periodontal ligament (PDL) of orthodontically treated teeth in cats. Differences in staining intensity for cAMP were observed between tension and compression sites. In areas of PDL compression intensely stained cells were found initially but disappeared completely after 24 hours, gradually increasing in staining intensity

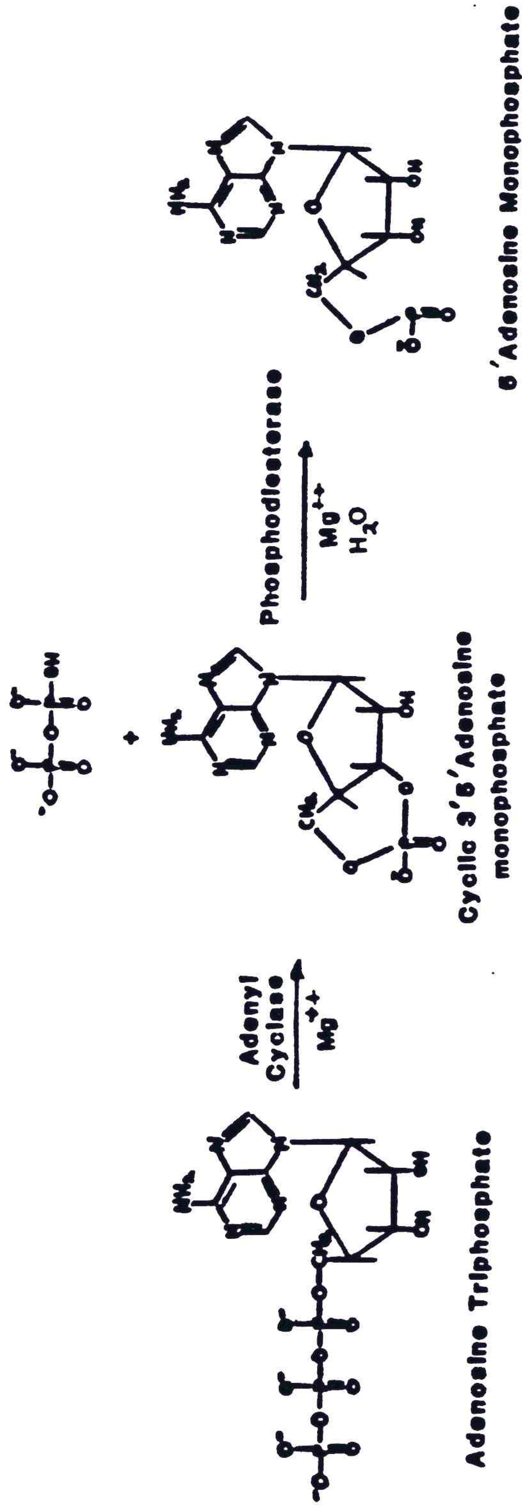
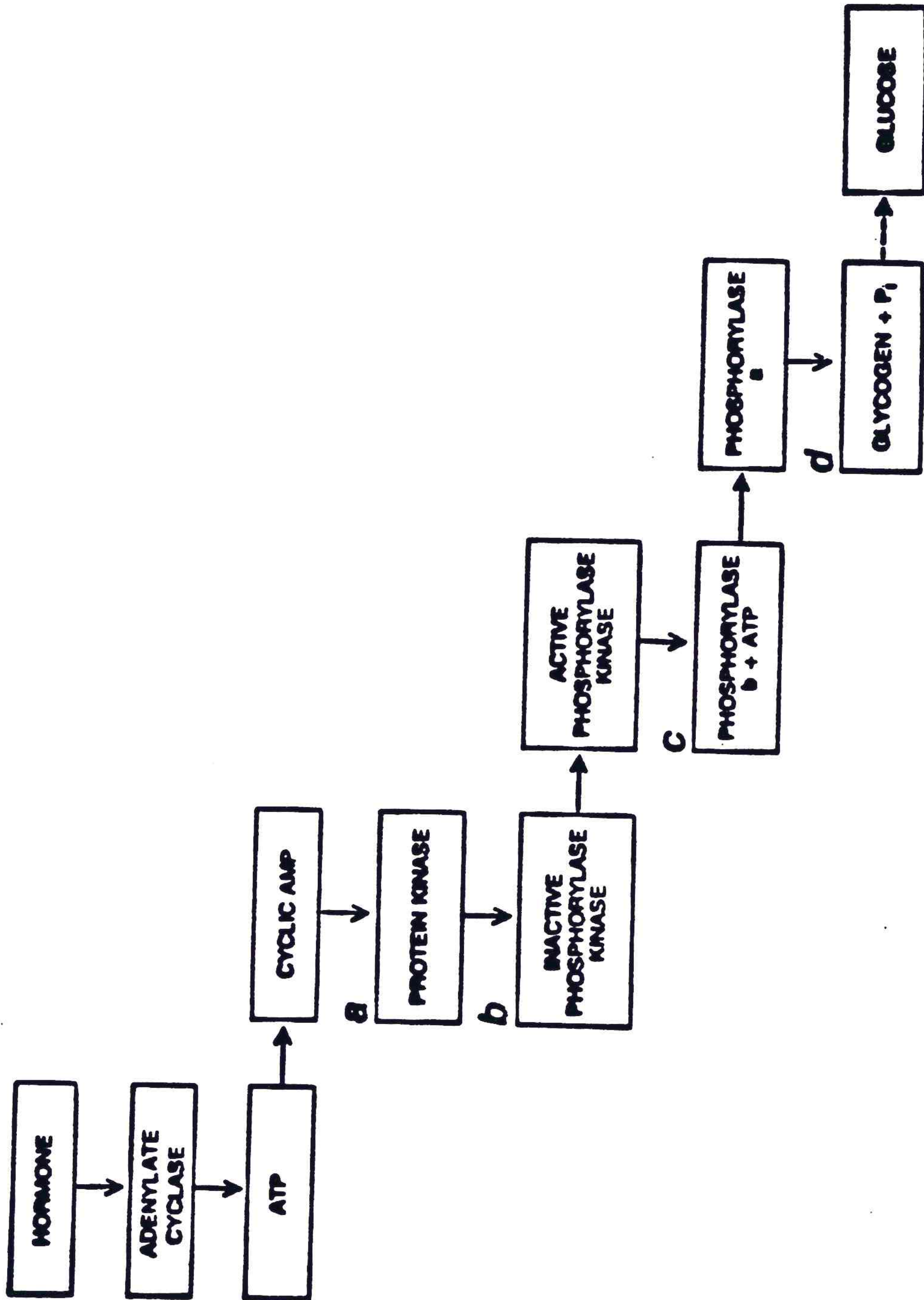

Adenosine Triphosphate
Adenyl Cyclase
Mg++
Cyclic 3'5'Adenosine monophosphate
Phosphodiesterase
Mg++
H2O
5'Adenosine Monophosphate

Figure 1b - Example pathway of cyclic AMP action. This schematic represents the initial sequences 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 tranformed phosphorylase then starts the sequence (d) that converts glycogen into glucose.


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

thereafter. In contrast, cAMP stained cells at tension sites were found to decrease initially, reaching minimal levels after 6 hours of appliance activation. Afterwards, cAMP staining intensity slowly increased up to 28 days. Using similar immunohistochemical techniques, Brin *et al*. (1981) studied the effect of rapid palatal expansion on the midpalatal suture in cats. They reported increased staining intensity for cAMP in osteoblasts, osteocytes, and osteoprogenitor cells at the suture site. These cells were located within areas of newly deposited bone.

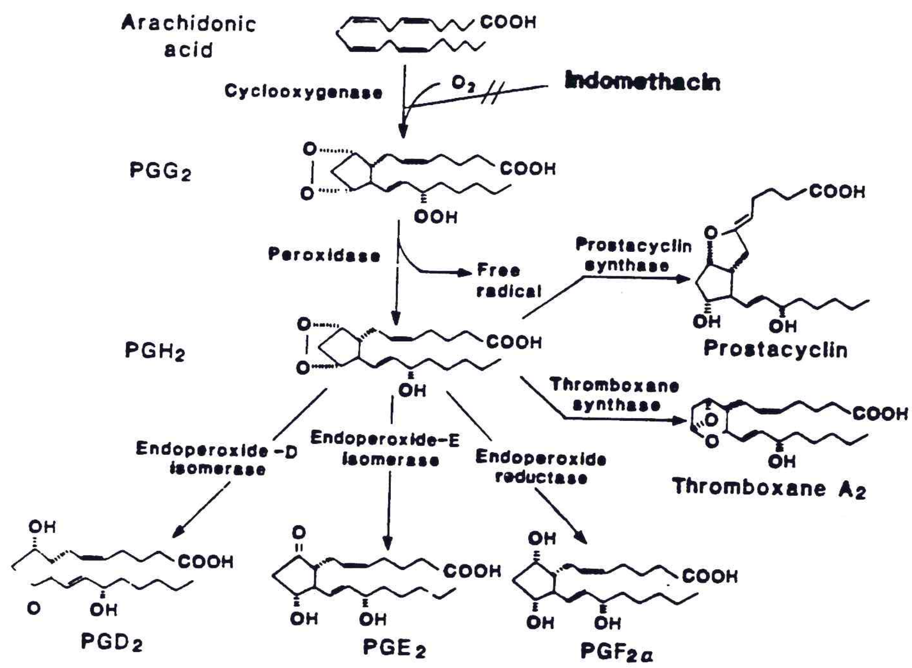
Benson (1983), using radioimmunoassay procedures, found a significant increase in cAMP and a decrease in cGMP when the midsagittal sutures of rats were subjected to mechanical tension. Theophylline, an inhibitor of phosphodiesterase activity, likewise increased cAMP levels and potentiated the effect of mechanical stress. Interestingly, a positive correlation between elevated cAMP levels, even when caused by theophylline alone, and sutural expansion was shown.

The available data supports the hypothesis that physical stress is able to induce changes in cellular cAMP concentration and that these changes are associated with the cytodifferentiation leading to bone remodeling. The evidence for cAMP as a modulator of the cellular response to mechanical stress appears quite strong.

That lipids may be involved in the cellular response in tooth movement was suggested by Buck *et al*. (1973). Before this, prostaglandin had been known to stimulate bone resorption *in vitro* (Klein

and Raisz, 1970; and Tashjian et al., 1972). Prostaglandin E_2 (PGE_2) has also been implicated as a causative factor in the inflammation and bone loss associated with periodontal disease (Goodson, 1974), and the resorptive process surrounding dental cysts (Harris et al., 1973). Since there was convincing evidence for prostaglandins as a local mediator of bone resorption, the notion followed that a relationship exists between prostaglandins and cAMP in skeletal tissue.

Although prostaglandins were known to stimulate cAMP levels in other tissues (Butcher and Baird, 1968), the first evidence that prostaglandins had a similar effect on bone cells was produced by Yu et al. (1976). They reported that PGE_2 caused a dose related increase in cAMP content when added to incubated bone cells isolated from rat calvaria. Lending further evidence to prostaglandin control of cAMP levels, Harell (1977), working with cultured rat periosteum, demonstrated an immediate increase in PGE_2 followed by an increase in cAMP after mechanically stressing the culture dish. The observed increase in PGE_2 concentration was shown to be the result of de novo synthesis of the prostaglandin, as indomethacin, when added to the culture medium, completely inhibited the PGE_2 response. Using the same experimental model as Harell, Somjen et al. (1980) uncovered a cause-effect relationship between PGE_2 and cAMP in cultured bone cells. They obtained decreased levels of cAMP when PGE_2 synthesis was inhibited by indomethacin and elevated cAMP levels when PGE_2 was added directly to the culture medium.

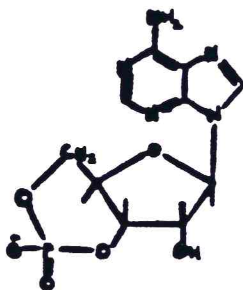

Arachidonic acid
COOH
Cyclooxygenase
O_2
Indomethacin
PGG2
COOH
OOH
Peroxidase
Free radical
PGH2
COOH
OH
Prostacyclin synthase
COOH
OH
OH
Prostacyclin
Thromboxane synthase
COOH
OH
Thromboxane A2
Endoperoxide -D isomerase
Endoperoxide-E isomerase
Endoperoxide reductase
OH
COOH
O
OH
PGD2
O
COOH
OH
OH
PGE2
OH
COOH
OH
OH
PGF2α

In vivo studies have also shown prostaglandins to be involved with the remodeling response. Inducing experimental tooth movement in rats and monkeys, Yamasaki *et al*. (1980, 1982b) demonstrated (1) a dose related inhibition of osteoclast formation when indomethacin was administered prior to and during tooth movement, (2) a dose dependent increase in osteoclast formation with local injection of PGE_1 or PGE_2, and (3) almost twice the rate of tooth movement when PGE_2 was injected into the submucosa adjacent to the teeth. Recently, Yamasaki (1984) extended this study to human subjects undergoing orthodontic tooth movement, again showing a two-fold increase in the rate of tooth movement when PGE_1 was injected into the adjacent submucosa. Davidovitch and Shanfeld (1980c) measured endogenous PGE_2 levels in alveolar bone of cats after orthodontic tooth movement. They found a persistent elevation of PGE_2 at compression sites but only transient elevation of PGE_2 levels at tension sites, suggesting the presence of two different mechanisms.

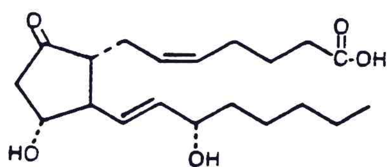
In contrast to the PG $\rightarrow$ cAMP $\rightarrow$ bone remodeling sequence of associations, Lerner and Gustafson (1980) presented evidence that cAMP actually inhibits PG induced bone resorption. Incubation of $C_1^{45}ca$ labeled rat calvarium with both PGE_2 and cAMP showed an inhibition of calcium mobilization when compared to incubation with PGE_2, alone. The inhibitory effect of cAMP was dose-related. The same result was found when PGE_2 was incubated with theophylline, which acts to elevate intracellular cAMP levels. Similarly, forskalin, which raises cAMP levels by activating the enzyme adenyl

cyclase, had a biphasic effect on calcium mobilization when added to incubated calvarial bones (Lerner *et al.*, 1984). An initial reduction was followed by a stimulation of calcium release into the medium. On the other hand, PTH, which is also known to increase intracellular cAMP levels, produced a rapid and sustained release of calcium from the bone tissue. The investigators concluded that cAMP could not mediate the initial action of PTH but may be involved with its delayed action. However, the forskalin treated cells had cAMP levels 20x those stimulated by PTH, which may account for the differences in calcium mobilization.

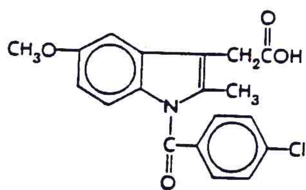
To further examine the role of cAMP and the Ca^{+} ion in bone resorption Yamasaki *et al.* (1983) tested the effects of a number of agents on the formation of osteoclasts during orthodontic tooth movement. Imidazole, which decreases intracellular cAMP by activating phosphodiesterase, and theophylline, which increases intracellular cAMP by inactivating the same enzyme, were shown to significantly decrease and increase, respectively, the appearance of osteoclasts and bone resorption. The effects of an analogue of cAMP, DBcAMP, and a Ca^{+2} ionophore, A-23187, were also tested. While neither agent alone altered the rate of mobilization of osteoclasts, when administered together a significant enhancement of osteoclasts was seen. When incubated with PTH or PGE_2, Ca^{+2} has been shown to enhance the cAMP response of bone cells in a dose dependent manner (Peck *et al.*, 1981). Also, Harell (1977), after mechanically stressing cultured bone cells, reported an increased



cAMP



PROSTAGLANDIN E_2



INDOMETHACIN

accumulation of intracellular calcium accompanying rising cAMP concentrations. In another study, a calcium antagonist, D600, inhibited the resorption effect of PGE_2 but did not alter the effect of increased intracellular cAMP (Yu et al., 1976). This suggested that the resorptive effect of PGE_2 was not a result of increased cAMP levels but rather to an increase in the influx of Ca^{+2} into the cell. Nonetheless, the majority of reported data suggest that the Ca^{+2} ion enhances both intracellular cAMP concentrations and the appearance of osteoclasts and subsequent bone remodeling.

Mechanical stress, when applied to bone cells, must be communicated to the cell membrane in order to trigger a response. Any of the following, either alone or in combination, must occur, (1) a flow of ions across the membrane, (2) deformation of structural proteins, exposing them to enzymatic activity, or (3) activation of receptor sites that initiate biochemical pathways (Binderman et al., 1984). These events then lead to the alterations in the chemical mediators responsible for the direction of cellular activity. A summary of the sequence of events in agreement with the works of most investigators is as follows:

Mechanical stress → perturbation of target tissues (membranes)→ prostaglandin synthesis → intracellular cAMP and Ca^{+2} accumulation → modulation and activation of cellular activity → bone remodeling.

STATEMENT OF THESIS

Recently, Benson (1983) found increased cAMP levels in cranial sutures subjected to an orthopedic force. Elevated cAMP levels in bone cells exposed to mechanical stress has been reported in other investigations (Davidovitch, 1976; 1984). The causative agent or agents responsible for the reported cAMP response to mechanical stress is not known.

Studies with cultured bone cells (Marcus and Orner, 1977; Yu *et al.*, 1976) have repeatedly produced results showing elevated cAMP levels when prostaglandins were added to the medium. Harell (1977) and Somjen (1980) demonstrated initial increases in PGE_2 followed by elevated cAMP levels in cultured bone cells subjected to mechanical stress. *In vivo* studies (Yamasaki *et al.*, 1980; 1982b) have also shown PGE_2 to be involved in the remodeling response. The majority of evidence for PGE_2 mediation of bone remodeling through a cAMP activated system has been derived from tissue culture. A substantial amount of work remains to be done with bone tissue *in situ* to determine the nature of the local control processes.

The objective of this study is to determine changes in PGE_2 and cAMP concentrations when the sagittal suture of rats is exposed to tensile or compressive forces, and how cAMP responds to drug induced changes in PGE_2 levels. To study these effects, springs were surgically implanted across the midsagittal suture, which was isolated from growth and structural influences of adjacent sutures with

osteotomies cut into the parietal bones. A sample of twenty-four Sprague Dawley rats was divided equally into tension, pressure, and control groups. Half the animals in each group received indomethacin, a potent inhibitor of prostaglandin synthesis, in order to prevent PGE_2 response. Resultant concentrations of PGE_2 and cAMP in each sample were quantitated by a radioimmune assay procedure specific for each substance. Non-collagen protein content of each sample, determined by a variant of the Folin protein assay, was used as a measure of active bone tissue and related to PGE_2 and cAMP changes.

MATERIALS AND METHODS

Animals

This study utilized 24 female Sprague Dawley rats (Zivic Miller Laboratories, Allison Park, PA), 21 days old at the beginning of the experiments. Female Sprague Dawley rats were chosen in order to correlate as closely as possible, previous data obtained by Benson (1983). The age of 21 days was chosen for the following reasons. This would supplement the findings of Benson (1983) by providing information on cAMP at an earlier period on the time-response curve. Second, at 21 days of age the rats are fully weaned from the mother. This circumvents the necessity for forced weaning and subsequent reduction in weight gain, which is an indicator of abnormal growth. Finally, the young age allows for easy handling of the animals during drug administration.

All animals were fed a diet of Purina Rat Chow (Ralston Purina Co., St. Louis, MO) and tap water *ad libitum*. They were kept under rhythmic lighting conditions for 11 hours of darkness per 24 hour period.

Experimental Design

The animal sample was divided into six groups of four animals each (Table I). The experiment started on the 21st day of life and the animals were sacrificed on the 26th day of life. One half of the animals received subcutaneous injections of (Indomethacin , Tarr

Industrial Drug Co., Cleveland, OH) at a dosage of 1 mg/kg body weight. The injections began ½ hour prior to surgery and were repeated every 12 hours for the entire 5-day experimental period (Yamasaki, 1976). To permit adjustments in drug dosage and allow monitoring of individual growth, the weight changes of each animal were recorded daily (Figure 11).

Groups A and D received tension springs across the midsagittal suture while groups B and E received pressive springs. Groups C and F served as controls. This allowed comparison between the variables of pressure and tension on PGE_2 and cAMP levels. Also, the effect of the third variable, indomethacin, within the pressure and tension groups could be examined. On experimental day 1 (animals 21 days of age), osteotomies within the parietal bones were cut to isolate the midsagittal suture (Figure 4). It permitted the elimination of influence from the surrounding cranial sutures and increased the probability that the force applied to the suture was purely expansive or compressive. Furthermore, perturbation of the target tissue at sacrifice was minimized, as tissue sample removal was quite simple.

At the end of the 5-day experimental period all animals were sacrificed. The portions of the parietal bones within the osteotomy were removed and immediately frozen in liquid nitrogen to preserve the biochemical composition. After lyophilization, the bone samples were powdered and frozen at -80°C for later cAMP, PGE_2, and protein analysis.

TABLE I

EXPERIMENTAL DESIGN

Variable(s)	Group	No. of Animals
Tension	A	4
Tension & Indomethacin	D	4
Pressure	B	4
Pressure & Indomethacin	E	4
No Force (Control)	C	4
No Force + Indomethacin (Control)	F	4

An experimental period of five days was chosen for a number of reasons. It was considered to be the minimum time necessary for meaningful morphometric and histologic changes to be recorded as a result of the experimental variables employed. During the span of five days, I was reasonably sure that the inserted springs would remain active, albeit some reduction in spring force through time was a certainty. However, had a longer period of time been used the springs may have lost all activation, mimicking synostosis of the suture. Finally, the short experimental period was a built-in safety factor that allowed repeats for premature death of experimental animals.

Appliance Construction

The fabrication of helical springs followed the design described by Benson (1983). 0.014 round 18:8 Austenitic stainless steel wire (Unitek Corporation, Monrovia, CA) was used for the spring construction. The helix consisted of four loops of wire formed around the large barrel of a Tweed loop forming plier (Dentronix Corp., Ivyland, PA). Extending from the helix were two arms of 7 mm length. At the end of each arm was a single helix of wire formed around the small barrel of the tweed loop forming plier. The distal legs of the helix were bent inward (compression) or outward (tension) to enhance retention of the spring (see Plate I).

All springs were calibrated with a precision force gauge (Haldex, Halmstad, Sweden) to deliver 20 gms of force at an arm separation of 5 mm.

Anesthesia

Anesthesia was induced in the rats by an intramuscular injection of 0.1 ml of a solution containing 90% ketamine hydrochloride (Ketaset , Bristal Labs, Syracuse, NY) and 10% acepromazine Maleate (Ayerst Laboratories Inc., New York, NY). Local subcutaneous injections of 2% Xylocaine hydrochloride with epinephine 1:100,000 (Astra Pharmaceutical Products, Inc. Worcester, MA) were given as needed throughout the surgical procedure.

Surgical Procedure

Once anesthesia was induced, the animal was weighed and the appropriate identification number was placed on the tail with a laboratory marker pen. Using a pair of scissors, the hair on the top of the head was cut in an area extending from the back of the neck to between the eyes. This area was swabbed with copious amount of Povidone-iodine (Betadine, The Purdue Fredrick Co., Yonker, NY) and blotted dry with a sterile 2x2 gauge. An incision was made with a scalpel in the midline from just behind the eyes extending a few millimeters behind the ears.

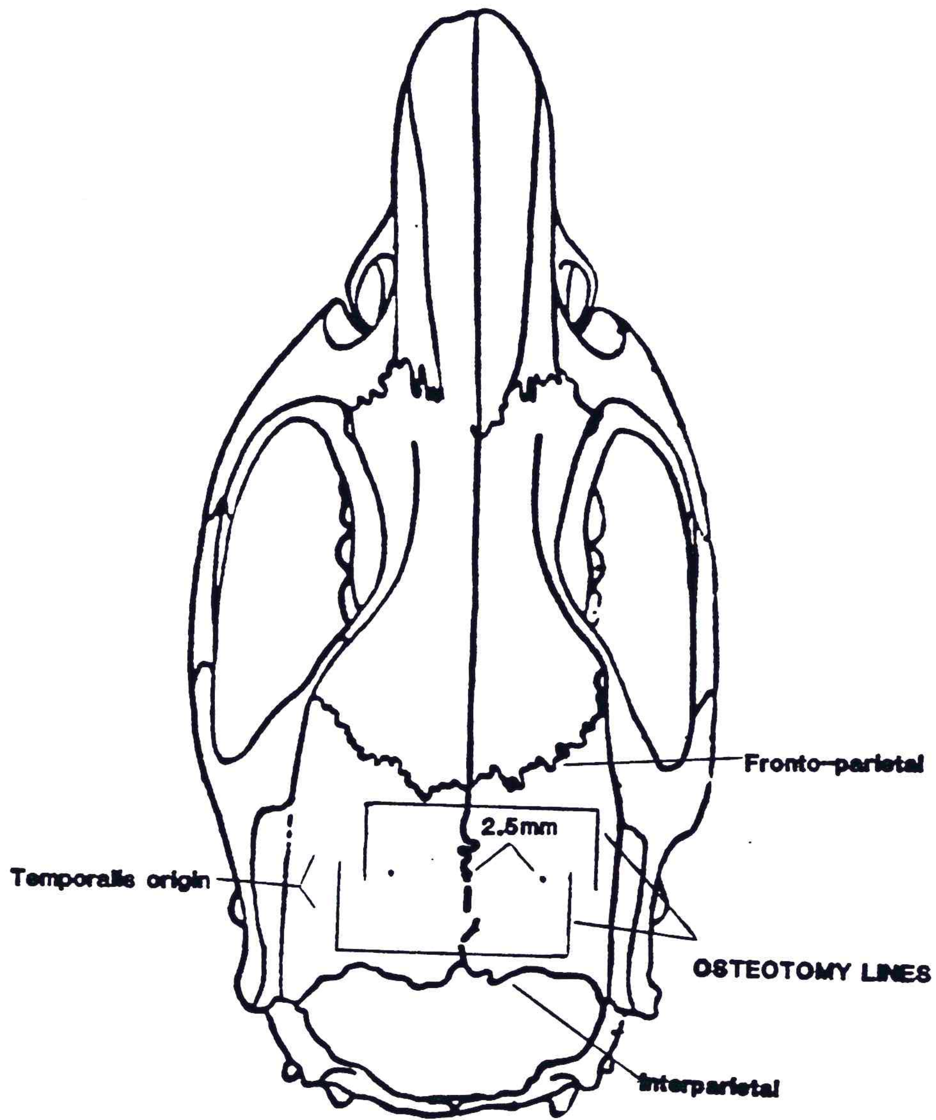
The tissue was reflected away from the underlying bone with a blunt no. 7 laboratory spatula. Care was taken to avoid damaging

the periostium of the parietal bones. The bone surface was then blotted dry with a sterile 2x2 gauge until all spot bleeding ceased.

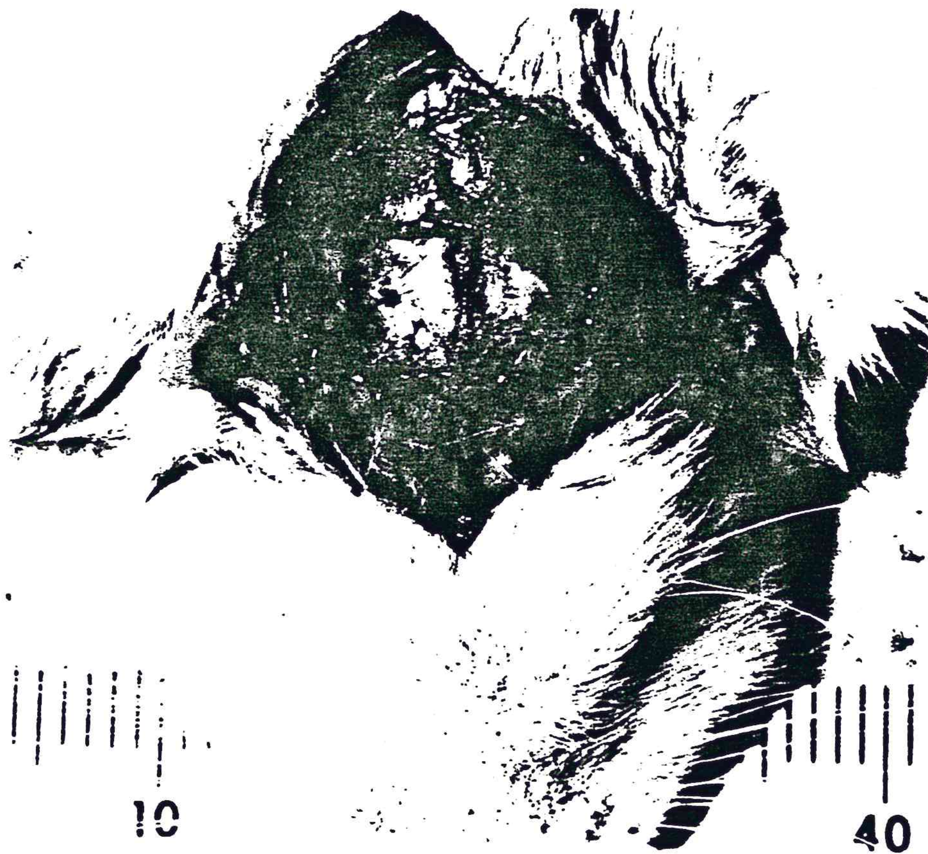
The arms of a Bowley gauge caliper were preset at a distance of 2.5 mm and the tips of the arms were dipped into black India ink. Aligning the caliper arms perpendicular to the sagittal suture, one tip was placed in the center of the suture at its midpoint between the frontoparietal and interparietal sutures. The other tip was then touched to the parietal bone. The procedure was repeated for the opposing parietal bone, using the mark left in the center of the sagittal suture as a reference point. This produced two marks for implant holes, each exactly 2.5 mm from the sagittal suture and 5.0 mm from each other.

At the marks, two holes for the spring arms were then drilled through the full thickness of the parietal bone with a ¼ round carbide bur in a slow speed dental handpiece. The osteotomy was then cut freehand through the parietal bones using the same carbide bur and dental handpiece. The dimensions and position of the osteotomy are diagrammed in Figure 4.

Before insertion each spring was checked to deliver 20 g of force when the spring legs were placed into the holes. This was accomplished by placing one leg in the implant hole and extending the other leg to the contralateral implant hole with the precision force gauge. Any final adjustments in spring tension were made at this time. After placement into the implant holes a drop of cyanoacrylate was placed over the holes to secure the springs.


Fronto-parietal
2.5mm
Temporalis origin
OSTEOTOMY LINES
Interparietal

M.M.


10
40

20

The surgical area was lightly dusted with Neosul II (Carson Chemical Inc., New Castle, IN) to help prevent postsurgical infection. The incision was closed with 4-0 silk suture and the surrounding area swabbed with betadine.

The animals were allowed to recover from the anesthesia in an isolated cage before being returned to their group mates.

Drug Administration

Indomethacin (Indocin, Tarr Industrial Drug. Co., Cleveland, OH) was chosen for this experiment because of its potential inhibition of prostaglandin synthesis. As indomethacin is insoluble in water, aqueous suspensions were prepared for administration.

The indomethacin was received in capsules of 25 mg. To prepare the suspensions, the contents of one capsule was placed in a 50 ml beaker on a magnetic stirrer. Appropriate dilution with normal saline (Abbott Laboratories, North Chicago, IL) were made to reach a final concentration of 0.4 mg Indomethacin/ml suspension for injection.

Injections were given to all rats one hour prior to the first surgery of the group and repeated each day at 8:00 pm and 8:00 am. Each rat was weighed at the p.m. injection and the dosage adjusted accordingly to keep it constant at 1 mg/kg. Initially, a dosage of 10 mg/kg of indomethacin was chosen for administration. At this dosage the rats became listless, resulting in daily weight loss (up to 3 grams/day) and the eventual death of two samples. Dosage was

reduced to 1 mg/kg and the surviving rats were killed. No rats that received a 10 mg/kg dosage were used in this study.

To immobilize the rats during drug administration they were carefully placed into a plastic animal restrainer containing a hole exposing the skin on the back of the animal. The appropriate volume of 0.4 mg/ml suspension was drawn into a 1 cc tuberculin syringe (Butler Co., Warren, OH). The drug suspension was quickly injected subcutaneously in the loose skin of the back. This proved to be a very simple, nontraumatic method of administration and well tolerated by the animals.

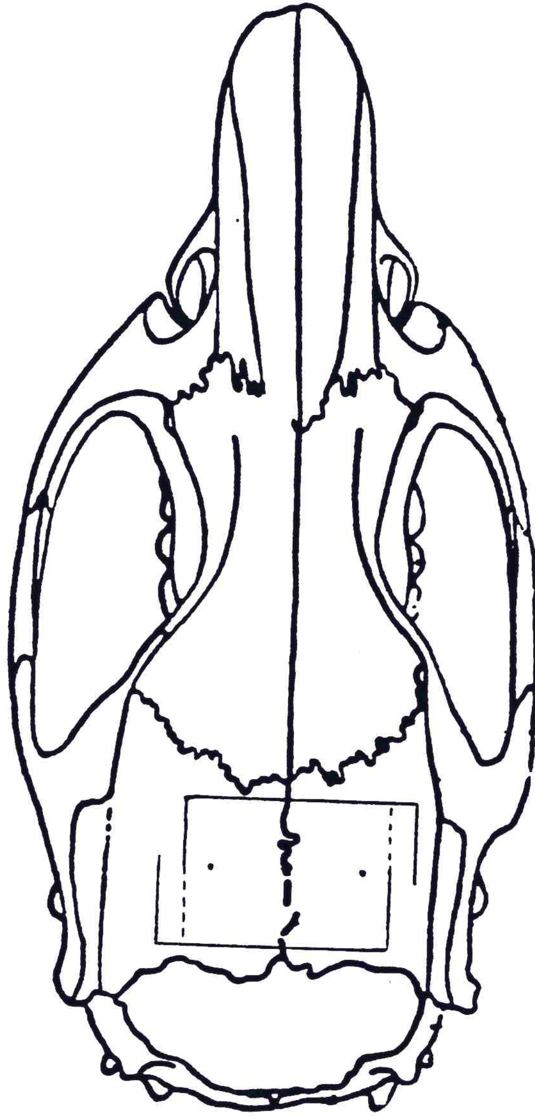
<u>Sacrifice of the Animals</u>

Sacrifice of the animals took place between 12:00 p.m. and 2:00 p.m. on the fifth day of the experimental period. This time was chosen in order that those groups receiving indomethacin would be sacrificed at the approximate midpoint between the 8:00 a.m. and 8:00 p.m. injection. Each was given a 0.2 ml Im injection of the ketamine/acepromazine mixture described previously and then weighed. The surgical site was reopened with a scalpel and the soft tissue was reflected to expose the spring and osteotomy. At this time the spring was inspected to ensure that it was not displaced during the experimental period. With a no. 7 laboratory spatula the spring and any granulation tissue present was quickly removed. Cuts were made along the indicated lines (see Figure 5) with a ¼ round bur in a slowspeed dental handpiece. The rectangular section of bone was

teased free from any underlying tissue, rinsed in saline to remove blood and extraneous tissue, blotted dry with a sterile 2x2 gauge, then immediately immersed in liquid N_2. The total elapsed time from administration of anesthesia to immersion in liquid N_2 was five minutes or less. When frozen, the bone samples were placed in a 15 ml corex glass centrifuge tube (Fisher Scientific) pre-cooled in liquid N_2 and lyophilized overnight (16-18 hours) (Virtis, Unitrap Model 10-100, Vistis Co., Gardiner, NY). All rats were then killed with an overdose of Sodium Pentabarbital (Diabutal, Diamond Laboratories, Des Moines, Iowa). After lyophilization was completed the samples were pulverized with a pestile in a porcelain mortor, sealed in 1 ml Corning-cryogenic tissue vials (25701, Corning Glass Works, Corning, NY) and frozen at -80°C until ready for assay.

Extraction of cAMP and Prosaglandin E_2

The frozen lyophilized bone samples (-80°C) were brought to room temperature in a desiccator containing Drierite Absorbent (Fisher Scientific). The samples were then weighed to the nearest 0.1 mg on an analytical balance and transferred to a siliconized (Sigmacote, Sigma Chemical Co.) 12x100 Nalgene polycarbonate centrifuge tube (Nalge Co., Rochester, NY). Through out the extraction and assay procedures all samples to be analyzed for PGE_2 content were kept in either siliconized glassware or polypropylene tubes to avoid PGE_2 adherence to the container wall. The extraction was performed according to the method of Benson (1983).



Samples were removed by connecting the osteotomies along the dotted lines

To the weighed bone samples was added the appropriate volume of 1 m Perchloric acid (PCA) to make an approximate sample concentration of 10 mg/ml (w/v). The extract was allowed to stand for half an hour in an ice both (0-4°C) with occasional stirring then spun at 20,000 rpm (SM-24 centrifuge head) for 15 minutes at 0-8°C (DuPont Sorval Superspeed Refrigerated Centrifuge). A measured volume of supernate was transferred by pipet to a 13x100 mm siliconized polycarbonate centrifuge tube. The acid-insoluble pellet was stored at -20°C until ready for assay of protein content.

The collected supernate was neutralized to pH 5.9 with saturated potassium carbonate (K_2CO_3). The appropriate volume ratios were determined by titration at the time the solutions were prepared. Neutralization was allowed to continue in an ice bath with periodic agitation until completed, as determined by pHydrian indicating paper. Neutralized extracts were centrifuged at 20,000 rpm for 10 minutes.

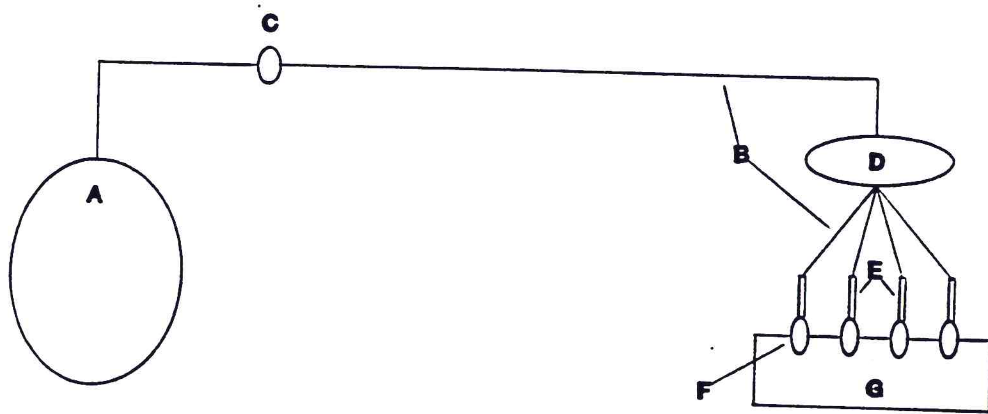
The maximum volume of supernate was removed by pipet and transferred to a 13x100 mm siliconized glass tube and the salt precipitate discarded. 250 µl to 350 µl of the neutralized extract was stored at -20°C for cAMP assay. Using tritiated nucleotide for recovery, Benson (1983) reported an extraction efficiency for cAMP obtained from this procedure to be 97.7%. This value was relatively constant for extraction concentrations between 10-100 mg bone/ml 1 M PCA.

Prostaglandin Column Separation

The remaining neutralized extract was diluted with four volumes of 0.1 M citric acid adjusted to pH 3.5 with 6 N NAOH column extraction. XAD-2 Quik-Sep columns, (Isolab, Akron, OH), prefilled with Amberlite resin, were utilized for PGE_2 extractions (Levine and Alan, 1979). Before use, the columns were washed with 25.0 ml of ddH_2O. The diluted extracts (pH 3.5) were then run through the columns followed by rinsing with 25.0 ml ddH_2O. The processed extract and wash water were discarded. The columns were eluted with 20 ml of absolute ethanol anhydrous ETOH which was collected in siliconized 50 ml erlenmeyer flasks.

Under N_2 gas, the ethanol was evaporated in a 30°C water bath (Figure 6). The flasks were rinsed 3X's with 0.5 ml volumes of absolute ethanol and the ethanol transferred to 4 ml capped vials (Wheaton Scientific, Millville, NJ). Evaporation was continued as before until dryness. The vials were capped, sealed with Parafilm, and stored at -20°C for PGE_2 assay.

I undertook a specific study to determine the extraction efficiency for PGE_2 using the XAD-2 columns. Recovery concentrations of 0.1, 1.0, and 10 ng PGE_2, prepared from a 1.0 mg/ml PGE_2 standard (Sigma Chemical Co.) or a 100 ng PGE_2/ml standard (supplied with PGE_2 ^{125}I RIA kit from New England Nuclear) were added to a volume of 400 µl of 1 M PCA and processed as per sample extraction. A mean recovery of 85% with a range from 65% to 100% was obtained as determined by RIA procedures (see Table II).


C
A
B
D
E
F
G

Assay for cAMP and PGE_2

The 250-350 µl extract fraction was assayed for cAMP content by radioimmunoassay (cAMP ^{125}I RIA kit cat. #NEK-033, NEN, Boston, MA). After addition of the cAMP precipitator, all tubes were centrifuged at 2,500 g (4,500 rpm) for 20 minutes to assure firm precipitate formation. Besides this modification, the procedure followed was that given in the kit instructions for the acetylated (plasma) protocol. An equivalent sample weight of 0.09 mg bone (unstimulated groups) and 0.009 mg bone (stimulated groups) per assay tube was adequate for measurable cAMP activity.

PGE_2 content was also determined by means of radioimmunoassay (PGE_2 ^{125}I RIA kit, cat.#NEK-020A, NEN, Boston, MA). A specific pilot study was undertaken using high pressure liquid chromatography to determine prostaglandin profiles of stimulated bone samples. Bone samples were prepared and extracted identical to experimental samples. Findings suggested PGE_2 as the major prostaglandin. This directed me in selecting PGE_2 as the target for radioimmunoassay.

The N_2 evaporated PGE_2 samples were reconstituted with assay buffer (supplied with NEN RIA PGE_2 kit) to give an equivalent concentration of 4.5 mg bone/ml assay buffer. After addition of the PGE_2 precipitator, all tubes were centrifuged at 2500 g for 30 minutes. The protocol given in the RIA PGE_2 kit insert was otherwise followed. An equivalent sample weight of 0.45 mg bone/tube was adequate to obtain measurable PGE_2 activity in unstimulated and indomethacin treated groups. However, due to increased PGE_2 in

TABLE II

Analysis of PGE_2 Extraction Efficiency

"0" std net cpm	Recovery no.	Concentration (ng PGE_2/ml)	Net cpm	pg PGE_2/tube assayed recovery value	pg PGE_2/tube diluted recovery value	% Recovery
4120	1	10	1455	3.2	3.7	86.5
	2	1.0	1713	2.4	3.7	64.9
	3	0.1	1314	3.6	3.7	97.3
	4	Blank	3919	-	-	-
4512	5	1.0	1944	3.4	3.7	91.9
	6	1.0	2267	2.6	3.7	70.3
	7	1.0	1817	3.7	3.7	100
	8	Blank	4128	-	-	—

Represents activity from PGE_2 recovery determination. Recovery samples of the indicated concentration were processed identical to the extraction procedure utilized for the experimental bone samples. Blanks were included to detect any interferences in the assay resulting from the extraction procedure. The experimental data was not corrected for extraction efficiency.

Figure 7 - 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)

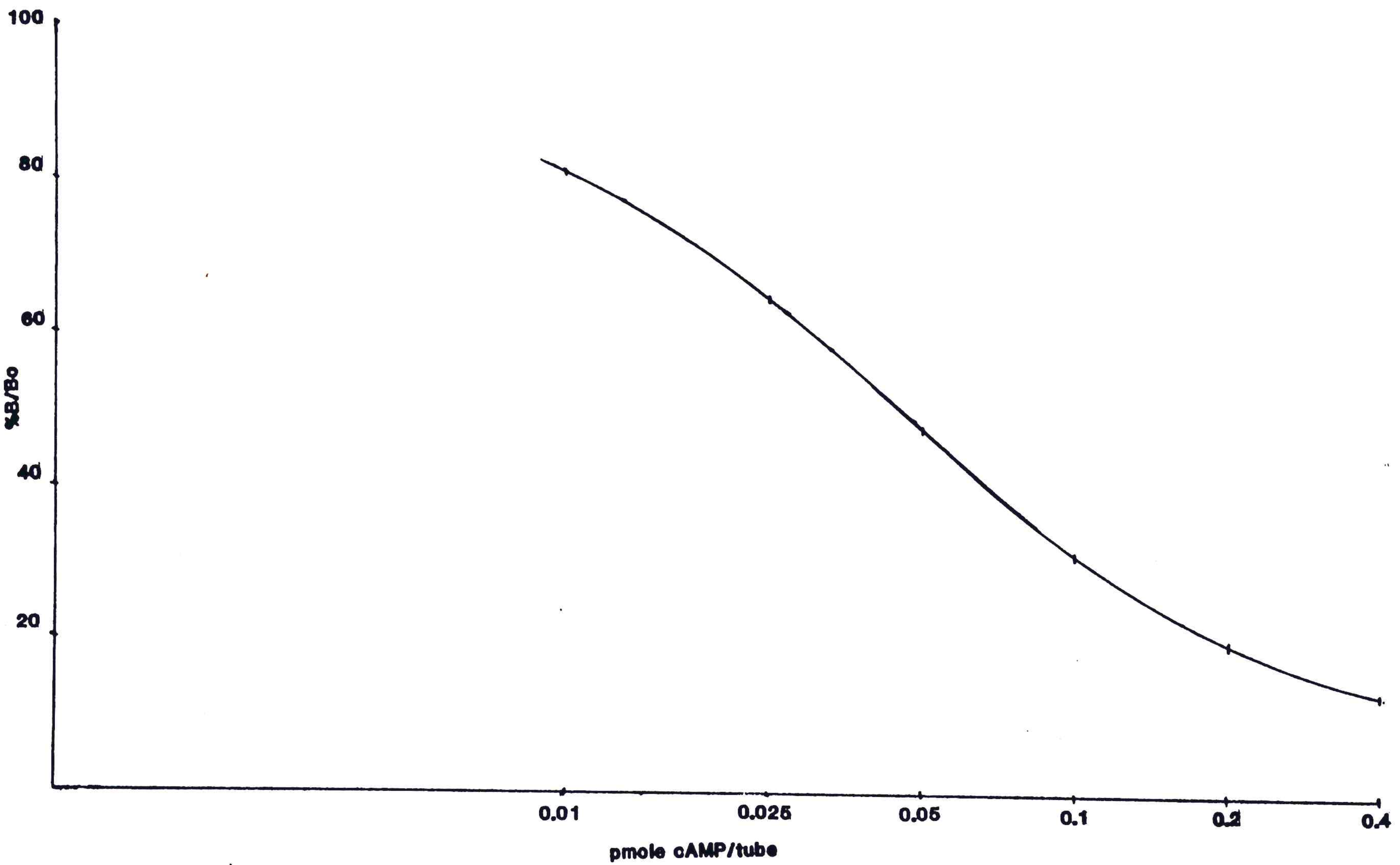
Labeled Antigen
Antibody Complex
(Ag*Ab)

Unlabeled Antigen
(Ag)

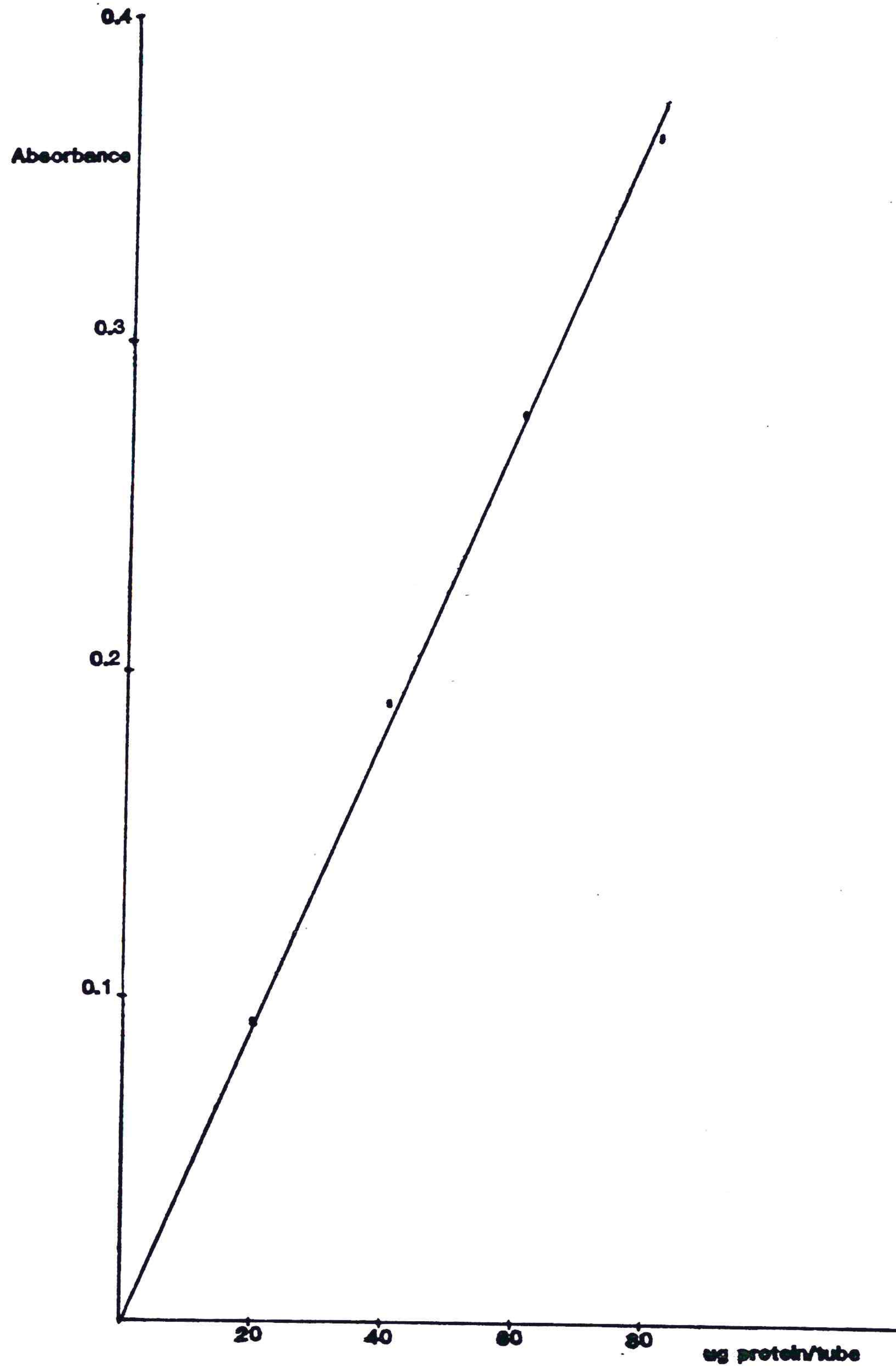
UnlabeledAntigen-AntibodyComplex
(AgAb)

Assay
(Ag*Ab) : (AgAb) Ratio

Figure 8 - Standard curve for assay of cAMP. Values of cAMP for unknown samples were interpolated from a standard curve determined by assaying known quantities of cAMP and plotting concentration (pmole) vrs% binding of the labeled antigen to the antibody. Due to the rapid decay rate of the ^{125}I label, a standard curve was determined with each sequence of unknown samples.


cAMP ASSAY STANDARD CURVE
100
80
60
%B/Bo
40
20
0.01
0.025
0.05
0.1
0.2
0.4
pmole cAMP/tube

PROTEIN ASSAY STANDARD CURVE



stimulated groups, it was necessary to dilute the samples from stressed animals as much as 10x in order to accurately measure PGE_2 activity.

Representative samples were chosen for reassay of both cAMP and PGE_2 levels. This was done to be certain that similar values would be obtained in consecutive assays. Duplicate assays were averaged and the values reported in Table V.

In both cAMP and PGE_2 determination, activity was counted for one minute in a gamma counter (Liquimat , Picker Nuclear).

Protein Assay

The protein content of all bone samples was determined from a variation of the Folin Protein assay of Lowry et al. (1951). Again, representative samples were chosen for reassay as done for cAMP and PGE_2. Dublicates were averaged and the values reported in Table III. The acid-insoluble pellet was extracted (20-24 hours) with periodic agitation at 2-8°C in 0.4 N NAOH at a concentration of 5.0 mg bone/ml NAOH. The samples were then centrifuged at 20,000 g for 10 minutes. The supernate was collected and stored at 2-8°C for assay. Before use, 2.0 ml of 2.68% NA-K tartrate solution and 2.0 ml of 1% ($CuSO_4$ solution were mixed and then diluted with 100 ml of 2% NA_2 CO_3-0.1 N NAOH. To 5.0 ml of the above solution was added 50 μl of the protein extract and 150 μl of 0.4 N NAOH. Standards were prepared from dilutions of a 1.0 mg/ml bovine serum albumin (Cohn Fraction V, Fisher Scientific, Warrensville, OH). After a 0.5 ml

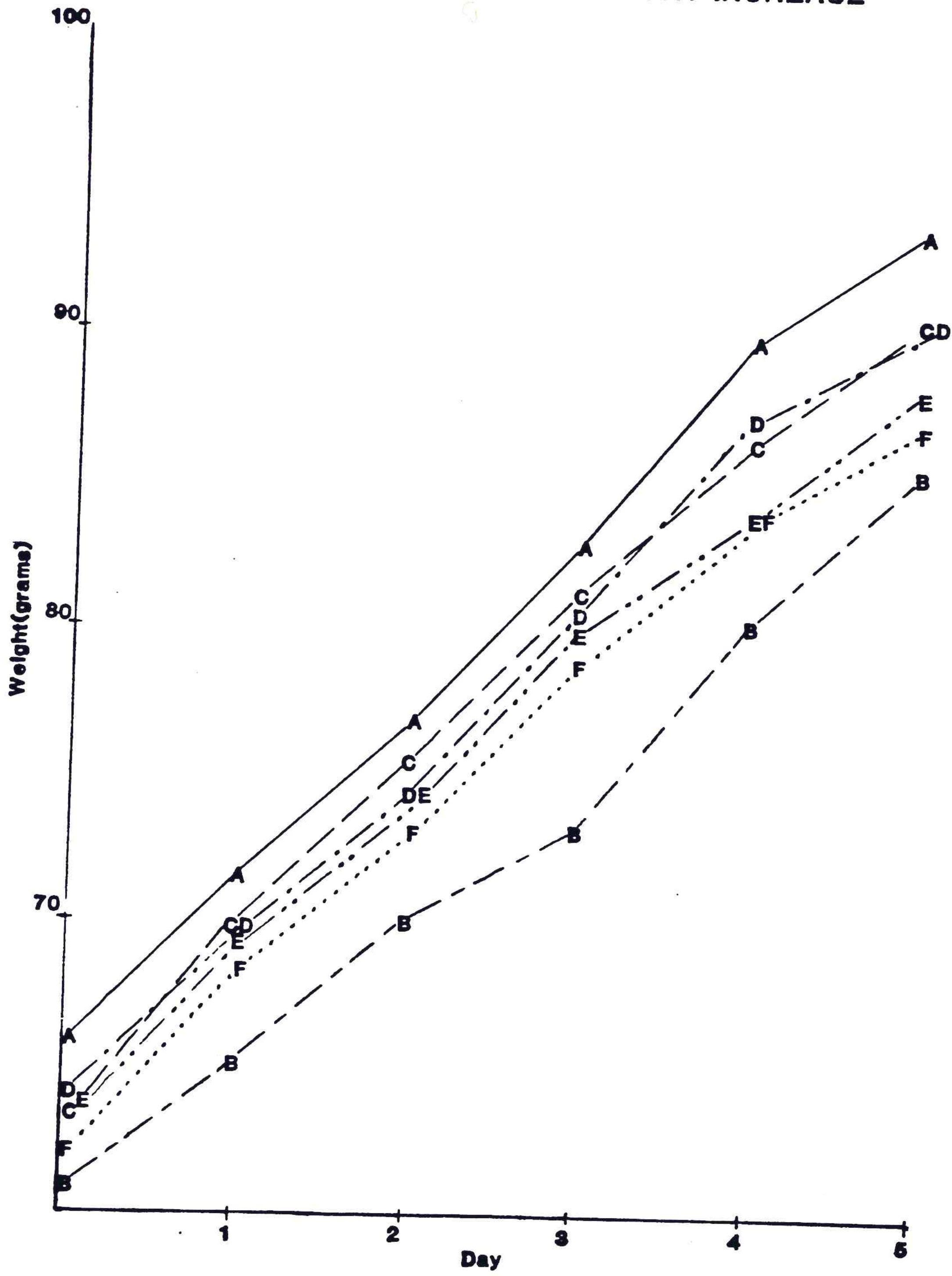
volume of diluted (1:1) phenol reagent (Fisher Scientific) was added to each standard and sample they were immediately vortexed. Following a development time of at least one hour samples and standards were read for absorbance at 750 nm in a 2400 Gilford spectrophotometer (Gilford Laboratories, Oberlin, OH).

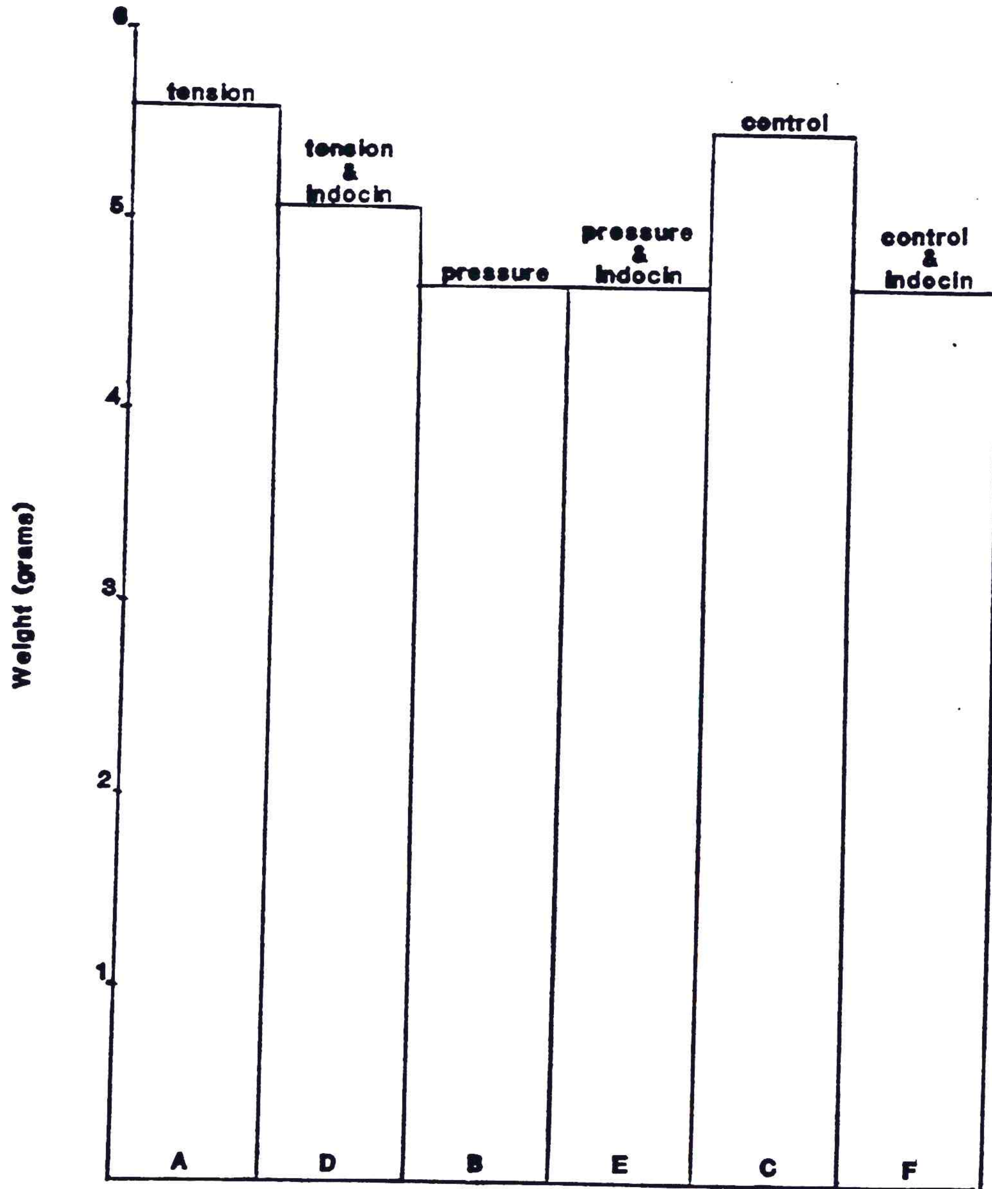
RESULTS

Response of the Animals to the Experimental Procedures

Recovery from the surgical procedure proved rapid, as the animals resumed active behavior shortly after regaining conscienceness. At no time during the experimental period did the animals show any gross signs of postoperative infection. Interaction with group mates did not interfere with the healing of the surgical wound and the five-day experimental period proceeded uneventfully.

Weight gain of the animals was recorded daily as an assessment of physical growth and to monitor the general response to the indomethacin dosage. As Figure 11 indicates, the animals tolerated both the drug administration and spring activation quite well. Group weights increased in a parallel manner throughout the experiment. Closer examination of the average daily weight increases (Figure 12) reveals slightly lower values for the groups receiving injections of indomethacin. Goodman and Gillman (1980) cite the occurrence of severe frontal headache in 25-50% of human subjects taking this drug. If a similar situation is operative in rats this could help to explain the tendency noted in Figure 12. Also, the trauma of repetitive drug administration could be a contributing factor. Those groups that received pressure springs also had slightly lower values than controls, suggesting that applied pressure was associated with the most physiological stress.


AVERAGE GROUP WEIGHT INCREASE
100
90
80
70
Weight (grams)
1
2
3
4
5
Day
A
B
C
D
E
F
CD
DE
EF


AVERAGE DAILY GROUP WEIGHT INCREASE
Weight (grams)
6
5
4
3
2
1
tension
tension & indocin
pressure
pressure & indocin
control
control & indocin
A
D
B
E
C
F

However, the order of average group weight at the beginning of the experiment remained unchanged at day 5 (see Figure 11). The range from the heavest group (A) to the lightest group (B) also remained relatively unchanged from day 0 to day 5. Animals from each group thus seemed to tolerate the experimental conditions equally well with only small variation.

Gross examination of the experimental site at the time of sacrifice revealed still patent osteotomy sites in all animals. Granulation tissue was present around the springs but was quite soft and should not have affected spring activation. All springs were firmly in place and were active (as indicated by lifting one leg of the spring out of its implant hole). Sutures subjected to tension were significantly wider than those of controls. On the other hand, sutures subjected to pressure appeared to have an exophytic-like appearance.

Protein and Total Bone Content

Both cAMP and PGE_2 content of the bone samples were quantitated utilizing radioimmunoassay procedures. Non-collagen protein content, as determined by the Folin protein assay procedure described previously, provided a means by which sample data could be expressed in terms of active bone tissue.

As shown in Table III, the protein content of the samples varied from 9.0% to 19.4% of total bone weight. This variation highlights the necessity for quantitative RIA data to be expressed

TABLE III

TOTAL BONE AND PROTEIN CONTENT OF THE EXPERIMENTAL SAMPLES

Condition	Sample	Animal wt (g) at sacrifice	Total Bone Wt. (mg)	Total Protein Wt. (mg)	mg Protein / mg Bone
Tension	A1	88.8	6.91	1.28	0.186
	A2	93.1	10.20	1.06	0.104
	A3	97.4	12.20	1.56	0.128
	A4	96.6	12.20	1.81	0.148
Tension + Indocin	D1	91.5	19.60	2.86	0.146
	D2	81.5	14.60	2.84	0.194
	D3	94.5	13.20	2.08	0.158
	D4	94.0	13.60	2.10	0.154
Pressure	B1	85.5	10.40	1.32	0.127
	B2	86.4	9.94	1.27	0.128
	B3	84.2	10.70	1.26	0.117
	B4	84.0	14.10	1.75	0.124
Pressure + Indocin	E1	86.5	12.90	1.45	0.112
	E2	86.5	11.00	1.21	0.110
	E3	94.0	13.20	1.53	0.116
	E4	80.5	13.80	1.44	0.104
No force (Control)	C1	91.5	9.93	0.89	0.090
	C2	82.0	9.76	1.22	0.125
	C3	94.6	10.80	1.25	0.116
	C4	93.5	11.80	1.46	0.124
No force (Control) + Indocin	F1	83.5	12.50	1.56	0.125
	F2	83.5	9.49	1.35	0.142
	F3	91.0	14.70	1.71	0.116
	F4	87.5	11.20	1.81	0.162

The protein/bone ratio was used to standardize PGE_2 and cAMP values to a measure of active bone tissue. The standardized values are presented in Table V.

TABLE IV

PGE_2 and cAMP Levels Per Total Bone Weight

Condition	Sample #	Mean $\frac{pgPGE_2}{mg\ bones}$	Mean $\frac{pmcAMP}{mg\ bone}$	Group mean $\frac{pgPGE_2}{mg\ bone}$	Group Mean $\frac{pmcAMP}{mg\ bone}$
Tension	A1	115.00	9.20	46.60	6.30
	A2	8.20	1.50		
	A3	20.30	3.60		
	A4	42.80	10.90		
Tension + Indocin	D1	2.50	1.65	5.50	3.60
	D2	5.50	5.60		
	D3	6.70	2.45		
	D4	7.20	4.80		
Pressure	B1	33.50	1.25	48.80	1.00
	B2	16.00	1.00		
	B3	4.10	0.54		
	B4	142.00	1.35		
Pressure + Indocin	E1	2.70	0.76	3.10	1.10
	E2	2.10	0.46		
	E3	2.70	1.75		
	E4	4.75	1.35		
No Force (Control)	C1	3.80	0.56	9.10	0.58
	C2	21.20	0.45		
	C3	2.65	0.38		
	C4	8.60	0.92		
No Force (Control) + Indocin	F1	3.60	0.74	4.20	0.68
	F2	5.10	0.80		
	F3	1.10	0.32		
	F4	7.00	0.88		

These values are presented only for comparison with Table V.

TABLE V

PGE_2 and cAMP Levels per Total Protein Weight

Condition	Sample #	Mean pgPGE_2 / mg protein	Mean pmcAMP / mg protein	Group mean pgPGE_2 / mg protein	Group Mean pmcAMP / mg protein	Ratio PGE_2/cAMP
Tension	A1	618.00	49.50	287.00	41.60	12.5
	A2	82.00	15.00			5.5
	A3	159.00	28.10			5.7
	A4	289.00	73.60			3.9
Tension + Indocin	D1	17.10	11.30	33.80	21.70	1.5
	D2	28.40	28.70			1.0
	D3	42.90	15.70			2.7
	D4	46.80	31.20			1.5
Pressure	B1	264.00	9.84	392.00	8.30	26.8
	B2	125.00	7.80			16.0
	B3	35.00	4.60			7.6
	B4	1145.00	10.90			150
Pressure + Indocin	E1	25.00	7.00	28.50	9.90	3.6
	E2	20.20	4.40			4.6
	E3	23.30	15.10			1.5
	E4	45.70	13.00			3.5
No Force (Control)	C1	45.20	6.70	76.80	5.20	6.7
	C2	170.00	3.60			47.2
	C3	22.80	3.30			6.9
	C4	69.40	7.40			9.4
No Force (Control) + Indocin	F1	28.80	5.90	29.50	5.00	4.9
	F2	35.90	5.60			6.4
	F3	9.50	2.80			3.4
	F4	43.70	5.50			7.9

Comparison of these values with those in Table IV reveal the importance of adjusting PGE_2 and cAMP data to a measure of active tissue. Only data expressed in terms of protein weight were used for statistical analyses.

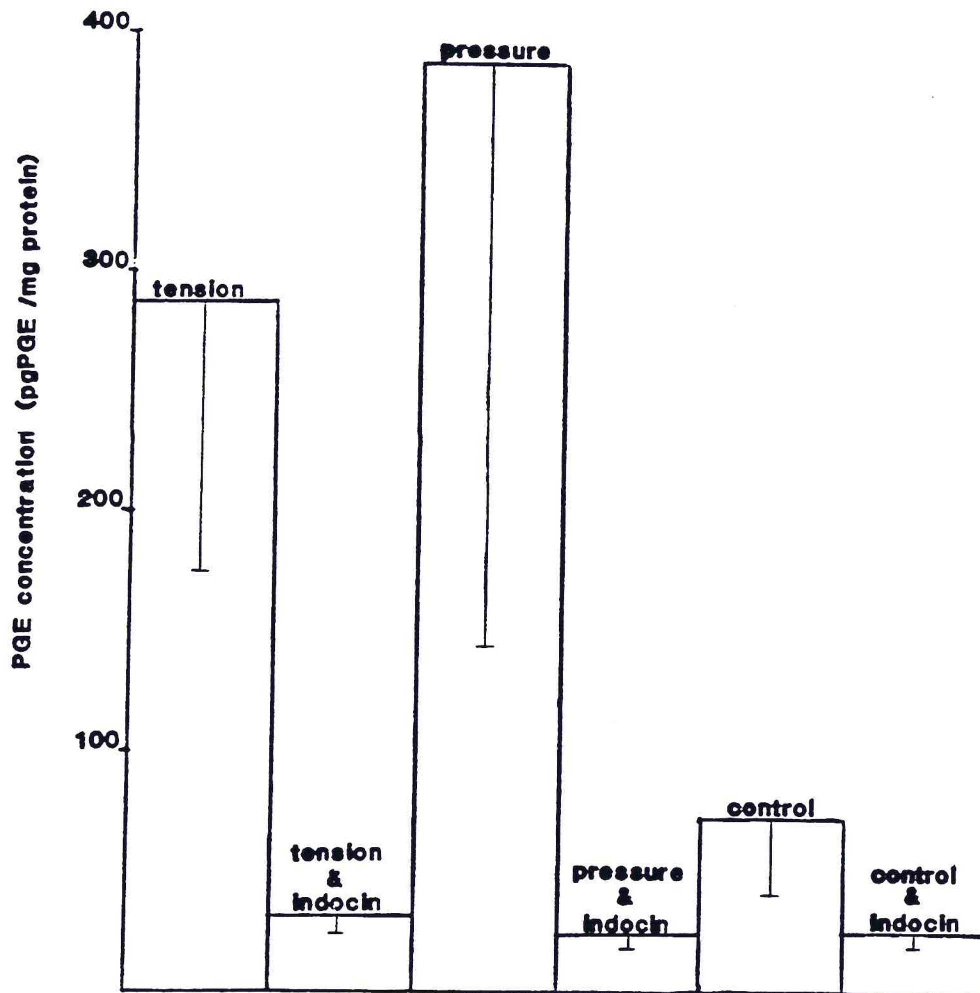
in relation to a relevant tissue component. The heterogeneity of bone tissue would otherwise preclude to error in the interpretation of results expressed only in terms of bone weight.

Analysis of Cyclic AMP and PGE_2 Levels

Table IV and Table V represent the cAMP and PGE_2 data for each sample expressed in terms of mg of bone and mg of protein, respectively. Due to the variation in non-collagen protein content shown in Table III, all data were adjusted to mg of protein for interpretation. These values are depicted graphically for PGE_2 and for cAMP in Figure 13 and Figure 14. PGE_2 levels increased dramatically in the tension and pressure groups over controls. Also, the administration of indomethacin in a dosage of 1 mg/kg reduced PGE_2 levels below that of controls even in stimulated groups. This may indicate that the dosage of indomethacin used was too high. The objective of drug administration is to obtain the desired response without unwanted side effects. Since a dosage of 1 mg/kg decreased PGE_2 levels below controls (Figure 13), it could be assumed that an even lower dosage would effect sufficient suppression of the PGE_2 response.

In contrast, cAMP levels increased eight-fold in the tension group but only doubled in the pressure group when compared to controls. It is interesting that indomethacin decreased cAMP levels in the tension group but had no effect in the pressure group (See Figure 14).

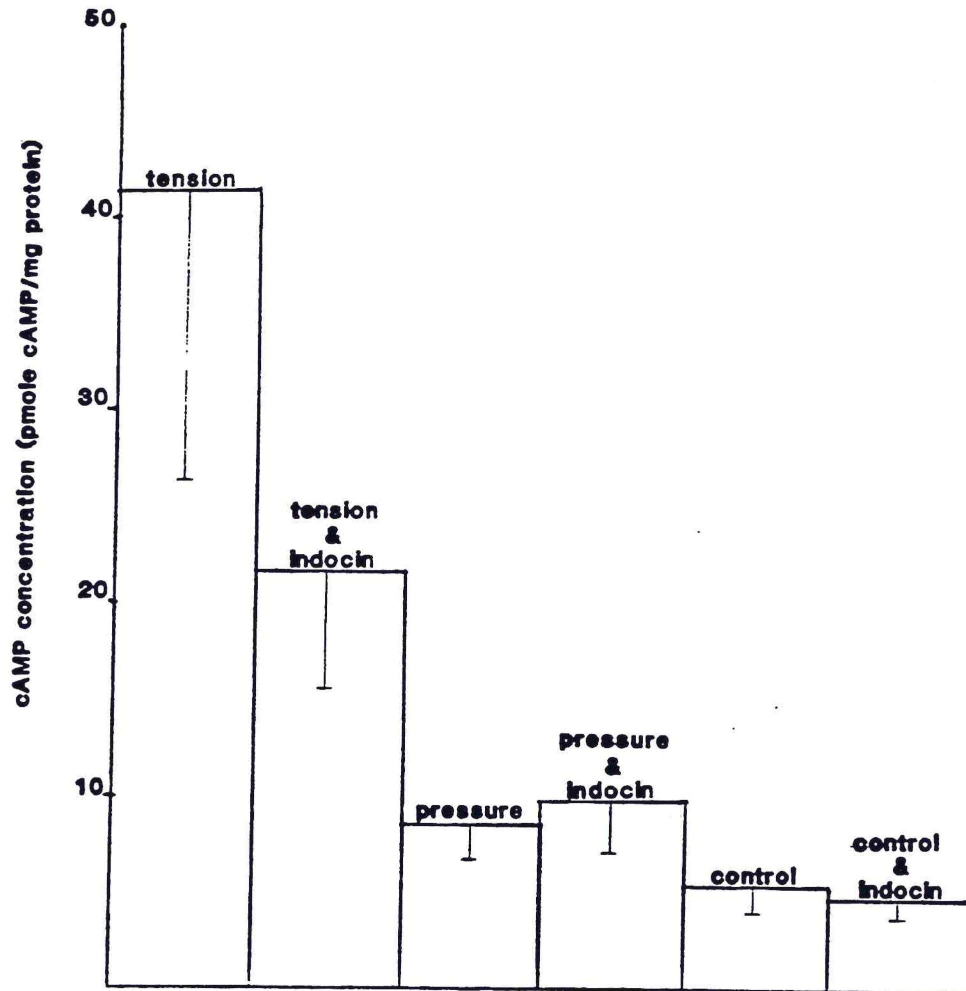
Figure 13 - Response of Calvarial PGE_2 to Stress. Error bars represent the standard deviation.



PGE2 CONCENTRATION
PGE concentration (pgPGE /mg protein)
400
300
200
100
tension
tension & indocin
pressure
pressure & indocin
control
control & indocin

Figure 14 - Response of Calvarial cAMP to Stress. Error Bars represent the standard deviation.

cAMP CONCENTRATION



For statistical analysis the Student - Newman - Keuls test was chosen for its use in the comparison of multiple group data. Because sample size was relatively small (n=4 for each group) and the variation in PGE_2 values was large, logarithmic transformation of all data was performed and used for statistical analysis.

Two approaches were taken in data analysis. One approach included the data taken from all twenty-four samples. The other eliminated the data obtained from three animals, namely A1, B4, and C2. Animals A2, A3, A4 and C1, C3, C4 were sacrificed on the same day, whereas A1 and C2 were sacrificed together on a day different from the other group members (see Appendix II). This occurred because of the premature death of some animals following surgery, necessitating the need for replacement animals at a subsequent date. Hence, there may have been variations in environmental conditions that altered PGE_2 levels, which were elevated in animals A1 and C2 when compared to other group members. Data from animal B4 was dropped due to the extraordinarily high levels of PGE_2. Iatrogenic handling of the sample during removal may have caused elevated PGE_2 levels not representative of the experimental conditions. Table VI summarizes the group means for cAMP and PGE_2 and includes both the values obtained when the afforementioned samples are eliminated (indicated as n=3) and the logarithmic transformations. For comparison, the log values for PGE_2 and cAMP as shown in Figure 15 and Figure 16. Another parameter, the PGE_2/cAMP ratio, was used as an indicator of interaction between PGE_2 and cAMP (Figure 17). If a

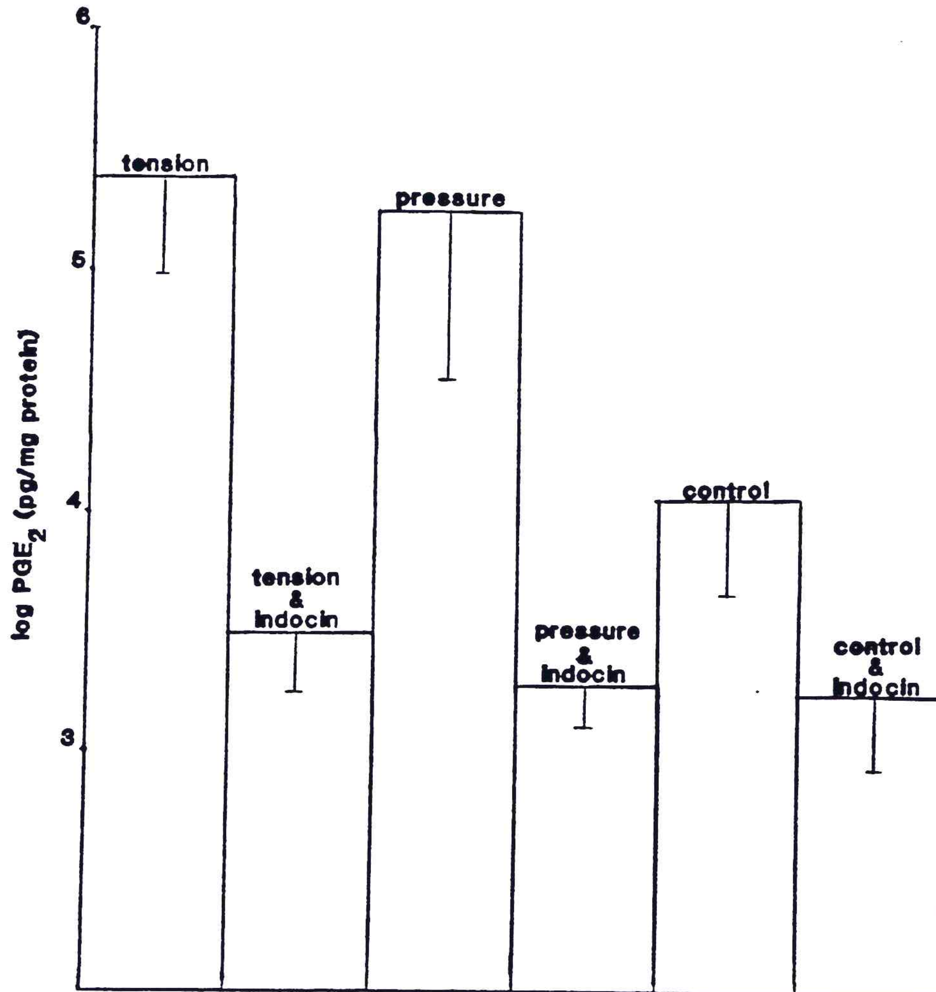
TABLE VI - SUMMARY OF PGE_2 AND cAMP ANALYSIS

Means ($\bar{X}$)

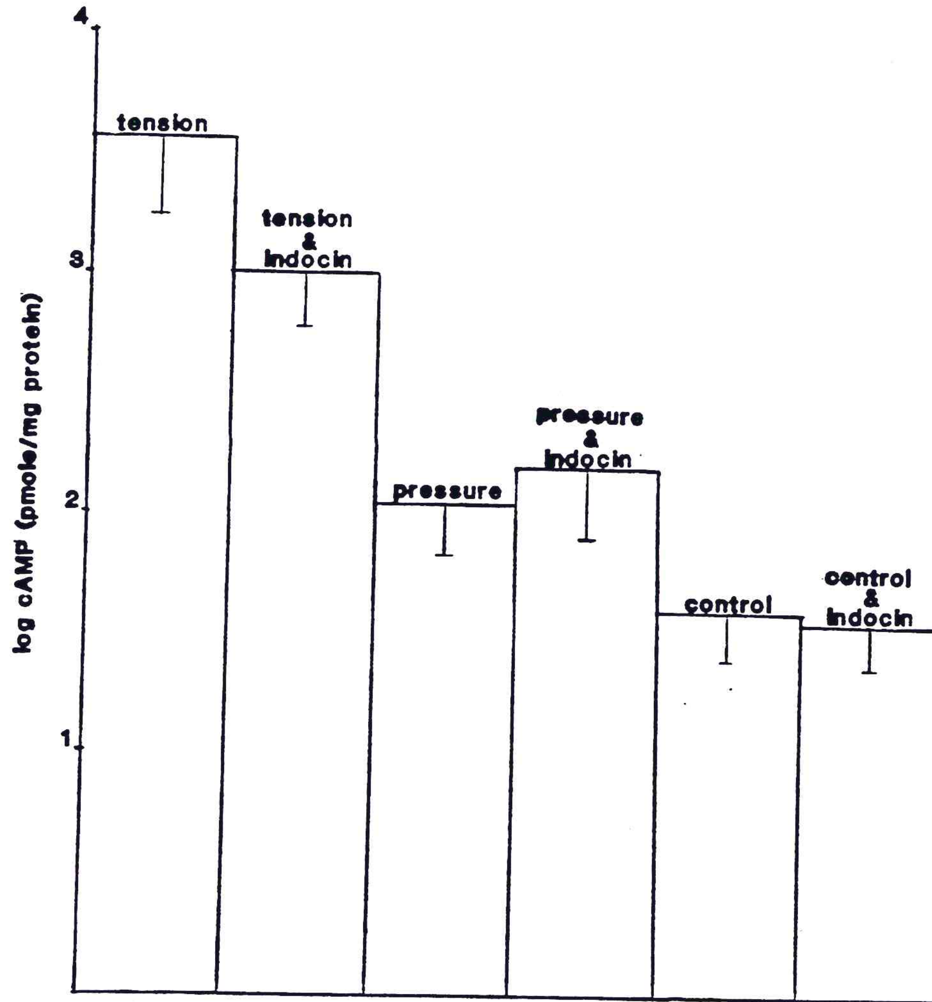
Condition \ Sample size	PGE_2 pg/mg Protein ±SD		cAMP pmole/mg Protein ±SD		Log PGE_2 pg/mg Protein ±SD		Log cAMP pmole/mg Protein ±SD		Log Ratio PGE_2/cAMP ±SD	
	n=3	N=4	n=3	n=4	n=3	n=4	n=3	n=4	n=3	n=4
Tension	177 ±105	287 ±237	38.9 ±30.8	41.6 ±25.7	5.0 ±0.63	5.4 ±0.86	3.4 ±0.80	3.6 ±0.69	160 ±20.2	183 ±49.1
Tension & Indocin	--	33.8 ±13.7	--	21.7 ±9.7	--	3.4 ±0.46	--	3.0 ±0.49	--	45.4 ±41.8
Pressure	141 ±115	392 ±511	7.4 ±2.6	8.3 ±2.8	4.7 ±1.0	5.2 ±1.5	2.0 ±0.39	2.1 ±0.38	270 ±63.6	319 ±111
Pressure + Indocin	--	28.6 ±11.6	--	9.9 ±5.0	--	3.3 ±0.36	--	2.2 ±0.57	--	112 ±47.5
Control	45.8 ±23.3	76.8 ±65.0	5.8 ±2.2	5.2 ±2.0	3.7 ±0.56	4.1 ±0.84	1.7 ±0.44	1.6 ±0.42	203 ±18.4	248 ±92.6
Control + Indocin	--	29.5 ±14.6	--	5.0 ±1.4	--	3.2 ±0.68	--	1.6 ±0.35	--	168 ±36.7

Table VI summarizes the average PGE_2 and cAMP values for each condition. Values for n=3 are included where appropriate for comparison. All logarithmic values are expressed as natural logs.

Figure 15 - Natural Logarithmic Transformation of PGE_2 Values. Error bars represent the standard deviation.

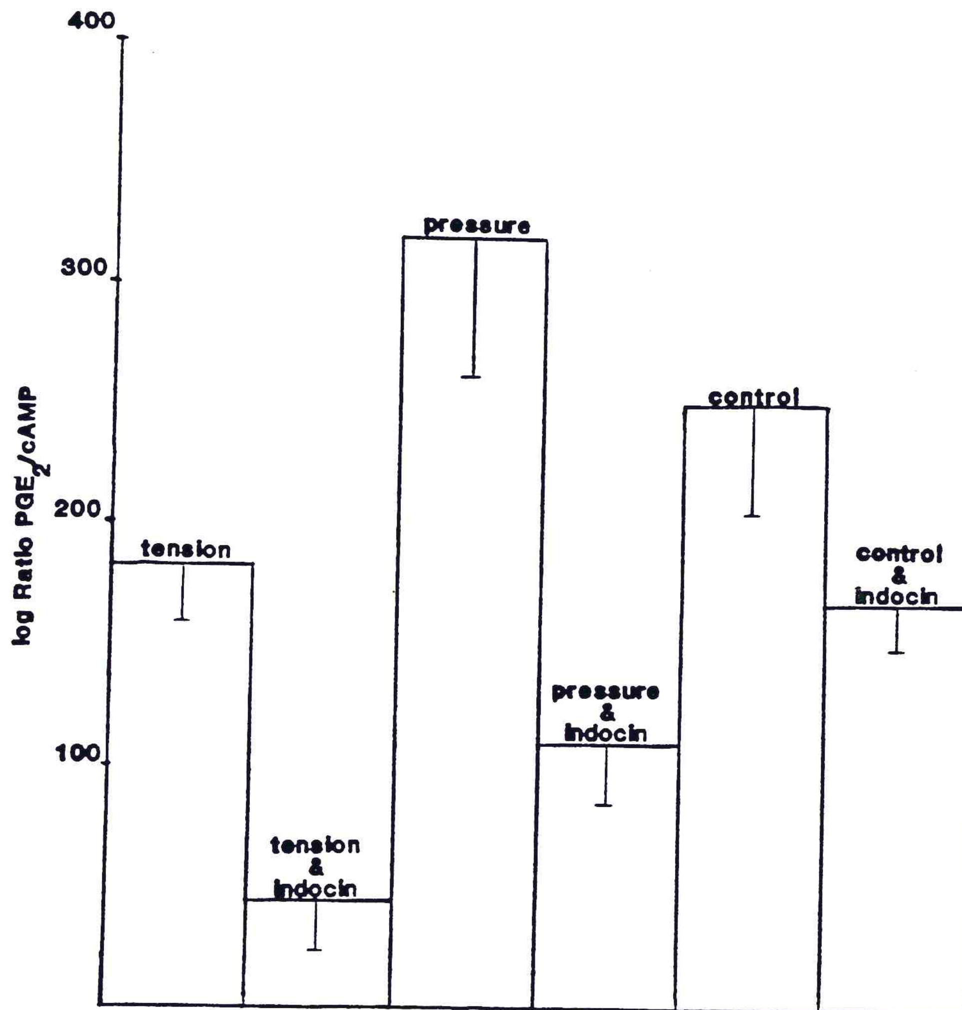


PGE2 CONCENTRATION
LOG VALUES
log PGE2 (pg/mg protein)
6
5
4
3
tension
tension & indocin
pressure
pressure & indocin
control
control & indocin



cAMP CONCENTRATION
LOG VALUES
log cAMP (pmole/mg protein)
4
3
2
1
tension
tension & indocin
pressure
pressure & indocin
control
control & indocin

RATIO PGE_2/cAMP LOG VALUES



direct and positive relationship exists between PGE_2 and cAMP, as suggested in the literature, then ratio values will remain relative constant as the level of PGE_2 is experimentally manipulated. Ratio values are also useful to compensate for any individual response variation within the groups.

No significant difference exists in the level of PGE_2 response between the tension and pressure groups (Table VII). The tension group has significantly higher ($p < 0.05$) PGE_2 levels than controls when sample A1 is eliminated (n=3). In the pressure group, PGE_2 levels tend to be increased at both n=3 and n=4 over controls, but not at a significant level. This trend would likely become significant if the sample number were greater. Tension or pressure applied to bone tissue _in situ_ thus increases PGE_2 levels to apparently equal degrees. Furthermore, indomethacin at a dosage of 1 mg/kg body weight significantly reduced ($p < 0.05$) PGE_2 levels regardless of the condition. The reduction was determined to be less than control levels (see Figure 15). A dose-response study to determine a minimum required dosage needed to reduce PGE_2 levels would be helpful, as even a lower dose than that used in this study may have been adequate.

Table VIII shows that cAMP levels were significantly higher ($p < 0.05$) in the tension group when compared to either pressure or control groups (n=3 or n=4). This difference suggests that cAMP plays different regulatory roles in the depository and resorptive process. That the function of cAMP may be different in these two

TABLE VII

Student - Newman - Keuls Test for Log PGE_2

	n	$\bar{X}$ (pg/mg Protein)	Grouping	N	$\bar{X}$ (pg/mg Protein)	Grouping
Tension	4	5.4	A	3	5.0	A
Tension + Indocin	4	3.4	B	4	3.4	B
Pressure	4	5.2	A	3	4.7	A B
Pressure + Indocin	4	3.3	B	4	3.3	B
Control	4	4.1	A B	3	3.7	B
Control + Indocin	4	3.2	B	4	3.2	B

Table VII Student - Newman - Keuls test for variable log PGE_2. A separate comparison of the data when values obtained from suspect animals are eliminated is included. Means having different letters are significant at the $p < 0.05$ level. Multiple letters indicate trends and belong to each group. The statistical grouping is not related to the animal group letters in Table I.

TABLE VIII

Student - Newman - Keuls Test for Log cAMP

	n	$\bar{X}$ (pmoles/ mg Protein)	Grouping	N	$\bar{X}$ (pmole/ mg Protein)	Grouping
Tension	4	3.6	A	3	3.4	A
Tension + Indocin	4	3.0	A	4	3.0	A B
Pressure	4	2.1	B	3	2.0	C
Pressure + Indocin	4	2.2	B	4	2.2	B C
Control	4	1.6	B	3	1.7	C
Control + Indocin	4	1.6	B	4	1.6	C

Table VIII Student - Newman - Keuls test for variable log cAMP. A separate comparison of the data when values obtained from suspect animals are eliminated is included. Means having different letters are significant at the $p < 0.05$ level. Multiple letters indicate trends and belong to each group. The statistical grouping is not related to the animal group letters in Table I.

TABLE IX

Student - Newman - Keuls Test for Log Ratio PGE_2/cAMP

Condition	N	$\bar{X}$	Grouping	N	$\bar{X}$	Grouping
Tension	4	183	B C	3	160	B
Tension + Indocin	4	45.4	C	4	45.4	C
Pressure	4	318	A	3	270	A
Pressure + Indocin	4	112	B C	4	112	B C
Control	4	248	A B	3	202	A B
Control + Indocin	4	168	B C	4	168	B

Student - Newman - Keuls test for variable log Ratio. A separate comparison of the data when values obtained from suspect animals are eliminated is included. Means having different letters are significant at the $p < 0.05$ level. Multiple letters indicate trends and belong to each group. The statistical grouping is not related to the animal group letters in Table I.

of the general increase in both PGE_2 and cAMP in respons to stress, yet are significantly different from each other, the regulation of cAMP levels is probably under the control of different mechanisms in deposition and resorption.

In summary, the findings of this study show: 1) the spring placement and indomethacin dosage was rather well tolerated by the animals, 2) the measurement of protein content of bone (or another measure of active tissue) is necessary for comparison of quantitative biochemical data between animals, 3) that PGE_2 levels increased equally in response to tension or pressure when compared to controls, 4) that indomethacin delivered subcutaneously in a dosage of 1 mg/kg decreased PGE_2 levels below controls in both tension and pressure groups, 5) that cAMP levels increased double that of controls in the pressure group but attained an eight-fold increase in the tension group, and 6) that indomethacin had no effect on cAMP levels in the pressure group but decreased cAMP levels by half in the tension group.

DISCUSSION

The purpose of this investigation was to examine the effects of applied pressure and tension on the concentration of PGE_2 and cAMP in rat calvarial tissue *in vivo*. Mechanical stress was applied across the midsagittal suture to compare the effects of force application upon normal rapid calcification occurring in the calvarium of young growing rats. Since samples of sutures undergoing active remodeling were needed, an experimental period of five days was chosen, as it was felt this was the earliest period that allowed for observable histologic change and, hence, a cell population representative of resorption or deposition.

Previous studies have utilized tooth movement for defining pressure and tension sites (Behringer *et al.*, 1985; Davidovitch and Shanfeld, 1980b). Samples taken from alveolar bone in the direction of tooth movement have been labeled as "resorption sites" (pressure) and those taken from the opposing surface as "deposition sites" (tension). Any surface of bone undergoing active remodeling (as in tooth movement) consists of a mosaic of resorptive and depository fields separated by distinct reversal lines (Enlow, 1982). The difficulty in removing a portion of alveolar bone adjacent to the root of a tooth that is representative of a purely resorptive or depository field need not be described. To overcome this problem the midsagittal suture in rats was chosen as the target area for force application (Benson, 1983). However, the study incorporated

the use of osteotomies (see Figure 5) within the parietal bones. This isolated the midsagittal suture from surrounding sutures while maintaining a patent blood supply. It was felt that this model was a more pure expression of resorption and deposition than that afforded by tooth movement.

The results show a significant increase in PGE_2 over controls when either pressure or tension was applied and these increases were negated by the administration of indomethacin. Also, there was no difference in PGE_2 levels between the pressure and tension groups. Cultured bone tissue has been shown to synthesize prostaglandins when the culture dish is subjected to physical stress (Binderman _et al._, 1984; Somjen _et al._, 1980), but these data cannot be accurately extrapolated to _in vivo_ PGE_2 responses. Davidovitch and Shanfeld (1980c) used radioimmunoassay techniques to determine PGE_2 levels in alveolar bone of orthodontically treated cats. They reported control levels of PGE_2 to be 7.1 pg/mg bone, which is consistent with the control levels of 9.1 pg/mg bone reported in this study. They also reported a four- fold increases in PGE_2 at tension sites after one day but a return to control levels at day 7 and an initial 30% increase at pressure sites that also returned to control levels at day 7. In contrast, this investigation showed a 5-fold increase in both tension and pressure groups after five days (Figure 13). These differences are likely the result of either the difficulty in acquiring a representative depository or resorptive sample from alveolar bone as described previously or species differences (cat

vs. rat). Other data on endogenous PGE_2 levels in stressed bone tissue as measured by direct radioimmunoassay procedures could not be found.

Unlike PGE_2, cAMP increases were dependent upon the condition. The tension group had eight times the concentration of cAMP than did controls while cAMP levels in the pressure group only doubled when compared to controls. This cAMP increase in the tension group was shown to be significant at the $p < 0.05$ level. In a similar experimental model, Benson (1983) reported cAMP levels in the midsagittal suture to be only 0.77 pmole/mg protein ± 0.10 in control animals, whereas this study found cAMP levels of 5.2 pmoles/mg protein ± 2.0. Likewise, Benson reported cAMP levels in his tension group to be only 1.4 pmole/mg protein ± 0.46 but the tension group in this study had cAMP levels of 41.6 pmoles/mg protein ± 25.7. A listing of the differences between this study and that of Benson which may have contributed such discrepancies in cAMP levels can be found in Appendix I. Of these differences, the inclusion of the frontoparietal suture, which was not a target suture and may have diluted the sample cAMP concentrations, and the prolonged period of spring activation (21 days), which was representative of a later date in the kinetics of the cellular response, are believed to be the major reasons for the lower cAMP values reported by Benson. No information could be found in the literature pertaining to pressure stimulated cAMP levels in cranial sutures.

The mechanism of PGE_2 action in the regulation of bone remodeling is not yet understood. Evidence from bone cells in tissue culture indicate that physical stress causes the de novo synthesis of PGE_2 and that the PGE_2 producing cells are osteoblast-like (Binderman et al, 1984; Yeh and Rodan, 1984). Evidence for PGE_2 production in response to stress by osteoclast-like cells is lacking (Raisz and Martin, 1983), although osteoclast-like cells do have the capability to respond to prostaglandins (Wong and Kocour, 1983). Chambers et al. (1984) report that PGE_2 specifically inhibits cytoplasmic spreading in osteoclasts, which is a phenomenon necessary for bone resorption. However, if the osteoclasts were cultured in the presence of osteoblasts, PGE_2 caused a marked stimulation of osteoclastic spreading. Because osteoblasts are known to contain receptors for bone-resorbing substances and their presence enhances osteoclastic activity, it has been postulated that osteoblastic cells regulate bone resorption (Rodan and Martin, 1981).

> Any theory of activation must account for the presence of receptors for many of the known stimuli of bone resorption in osteoblasts, but not in osteoclasts, and must provide for specific local instruction of the osteoclasts (Parfitt, 1984).

In leu of current understanding, the question arises as to why cAMP levels were enormously increased in the tension groups but only mildly elevated in the pressure groups. Bone resorption is a function of osteoclastic cells, which should have been the predominate cell type in the pressure samples. Such low levels of cAMP in the pressure groups is puzzling, since PGE_2 has been shown to elevate

cAMP levels in cultured osteoclasts (Wong and Kocour, 1983). A related prostaglandin, PGE_1, can inhibit adenylate cyclase in adipocytes thru regulation of an adenosine receptor (Londos *et al.*, 1981). Similar mechanisms in osteoclastic cells, though not yet found, may explain the low cAMP values. Also, osteoblasts may regulate osteoclastic activity by either the release of positive mediators or cessation of the synthesis of a osteoclast inhibitory substance (Rodan and Rodan, 1983), both of which may regulate osteoclastic activity without effecting increased cAMP levels. Another explanation is that the low cAMP levels are representative of only one time period within the total kinetic response. Both PGE_2 and cAMP levels have been shown to fluctuate over time in similar *in vivo* studies (Davidovitch and Shanfeld, 1984, 1980c). This is a consideration in the interpretation of all data from this study. A time-response experimental model is needed to resolve the kinetics of bone remodeling.

The significant elevation of cAMP levels in the tension groups is thought to be a reflection of the differences in the regulatory process between resorption and deposition modes. The tension groups, being a depository model, should have osteoblasts as the primary cell type. In regard to PGE_2 activity, two subtypes of osteoblasts have been identified in tissue culture. One population shows an enhanced capacity for PG production with a diminished capacity for PG responsiveness (Partridge *et al.*, 1981) and the other cell line exhibits typical adenylate cyclase activation when

stimulated by PG's (Partridge _et al._, 1982). Whether these cell types, be they different subtypes of osteoblasts or the same cell type in different maturation stages, can function in a similar manner _in vivo_ is not known. However, their assumed presence can help to explain the observed cAMP changes.

PG-producing osteoblasts could respond to the physical stimulus by increasing PG synthesis, which in term would increase adenylate cyclase activity and cAMP levels in the PG-responsive cells. These PG responsive cells may then be activated to begin new bone formation. PGE_2 stimulation, followed by elevated cAMP levels, has been demonstrated to affect increased alkaline phosphatase activity in cultured bone cells (Kumegawa _et al._, 1984). Rodan and Rodan (1983) also report that PGE_2 has a stimulatory effect on collagen synthesis in cultured bone. Considering the evidence, the elevated cAMP levels seen in the tension group may be the result of cell type, function, and numbers.

However, this explanation is incomplete in that the tension + indocin group maintained elevated cAMP levels even after the PGE_2 response was halted. Although a portion of the cAMP response is under the influence of PGE_2, possibly by the mechanism described, the remaining cAMP response must be under control of mechanisms other than PGE_2. Cyclic nucleotides are responsive to a number of stimuli (Peck and Klahr, 1979), including parathyroid hormone, calcitonin, and extracellular Ca^{++}. Other products of arachidonic acid metabolism may also be involved. Indomethacin inhibits the

cyclooxygenase pathway but not the lipoxygenase pathway of arachidonic acid metabolism, the latter leading to the synthesis of leukotrienes, substances thought to be modulators of inflammation (Kuehl and Egan, 1980). Inflammation has been shown to be a part of the bone remodeling response (Yamasaki, 1982a). Further studies are needed to complete the picture of bone cell activation and regulation in response to stress.

In an attempt to relate changes in PGE_2 concentration to cAMP levels the drug indomethacin, a potent inhibitor of the cyclooxygenase enzyme, was used. A disadvantage to its use is that it inhibits production of all arachidonic acid products via the cyclooxygenase pathway (see Figure 3). What effects the absence of the other prostaglandins would have on this system should be investigated with the use of more specific prostaglandin synthetase inhibitors. In this study it is assumed that PGE_2 is a substantial effector prostaglandin.

Indomethacin is also known to be a potent inhibitor of cAMP dependent protein kinase in rabbit ileal mucosa (Kantor and Hampton, 1978). The inhibition of this enzyme would block the action of cAMP regardless of the stimulating agent. If indomethacin inhibits protein kinase in bone cells it should not have interfered with the quantitation of cAMP levels in this study, since the cellular concentration of cAMP is dependent upon the enzymes adenyl cyclase and phosphodiesterase (Figure I). However, the findings of this study need to be correlated with changes in the direction of

calcium flow (i.e. measureable histologic responses, via vital dyes, to tension and pressure). In such a study it would be prudent to consider the use of another prostaglandin synthetase inhibitor, because indomethacin may interfere with cAMP modulation of bone cell activity.

Kong _et al_. (1976) suggested that elevated prostaglandin levels in response to mechanical stress were the result of perturbations in the cell membrane. This exposes membrane phospholipids to phospholipase enzymes, making arachidonic acid available for prostaglandin production. The cells responsible for this prostaglandin synthesis are probably the PG-producing osteoblasts described by Partridge _et al_. (1981). Another explanation for increased PG levels in areas of mechanical stress is the induction of an inflammatory response. Prostaglandins are known to be a mediator of the inflammatory process (Willoughly, 1972). Also, Yamasaki (1982a) has shown that mechanical stress significantly increases the appearance of mast cells, a cell known to be involved with the initiation of the inflammatory response. The increased PGE_2 levels in response to tension or pressure is likely a result of a combination of the above two processes.

PGE_2 levels were not significantly different between tension and pressure groups (Table VII), suggesting either that PGE_2 is not the mediator responsible for directing bone cell activity into a depository or resorptive mode or that PGE_2 elevation is a reflection of a common process, possibly inflammation, occurring in both modes.

It would be helpful to obtain a profile of prostaglandin concentrations by means of high pressure liquid chromatography. With such techniques, Ohm _et al_. (1984) reported on the concentration of various prostaglandins in human periodontal disease. Of the eight prostaglandins tested, thromboxane A_2 and PGI_2 were most prevalent, followed by PGE_2. Similar differences may exist between the tension and pressure groups.

Figure 13 shows the reduction of PGE_2 levels in groups treated with indomethacin to below control levels. A dose-response study would be helpful in determining the minimum dosage of indomethacin needed to adequately suppress prostaglandin syntheses, as the dosage used in this study may have been too high. Kuehl and Egan (1980) report that indomethacin inhibits a number of enzyme systems but at much higher concentrations than that needed to suppress prostaglandin synthesis. However, indomethacin induced inhibition of the protein kinase enzyme (Kantor and Hampton, 1978) is of concern. Therefore, the lowest dosage of indomethacin needed for inhibiting the PGE_2 response should be used for future studies.

In an attempt to relate increasing PGE_2 synthesis with increasing cAMP production a comparison of the ratio PGE_2/cAMP was made between experimental groups. The ratio values for tension or pressure were not different than controls (Table IX), showing that both PGE_2 and cAMP are responding in kind to physical stress. Comparison of the ratio values between tension and pressure, however, show a significant difference. This is indicative of the different

regulatory process between deposition and resorption already described. Recent studies (Behringer _et al._, 1985 and Johnson _et al._, 1985) report higher concentrations of cAMP in osteoblasts at deposition sites than in osteoclasts at resorption sites as measured by immunohistochemical techniques. This is similar to the findings of this study, as the ratio differences between tension and pressure were a result of differences in cAMP levels not PGE_2 (compare Figure 15 and Figure 16). Of particular interest is the significant difference between the pressure and pressure + indocin groups (n=3 and n=4) and the weaker statistical difference between tension and tension + indocin (n=3 only). This finding indicates that PGE_2 has no regulatory role in cAMP production in the resorption model but does influence cAMP levels during bone formation. This is probably a result of the different predominate cell populations and their particular membrane receptor/enzymatic activation systems.

The non-collagen protein content of all samples was used as an estimate of cellular mass in order to relate PGE_2 and cAMP levels to a measure of active bone tissue. Protein content of the bone sample was shown to vary between 9.0 and 19.4% of total bone weight (Table III). Ham (1974) reported the protein content of bone samples to vary from 1-13% and Benson (1983) showed a more constant value of 22%. The values determined in this study lie between these two figures and are probably an accurate assessment of protein content. The wide variation between samples demands that future investiga-

tions include some determination of cellular mass in order to "fine-tune" the experimental data.

Findings of this investigation show that cAMP is a likely mediator of bone cell function during the remodeling process. The role of PGE_2, however, is less certain. Studies using immunohistochemical techniques to identify the sites of mediator concentrations and correlated with measureable histologic change are needed. Also, more specific enzyme inhibitors would be helpful in identifying the actions of specific arachidonic acid products, as these compounds are many and their actions yet still undefined.

Control of cellular response to tension and pressure is quite complex, the dynamics of which cannot be uncovered without a time-response experimental model capable of following the concentrations of biochemical mediators thru time. Examining the PGE_2 and cAMP levels at one time point is an inadequate representation of the complexity of the total response. Nonetheless, these types of investigations do give a glimpse at the total picture and are invaluable in directing the course of future research.

SUMMARY & CONCLUSIONS

The biochemical mediators in bone tissue responsible for the recognition of mechanical stress and its subsequent translation into a cellular response remain undefined. PGE_2 effects in other tissues have been widely studied, but its role in the local control process of bone remodeling is receiving increased attention. Likewise, cAMP is known to be a common "second messenger" to a variety of stimulating agents in an equally large variety of cell types. That these two substances interact along the pathway leading from stimulus recognition to cell activation in the bone remodeling response is a postulation formulated, for the most part, from tissue culture research.

The present study was initiated to examine the effects of mechanical forces upon the levels of PGE_2 and cAMP *in situ*. Springs calibrated to deliver 20 g of tensile or compressive force were surgically implanted across the midsagittal sutures of 21 day old female Sprague Dawley rats. Osteotomies within the parietal bones were used in all animals to minimize the influence of surrounding cranial regions. Indomethacin, as potent inhibitor of cyclo-oxygenase, was used in selected groups to suppress the PGE_2 response in order to determine any related changes in cAMP response. A sample of 24 animals were utilized and divided into the following groups of four animals each:

Group A - received tension springs.

Group B - received pressure springs.

Group C - no springs (controls).

Group D - received tension springs plus subcutaneous injection of indomethacin (1 mg/kg).

Group E - received pressure springs plus subcutaneous injections of indomethacin (1 mg/kg).

Group F - no springs but subcutaneous injections of indomethacin (1 mg/kg) (controls).

At the end of a five-day experimental period, the bone samples containing the target suture were removed under general anesthesia, immediately frozen in liquid nitrogen, and lyophilized. Samples were extracted and individually quantitated for PGE_2 and cAMP content by specific radioimmunoassay procedures. These values were related to a measure of active bone tissue as determined by analysis of the non-collagen protein content for each sample.

Analysis of PGE_2 concentration showed no difference between tension and pressure groups but both were significantly elevated over controls. The use of indomethacin significantly inhibited PGE_2 levels equally in all groups to levels below that of the control group.

Analysis of cAMP concentration showed elevated levels in both tension and pressure groups. However, the tension group had a significant eight-fold increase over controls while cAMP levels were only double control values in the pressure group. Indomethacin-induced PGE_2 inhibition had no effect on cAMP in the pressure group but reduced levels by one-half in the tension group.

The most significant findings of this investigation were:

(1) A simple, well tolerated surgical procedure, that provided a good model of the bone remodeling response;

(2) An extraction procedure that allowed recovery of PGE_2, cAMP, and cellular proteins from the same bone sample at an efficiency suitable for quantitative measurement;

(3) No difference in PGE_2 levels between tension and pressure groups, but both were significantly elevated over controls;

(4) Indomethacin, administered subcutaneously in a dosage of 1 mg/kg body weight, significantly decreased PGE_2 levels below controls even in stressed groups;

(5) cAMP levels were elevated eight-fold above controls with applied tension;

(6) cAMP levels were elevated two-fold above controls with applied pressure;

(7) Suppression of the PGE_2 response reduced cAMP levels by half in tension groups but had no effect in pressure groups;

(8) The non-collagen protein content of the bone samples, as determined by a variant of the Folin protein assay, was shown to vary between 9.0% and 19.4% of the total bone weight.

Findings of this investigation show that cAMP is a likely mediator of bone cell function during the remodeling process. The

role of PGE_2, however, is less certain. These results represent PGE_2 and cAMP levels at a single time period during the remodeling response. As the local mechanisms function to return the stressed condition to a state of equilibrium, the concentrations of these substances may fluctuate. A time-response study to gather information on the kinetics of the remodeling response (how it happens) with corollary histologic representation (what happens) is needed.

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APPENDIX I

Experimental Design Differences Between This Study and That of Benson (1983).

Condition	Radulovich (1985)	Benson (1983)
Age at Sacrifice	26 days	49 days
Average Animal Wt. at Sacrifice	88 gm	185 gms
Total Wt. of Bone Sample	10-20 mg	80-100 mg
Total Days of Spring Activation	5 days	21 days
Force of Spring Activation	20 g	60 g
Use of Osteotomy	yes	no
Sutures Included in Sample	midsagittal	midsagittal and fronto-parietal

APPENDIX II

Surgical Groupings of Experimental Samples.

Surgical Group No.	Experimental Samples Included	Day of Surgery	Day of Sacrifice
I	A1; B1; B2; C2	12/4/84	12/9/84
II	A2; A3; A4; B3; B4; C1; C3; C4	12/8/84	12/13/84
III	D3; D4; E3; E4; F3; F4	12/13/84	12/18/84
IV	D1; D2; E1; E2; F1; F2	12/16/84	12/21/84

Appendix III. Experimental sample identifications correspond to those found in Tables III, IV, and V. Premature death of animals A_2 and C_1 in surgical Group I necessitated replacement animals in surgical Group II