CASE WESTERN RESERVE UNIVERSITY

GRADUATE STUDIES

We hereby approve the thesis of

JEFFREY LEE COLLINS

candidate for the MASTER OF SCIENCE IN DENTISTRY

degree.

Signed: _____
(Chairman)

_____

Donald H. Enlow

John W Daniel

_____

Date MAY 20, 1986

THE EFFECTS OF MECHANICAL STRESS UPON THE CONTENT OF LEUKOTRIENE B_4, C_4 AND PROSTAGLANDIN E_2 IN THE MIDSAGITTAL SUTURE OF THE RAT

by

JEFFREY LEE COLLINS

Submitted in partial fulfillment of the requirements

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Department of Orthodontics

School of Dentistry

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THE EFFECTS OF MECHANICAL STRESS UPON THE CONTENT OF LEUKOTRIENE B_4, C_4 AND PROSTAGLANDIN E_2 IN THE MIDSAGITTAL SUTURE OF THE RAT

Abstract

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JEFFREY LEE COLLINS

A group of compounds which has received considerable investigative attention as possible mediators of mechanical stress in bone tissue is the cyclooxygenase products, especially prostaglandin E_2 (PGE_2), an arachidonic acid derivative. Recently another branch of the arachidonic acid cascade known as the leukotrienes has been identified. The possible presence of these compounds in skeletal tissue indicates that they too may have regulatory significance.

The purpose of this study was to evaluate the mediating role of leukotrienes B_4 and C_4 (LTB_4 and LTC_4) in the sutural remodeling induced by mechanical stress and a possible interrelationship between these leukotrienes and PGE_2 in the response mechanism to the applied stress.

A sample of thirty-six growing Sprague-Dawley rats was divided equally into one control group (shame operated) and two experimental groups. Osteotomies were cut into the parietal bones to isolate the midsagittal suture from biomechanical influences from surrounding areas. Springs were implanted across the suture to exert

twenty grams of pressure in one experimental group and twenty grams of tensile force in the other. One half of the animals received subcutaneous injections of piriprost, a specific inhibitor of the 5-'leukotriene pathway.

At the end of the five day experimental period levels of LTB_4, LTC_4 and PGE_2, expressed as weight/mg of non-collagenous protein, were measured by radioimmunoassay procedures, specific for each metabolite. Histologic descriptive analysis was performed to yield corollary histological data with the changes in metabolite concentrations.

This investigation demonstrated a simple surgical procedure that provided a good model of the bone remodeling response. Findings showed that applied pressure caused strong reduction in LTB_4 levels, a marked increase in LTC_4 levels, and little change in PGE_2 levels when compared to controls. Applied tension caused strong reduction in LTB_4 levels, a two-fold increase in PGE_2 levels, and little change in LTC_4 levels when compared to controls. Furthermore, subcutaneous administration of piriprost, a specific inhibitor of the 5'-leukotriene pathway, caused reduction in all LTB_4 responses and in the pressure induced LTC_4 response. Piriprost stimulated the pressure induced response of PGE_2 but had no effect on the sham-operated controls.

Histologic data showed no evidence of inflammation or necrosis in any sample. Histology allowed correlation of the bone remodeling response with fluctuations in metabolite response. The com-

bined data suggests that all mechanical-stress induced remodeling were correlated with a reduced LTB_4 level. In addition PGE_2 appears to be a potential mediator of bone formation and LTC_4 maybe a potential mediator of bone resorption.

DEDICATED

TO

Emily Maxwell MacMichael, 1900-1986; my grandmother, who passed away recently and never had the opportunity to see my final graduation. It was her love and inspiration which led to my success in education. She will be missed by many, especially me.

ACKNOWLEDGEMENTS

A project of this magnitude could not have been completed by a single individual. The author wishes to thank those people who participated in the completion of this thesis.

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To the entire staff and clincial instructors in the Department of Orthodontics, who provided a wonderful environment for education.

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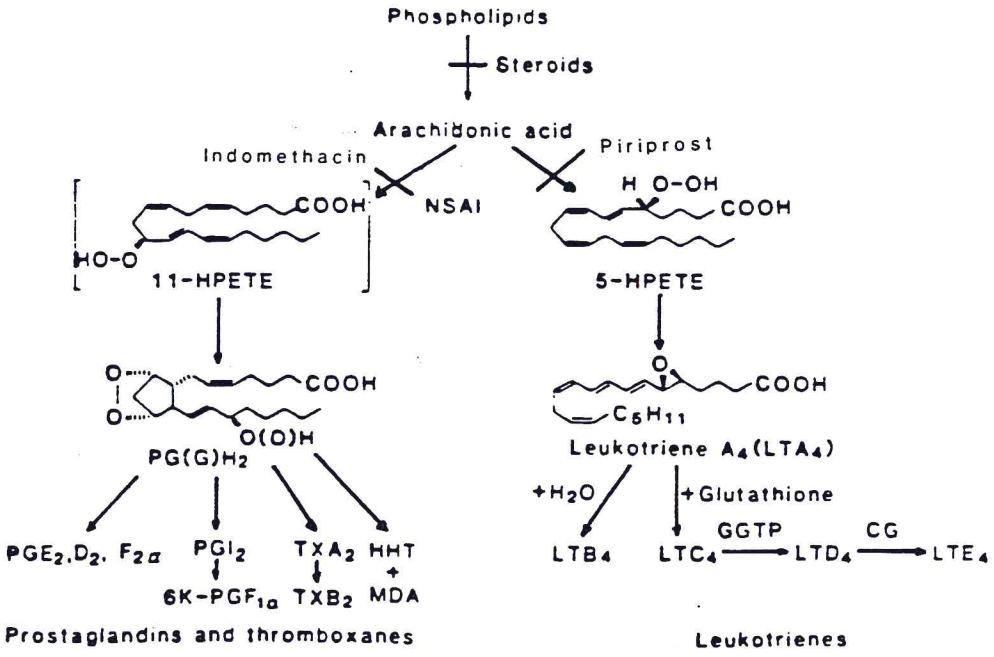
INTRODUCTION

A fundamental concept in dentofacial orthopedics is that when a mechanical force is applied to a skeletal articulation, the remodeling pattern may be altered. However, how response mechanisms to mechanical stress are switched on and controlled are poorly understood. As a result, the cellular regulation of bone remodeling is an area which is attracting a great deal of study. A group of compounds which has attracted a great deal of attention recently are the cyclooxygenase products, especially prostaglandin E_2 (PGE_2) an arachidonic acid derivative (Figures 1 & 3). However, the effects of arachidonic acid metabolites other than the cyclooxygenase products on bone remodeling are presently unknown. While studying inhibitory drugs of the cyclooxygenase enzyme pathway (aspirin, indomethacin and other non-steroidal anti-inflammatory (NSAI) drugs) it was found that there were pronounced differences in the anti-inflammatory response compared to the anti-inflammatory steroidal compounds (Borgeat et al., 1976). These results prompted the search for another group of arachidonic acid metabolites. To confirm the presence of these compounds Borgeat et al. (1976) conducted experiments with ^{14}C-labeled polymorphonuclear leukocytes. The result was the identification of a family of lipoxygenase products known as the leukotrienes (Figures 1 & 4).

To date there are little published data on leukotriene levels and their possible effects on bone remodeling. However, the presence of these compounds indicates that they may have regulatory

significance. Not only is there a possibility that the leukotrienes participate in the control mechanism influencing the levels of cyclooxygenase products (Feuerstein et al., 1981), but also the potential exists that leukotrienes might affect bone formation or bone resorption directly.

Thus, the purpose of this study was to assess possible changes in levels of leukotrienes B_4 and C_4 (LTB_4 and LTC_4) in response to induced bone remodeling and to detect any interrelationship between LTB_4, LTC_4 and PGE_2 in the regulation of sutural remodeling.

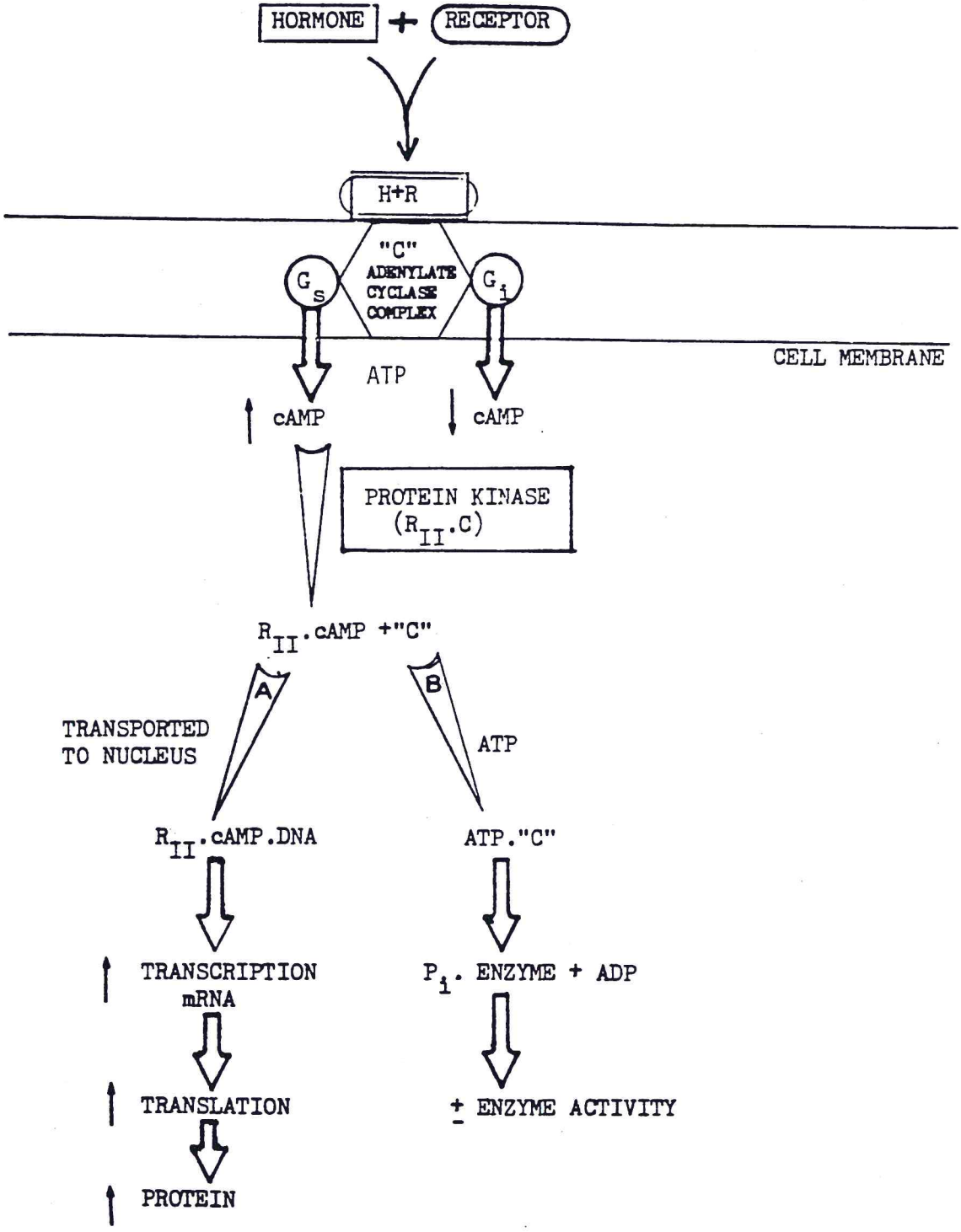

Phospholipids
Steroids
Arachidonic acid
Indomethacin
NSAI
Piriprost
COOH
HO-O
11-HPETE
H O-OH
COOH
5-HPETE
O
O
COOH
O(O)H
PG(G)H2
O
COOH
C5H11
Leukotriene A4(LTA4)
+H2O
+Glutathione
GGTP
CG
LTB4
LTC4
LTD4
LTE4
PGE2,D2, F2α
PGI2
TXA2
HHT
+
MDA
6K-PGF1α
TXB2
Prostaglandins and thromboxanes
Leukotrienes

BACKGROUND

Descriptive histological analyses of tooth movement in response to force application have been undertaken by many investigators, e.g. Oppenheim (1911), Noyes (1942) and Reitan (1947). They reported that orthodontic forces brought about reorganization of bone tissue cells resulting in movement of the teeth. Although the histological picture of bone remodeling has been detailed, the mechanism of how an applied force is recognized at the cellular and molecular level and the nature of the resulting response are unknown in skeletal tissue.

Of great importance in molecular biology was the finding of Sutherland and Rall (1960) that the enzyme adenylate cyclase is present in cell membranes and transforms adenosine triphosphate (ATP) into cyclic - 3'5' - monophosphate (cAMP). A hormone, acting as a first messenger, travels to the target cell, binds to the specific receptor (adenylate cyclase). The cAMP is released intracellularly where it acts as a second messenger activating protein kinase which catalyzes the phosphorylation of a broad spectrum of proteins directing cell function as well as controlling DNA transcription (Figure 2).

Rodan et al. (1975) were the first to correlate the levels of cyclic nucleotides, cAMP and cGMP, with application of mechanical stress on cultured bone tissue cells. At the same time Davidovitch and Shanfeld (1975) were examining cAMP levels in orthodontically treated cats. They found results which indicated a significant


HORMONE
RECEPTOR
H+R
"C"
ADENYLATE CYCLASE COMPLEX
G_s
G_i
CELL MEMBRANE
ATP
cAMP
cAMP
PROTEIN KINASE (R_{II}.C)
R_{II}.cAMP +"C"
A
B
TRANSPORTED TO NUCLEUS
ATP
R_{II}.cAMP.DNA
ATP."C"
TRANSCRIPTION mRNA
P_i. ENZYME + ADP
TRANSLATION
± ENZYME ACTIVITY
PROTEIN

increase in cAMP levels over controls. Benson (1983) found that mechanical stress applied to the midsagittal suture of rats induced a significant increase in cAMP and a decrease in cGMP. Theophylline, a phosphodiesterase inhibitor, resulted in a similar increase of cAMP. When treatment with this drug was combined with mechanical stress the effects were enhanced. In addition, a positive association was found between levels of cAMP and amount of sutural expansion. From these projects it was concluded that mechanical force can activate the cyclic nucleotide system and that this effect is associated with increases in bone formation and/or bone resorption.

PGE_2 was first implicated in the bone remodeling system when it was observed by Goodson et al. (1974) that patients with bone loss due to periodontal disease had a 10 fold increase in the levels of PGE_2. It was shown by Yu et al. (1976) that prostaglandins, suspected to be involved in localized bone loss, increase cAMP levels in bone cells cultured from rat calvaria. Yu and coworkers suggested that the bone response to prostaglandins, primarily PGE_2, was mediated via cAMP. Providing further evidence for a mechanism of prostaglandin control of cAMP levels, Somjen et al. (1980) described a cause-effect relationship between PGE_2 and cAMP in cultured rat periosteum stimulated by physical stress. His findings showed that when PGE_2 synthesis was blocked with indomethacin, a potent inhibitor of the cyclooxygenase enzyme, it

resulted in a decrease in cAMP levels. In addition, when PGE_2 levels were increased, cAMP levels also increased.

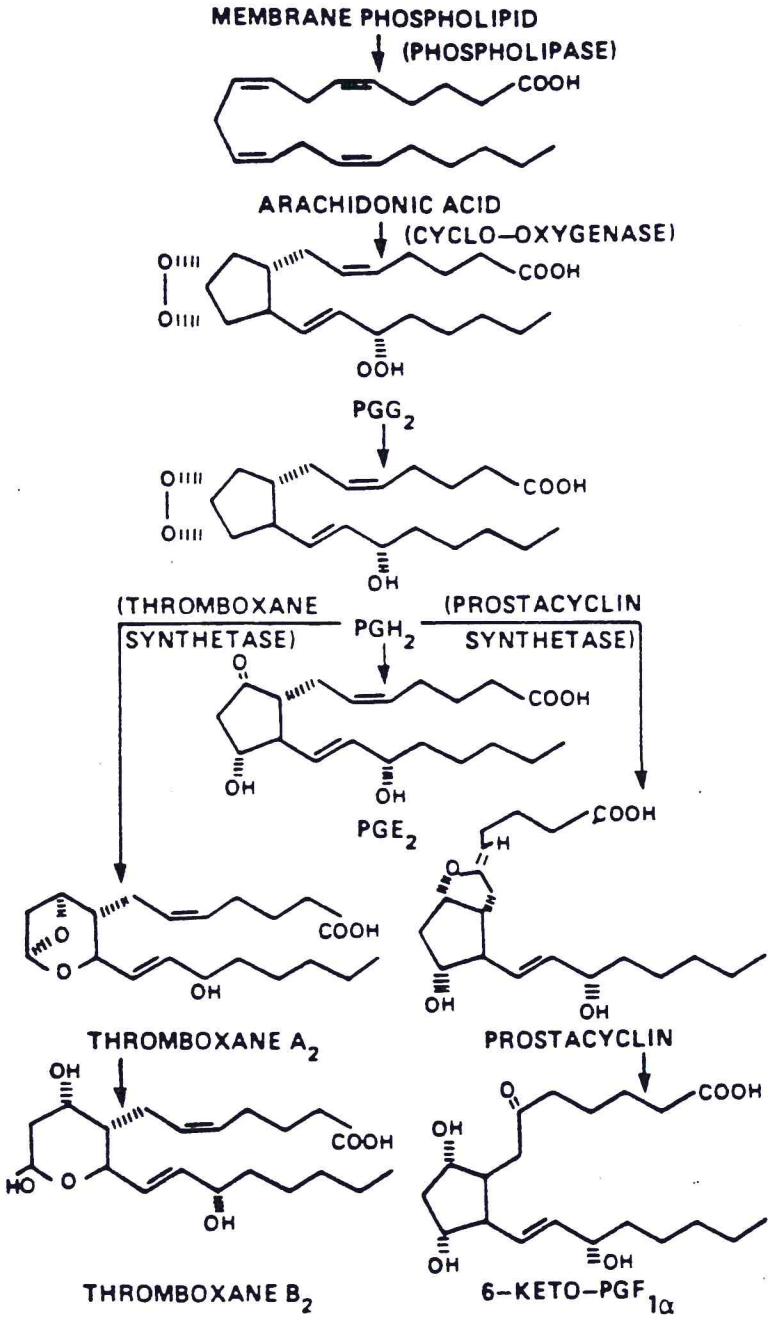
In vivo studies have suggested that prostaglandins may be involved in the bone remodeling response. Through experimental tooth movement in rats, Yamasaki et al. (1980) demonstrated (1) a dose-related inhibition of osteoclast formation when indomethacin was administered prior to and during tooth movement, and (2) a dose-dependent increase in osteoclast formation with local injections of PGE_2 adjacent to the teeth. In addition to this study, Yamasaki et al. (1982,1984) measured a doubling in the rate of tooth movement with local administration of PGE_1 and PGE_2 in monkeys and humans. Recently, Chumbley and Tuncay (1986) showed that when orthodontically treated cats were injected with indomethacin they observed the rate of tooth movement in the experimental animals was approximately one half of their controls.

Radulovich (1985) found by placing pressure and tension springs across the midsagittal suture of 21 day old Sprague-Dawley rats a significant increase in the levels of PGE_2 in both pressure and tension. Concomitantly he found an eight fold increase of cAMP in the tension group and a two fold increase in the pressure group. He found suppression of PGE_2 with indomethacin injections resulting in 50% reduction of cAMP response in tension rats. However, indomethacin had little effect on cAMP levels in pressure treated rats compared to controls.

Lipoxygenase products have not been examined in the bone remodeling system. However, there are several possible mechanisms by which leukotrienes might be involved: (1) Some lipoxygenase products participate directly by altering processes which can control cyclooxygenase products. Studies by Feuerstein et al. (1981) with rat peritoneal macrophages showed that macrophages incubated with LTC_4 resulted in a significant increase in the release of PGE_2. Also, Seal and Piper (1978) reported that LTC_4, isolated from human lung tissue and exposed to Guinea pig lung tissue, resulted in release of prostaglandin-like substances. Folco and Hansson (1981) isolated sensitized guinea pig lungs and injected LTC_4. This administration stimulated formation of thromboxane A_2. These results indicate the presence of a control interaction between the two arachidonic acid pathways rather then just a sharing of arachidonic acid precursors. (2) Another possible mechanism of leukotriene involvement in bone remodeling is the phenomenom of chemotaxis. The cells involved in the local differentiation of osteoclast progenitors seem to be from the hematopoietic lineage, the most likely being the monocyte and mononuclear phagocyte (Tinker et al., 1982; Burger et al., 1984). Studies by Ford-Hutchinson et al. (1980) illustrated that when polymorphonuclear leukocytes were stimulated by a calcium ionophore, they released LTB_4. This leukotriene has been shown to have a substantial chemotactic activity towards polymorphonuclear leukocytes and eosinophils. (3) Leukotrienes might be responsible in the control of microcirculation during bone

remodeling. Perhaps leukotrienes are a trigger responsible for bringing nutrients into the area. Dahlén et al. (1980) showed in experiments with hamster cheek pouches that LTC_4 and LTD_4 not only showed bronchoconstrictive activity but also produced a significant dose-dependent increase in the permeability of the capillary system.

Because metabolites of the lipoxygenase pathway probably play a role in the pathogenesis of several diseases, an extensive search is in progress to find drugs which have specific inhibitory effects on the lipoxygenase pathways. An effective compound has been shown to be piriprost, a prostacyclin derivative. Bach (1982) found that piriprost is an inhibitor of the enzyme 5'-lipoxygenase which converts arachidonic acid into 5'HPETE. However, it does not inhibit 12' and 15' lipoxygenase or cyclooxygenase. This is important because the derivatives of these enzymes influence the levels of LTB_4 and LTC_4 (Vanderhoek et al., 1982). A secondary mode of action of piriprost according to Bach et al. (1984) is the inhibition of glutathione S-transferase which converts LTA_4 to LTC_4.


MEMBRANE PHOSPHOLIPID
(PHOSPHOLIPASE)
COOH
ARACHIDONIC ACID
(CYCLO–OXYGENASE)
COOH
OOH
PGG2
COOH
OH
(THROMBOXANE SYNTHETASE)
PGH2
(PROSTACYCLIN SYNTHETASE)
COOH
OH
OH
PGE2
COOH
COOH
OH
OH
THROMBOXANE A2
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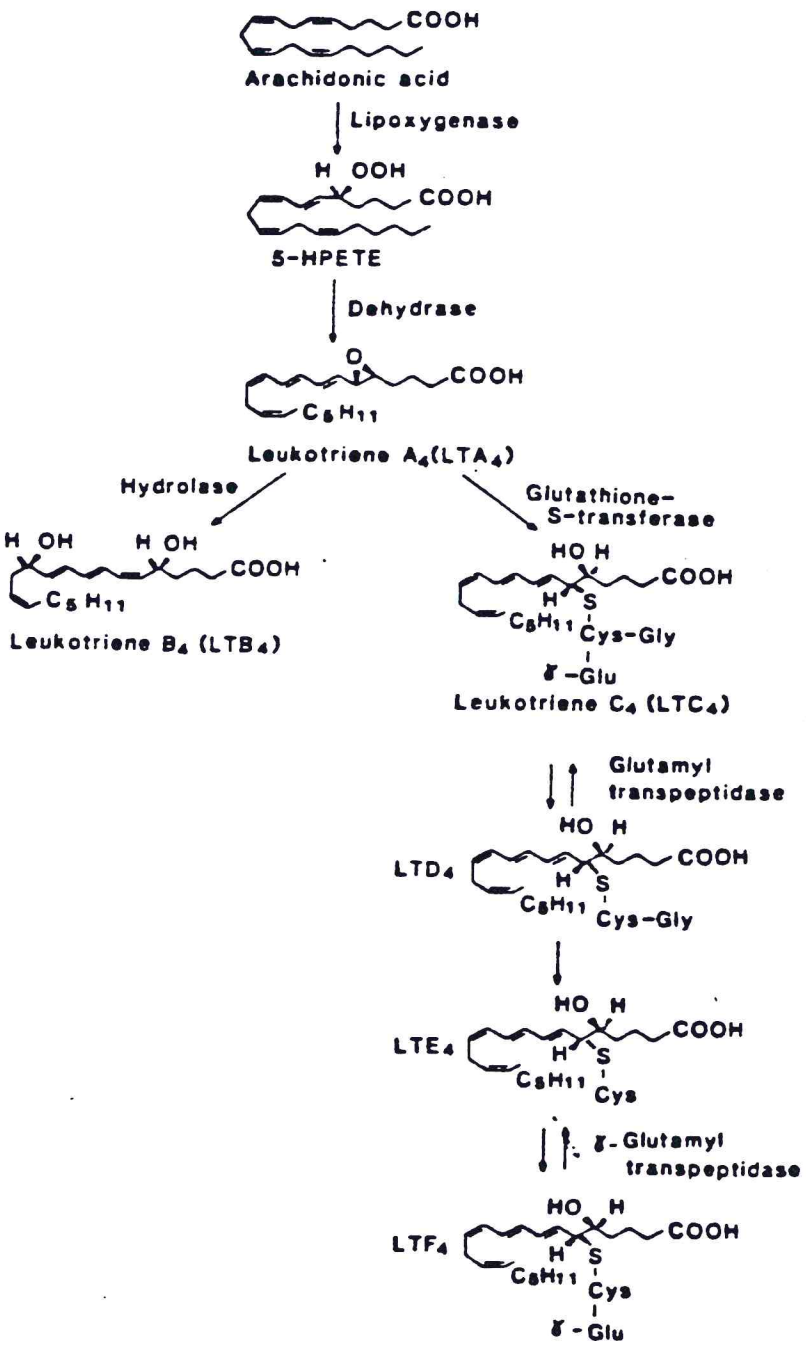
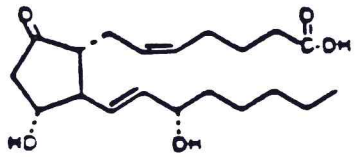
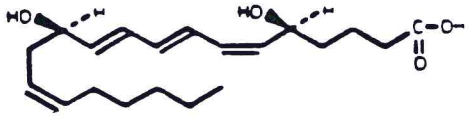
OH
THROMBOXANE B2
6–KETO–PGF1α


COOH
Arachidonic acid
Lipoxygenase
H OOH
COOH
5-HPETE
Dehydrase
O
COOH
C_5H_{11}
Leukotriene A_4(LTA$_4$)
Hydrolase
Glutathione-
S-transferase
H OH H OH
COOH
C_5H_{11}
Leukotriene B_4 (LTB$_4$)
HO H
COOH
H S
C_5H_{11} Cys-Gly
γ-Glu
Leukotriene C_4 (LTC$_4$)
Glutamyl
transpeptidase
LTD$_4$
HO H
COOH
H S
C_5H_{11} Cys-Gly
LTE$_4$
HO H
COOH
H S
C_5H_{11} Cys
γ-Glutamyl
transpeptidase
LTF$_4$
HO H
COOH
H S
C_5H_{11} Cys
γ-Glu

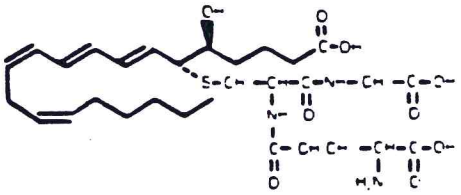
PROSTAGLANDIN E_2



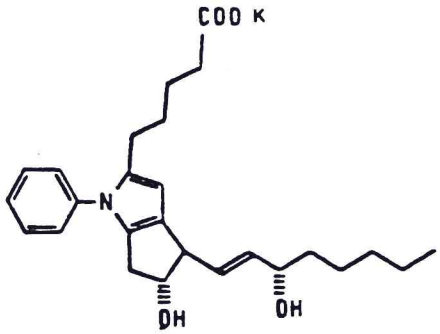
Leukotriene B_4



Leukotriene C_4



Piriprost



STATEMENT OF THESIS

The purpose of this study was to evaluate the mediating role of leukotrienes in sutural remodeling induced by mechanical stress and a possible interrelationship between leukotrienes and prostaglandins in the response mechanism to the applied stress.

A sample of thirty-six growing Sprague-Dawley rats was divided equally into a control and two experimental groups. Osteotomies were cut into the parietal bones to isolate the midsagittal suture from biomechanical influences from surrounding areas. Springs were implanted across the suture to exert pressure in one experimental group and tensile forces in the other. One half of the animals received subcutaneous injections of piriprost, a specific inhibitor of the 5'-leukotriene pathway.

Resultant levels of leukotrienes B_4 and C_4 (LTB_4 and LTC_4) and prostaglandins E_2 (PGE_2) in each bone sample were measured by radioimmunoassay procedures specific for each metabolite. Levels of non-collagenous protein were determined for each sample by a variation of the Folin protein assay to normalize the bone samples in terms of active bone tissue.

Histologic descriptive analysis was performed to yield corollary histological data with the changes in metabolite concentrations.

METHODS AND MATERIALS

Animals

The present study utilized 36 female Sprague-Dawley rats. They were delivered at 14 days of age from Zivic Miller Laboratories (Allison Park, PA) one week before the start of the experiment. For consistency of growth the animals were special ordered to weight approximately 25-30 gms on delivery (12-13 days old). Weights below this showed an irregular growth pattern in an initial pilot study. The seven days between delivery and the start of the project allowed the animals to recover from the trauma from shipping and to adjust to their new environment. The pups were force-weaned at 19 days of age to prepare them for independency from the mother at the beginning of the experiment.

The experimental period began when the animals were 21 days of age. This age was chosen to resemble the experiments of Radulovich (1985). This is a time when the pups have left their mother but are growing rapidly. Their weights at this time ranged from 47 to 66 grams. Care was followed to attempt to maintain a constant circadian rhythm of the animals with a consistent light-dark cycle (6:30 am - 6:30 pm) and insuring minimal disturbance of the pups during the experimental period. The animals were weighed daily just prior to dark cycle and the bedding (Wood Particles 1, American Excelsior Co., Arlington, TX) was changed at the this same time every third day.

Each cage (17"x13"x8") housed six animals in a temperature (23-25°C) and humidity (10-20%) controlled environment. The pups were fed a diet of Purina Rat Chow (Ralston Purina Co., St. Louis, MO) and tap water ad libitum.

Experimental Design

The procedures to isolate compressive and tensile forces on the midsagittal suture were similar to those used by Radulovich (1985). The present study utilized 36 growing female Sprague-Dawley rats. At the start of the experimental period, they were 21 days old and were killed five days later, at 26 days of age. The animals were equally divided into three groups: control, sutural pressure and sutural tension. Each of these groups were further divided equally into two subgroups. Thus, six groups were created with six animals in each. Groups D, E and F received subcutaneous injections of piriprost, a prostacylin derivative, (The Upjohn Company, Kalamazoo, MI) which is a specific inhibitor of the 5'-lipoxygenase pathway, while groups A, B and C received no drug therapy (Table 1).

The injections began two hours prior to the operations and were repeated immediately before and after the dark cycle of each 24 hour period during the entire five day experimental period. The dosage of piriprost was 10 mg/kg body weight. The animals were weighed daily and the amount of drug administered was adjusted accordingly.

EXPERIMENTAL DESIGN

EXPERIMENTAL CONDITION	GROUP	BIOCHEMICAL ANALYSIS # OF ANIMALS	HISTOLOGIC EVALUATION # OF ANIMALS
CONTROL	A	4	2
PRESSURE	B	4	2
TENSION	C	4	2
CONTROL & PIRIPROST	D	4	2
PRESSURE & PIRIPROST	E	4	2
TENSION & PIRIPROST	F	4	2

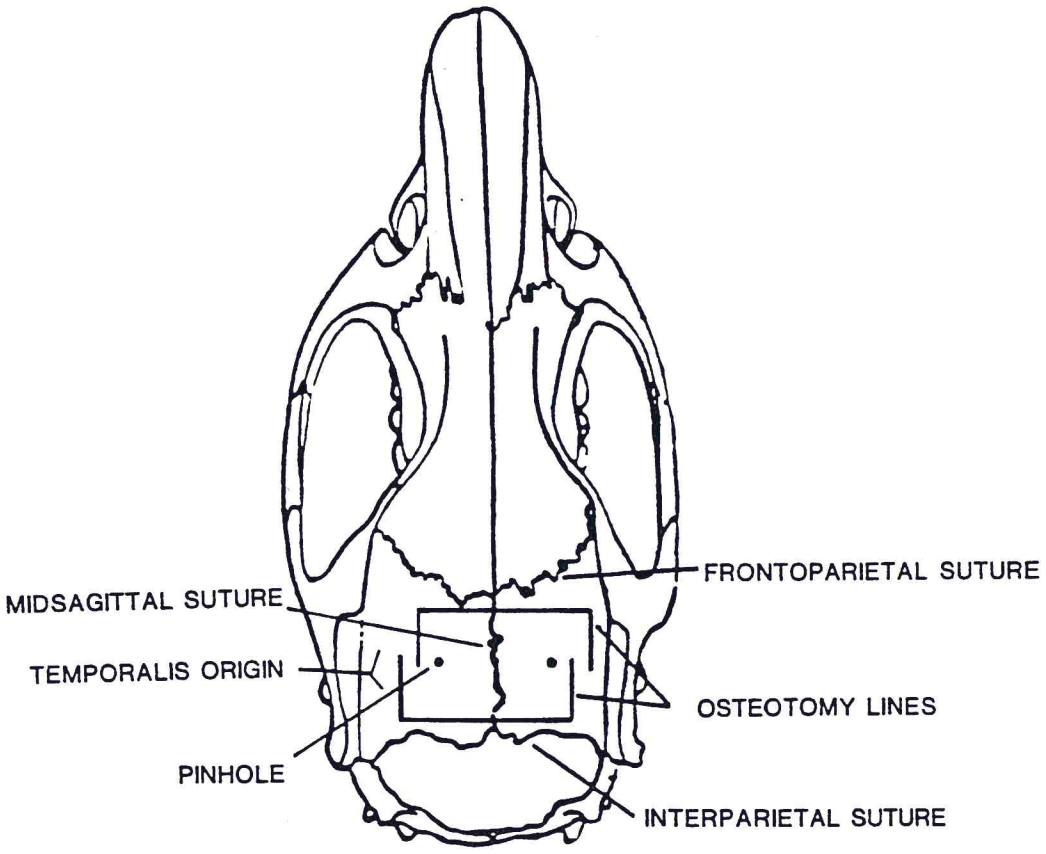
Four animals within each group were randomly selected for biochemical analysis and two for histological evaluation. Groups A and D were the control groups. These animals underwent surgical procedures consisting of calvarial osteotomies in the parietal bones (Figure 6). They received no experimental pressure or tensile forces. Groups B and E were the pressure groups. Following osteotomies a coil spring was implanted in the parietal bones of these animals which elicited twenty grams of pressive force across the midsagittal suture. The design of the osteotomy allows the bone segment to maintain continuity with the surrounding structures, yet produces a segment capable of isolating pressure or tensile forces. Finally, groups C and F had implanted in the osteotomized parietal bone, coil springs with twenty grams of tensile force across the midsagittal suture.

The described grouping of the animals made it possible to quantitate the effect of pressure and tension with and without a leukotriene inhibitor, as well as the effect of the inhibition itself. The metabolite levels were obtained by protein assay and radioimmunoassay of LTB_4, LTC_4 and PGE_2.

The levels of these metabolites were used to determine the degree of participation of leukotrienes and any interrelationship between leukotrienes and PGE_2 in the regulation of sutural remodeling.

At the end of the five day experimental period the bone segments were removed. Granulation tissue and blood products sur-

DORSAL VIEW OF RAT CALVARIA



rounding the bone segment were gently scraped and rinsed, the edges of the osteotomy were removed to eliminate any possible increase in the biochemical mediators from fracture healing.

The bone specimens were than quick frozen in liquid nitrogen to preserve and minimize biochemical changes at the time of removal. The samples were lyophilized overnight ($\sim$ 12 hours) and than stored in a freezer at -80°C for future extraction and analysis for LTB_4, LTC_4, PGE_2 and protein assay.

The two animals within each group used for histologic evaluation receive identical treatment to the other animals in that group. At the end of the five day experimental period the animals were perfused using one half strength Karnofsky solution. Once perfusion was complete the osteotomy was removed and divided in half. One half was placed in 10% formalin to be processed for light microscopy. The other half was placed in one half strength Karnovsky solution for future electron microscopy preparation.

The bone segment for light microscopy was placed in a formalin/alcohol series, decalcified in EDTA, embedded in paraffin, sectioned and stained with hematoxylin and eosin and examined by light microscopy. Histological descriptive analysis was performed to yield corollary histologic evidence with the biochemical analysis.

Appliance Construction

The appliances used to deliver the compressive and tensile forces were designed and used by Benson (1983). Radulovich (1985) used similar appliances. They consisted of springs constructed

from 0.014 inch round austenitic stainless steel wire (Unitek Corporation, Monrovia, CA) (Plate 1).

All springs were fabricated by first placing a single coil with a one half mm extension in the wire. This was accomplished by wrapping the wire around the small barrel of the tweed loop forming plier (Unitek Corporation). These extensions were used to anchor the spring to the calvaria by placing them in bilateral pinholes drilled in the osteotomy (Figure 2). Further retention was maintained by bending the extensions either inward (pressure spring) or outward (tension spring) approximately 60° from the horizontal. The single coil was included to prevent excessive penetration of the wire into the underlying tissues.

The next step in the fabrication was to make two 7 mm arms separated by a multihelical coil. This coil was formed by placing four loops of wire around the large barrel of a Tweed loop forming plier. This coil provided the flexibility in the spring to elicit a reasonably constant force of 20 gms.

All springs were preadjusted with a precision force gauge (Haldex, Halmstad, Sweden) to produce 20 grams of force when either compressed or expanded to five mm. Placement of springs and final calibration is discussed in the surgical section.

Anesthesia

A. Surgical Procedure Anesthesia

Induction of anesthesia for implant surgery was produced by a intramuscular gluteal injection of 0.1 ml 90% Ketamine hydrochlo-

ride solution, (100 mg/ml Ketaset , Bristol Labs, Syracuse, NY) and 10% Acepromazine Maleate (10 mg/ml Ayerst Laboratories Inc., New York, NY). These drugs were used in combination to facilitate handling of the animal and to increase the margin of safety over and above the sole use of a single drug. The Ketaset was used to produce anesthesia which causes the animal to be immobile and free of pain. The Acepromazine was used as a tranquilizer which renders the animal relaxed. In addition to these two drugs a 0.05 ml local subcutaneous infiltration injection of 2% Xylocain hydrochloride with epinephrine 1:100,000 (Astra Pharmaceutical Products, Inc., Worcester, MA) was given. This reduced sensation in the surgical area and prevented any sudden jerks during the surgical procedures.

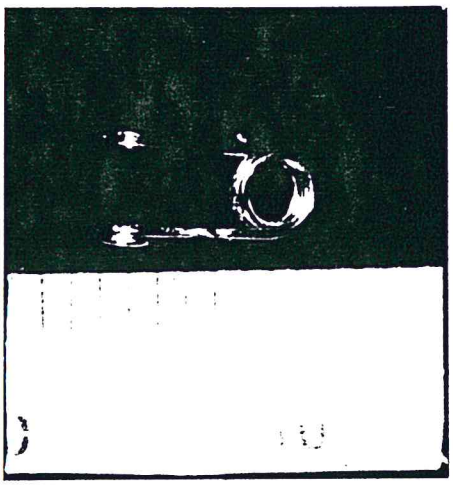
B. Sacrifice Anesthesia

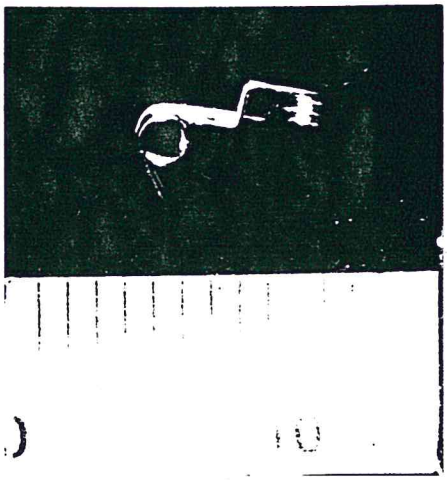
The animals in the biochemical assay group were given a 0.2 ml intramuscular injection of anesthetic prior to sacrifice. At this time no lidocaine was given because it was felt it might have an adverse effect on the metabolites. The animals that were prepared for histologic evaluation were injected with 0.2 ml Ketamine/ Acepromazine solution at the time of sacrifice. In addition the pups were also given approximately 0.2 ml intraperitoneal injections of sodium pentobarbital 60 mg/ml (Diabutal, Diamond Laboratories, Des Moines, IA) in increments of 0.05 ml until the animal no longer responded to tail pinching.

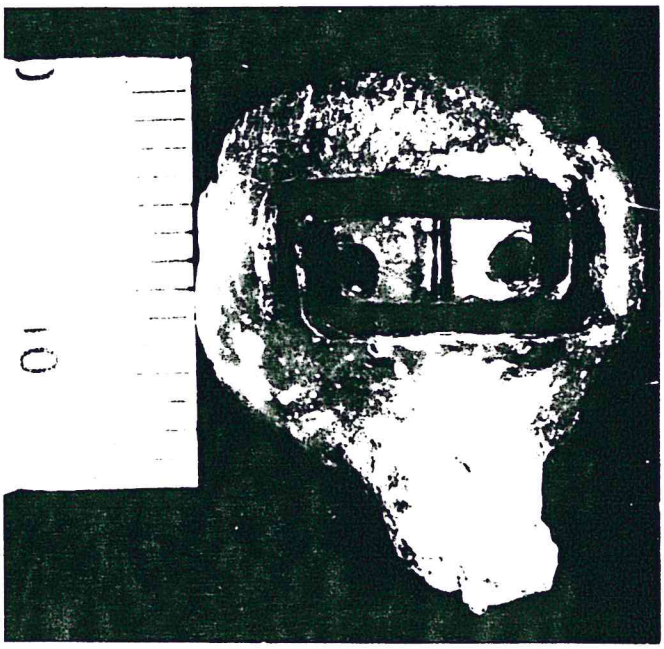
Surgical Procedure

The purpose of this procedure was to isolate a bone segment and to implant coil springs applying either pressure or tension to the midsagittal suture. Once the animals were anesthetized, they were weighed and prepared for surgery. The hair on the top of the head was removed with scissors from the back of the neck to between the eyes. Copious amounts of Povidone-iodine (Betadine , The Purdue Frederick Co., Yonkers, NY) was applied to the surgical field with a cotton tip applicator and blotted dry with a sterile 2"x2" gauze. An incision approximately 20 mm from the back of the neck to between the eyes was made with a #15 scalpel. The periosteal tissue covering the osteotomy was carefully deflected with the scalpel. The calvaria was rinsed with normal saline (0.9% sodium chloride, pH 5.7, Butler Co., Warren, OH) and blotted dry with a sterile 2"x2" gauze.

To obtain congruent osteotomies in all animals an acrylic template (Orthoacryl , Stratford-Cookson Co., College Park, GA) was constructed (Plate 3). It contained the outline of the osteotomy and the two pinholes for anchorage of the spring. The template was aligned with the midsagittal suture and placed midway between the frontoparietal suture and the interparietal suture. An outline was traced onto the calvaria utilizing a razor point felt tip marker, in addition two small dots were placed for location of the pinholes. Each hole was exactly 2.5 mm on either side of the





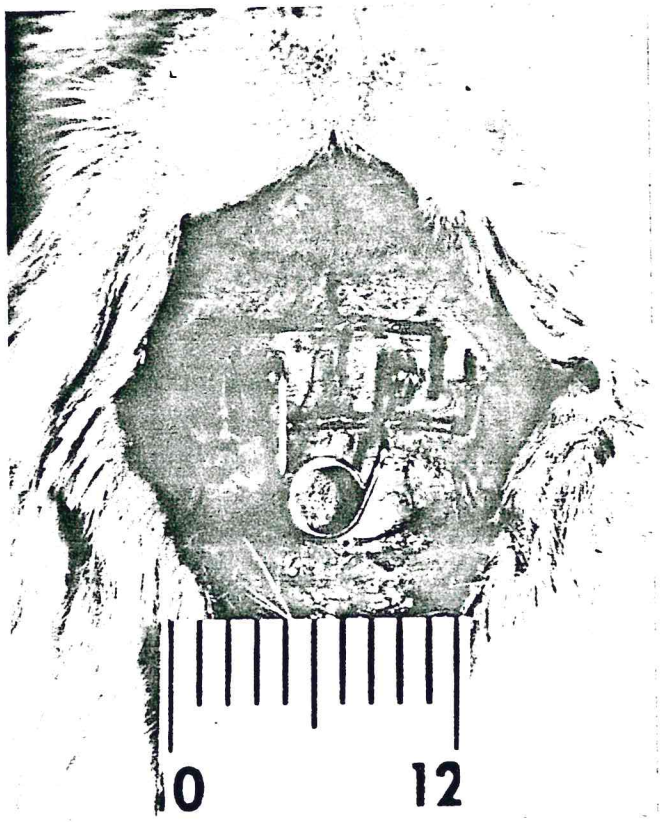


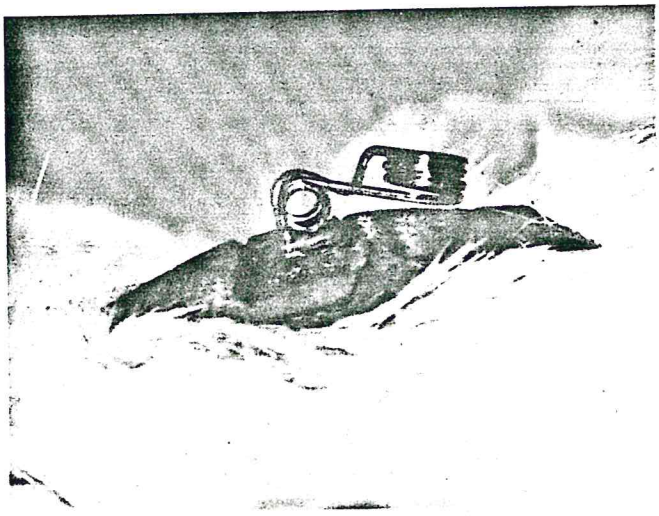
midsagittal suture, thus 5 mm of separation existed between the holes.

The pinholes were drilled into the bone utilizing a ¼ inch round carbide bur in a slow speed dental engine (maximum 14,000 rpm). Following placement of the pinholes the outline of the osteotomy was cut into the parietal bone using the same dental bur. The cuts were made completely through the bone, however special care was taken not to injure the underlying dura mater or to puncture the sagittal sinus.

While drilling the osteotomy the bone was irrigated frequently and thoroughly with saline solution. This was done to prevent the bone tissue from overheating due to friction of the cutting instrument and to remove any pulverized bone tissue which might initiate or accelerate fracture closure.

When the osteotomies were completed all animals except the controls had implanted in them either a pressure or tension producing spring (Plates 4 & 5). All springs were adjusted to 20 gms of force by inserting one wire extension of the spring into a pinhole while the other wire extension was placed into the opposite hole with the precision force gauge. Because all springs were preadjusted to deliver a force of 20 gms at a separation of 5 mm most of the springs were ready for cementation. However, a few springs needed to be slightly adjusted and checked again with the force gauge. Once the springs were in place a small drop of cyanoacrylate (910 Adhesive, Permabond International Division, Englewood,


0
12



NJ) was applied with a dental explorer around the hole to retain the spring. After 30 seconds the bond strength was tested by placing a dental explorer under the spring and raising the head of the animal off the surgical table using the spring as a handle.

The surgical area was then lightly dusted with neomysin sulfate (Neosul II , Carson Chemical Inc., New Castle, IN) to prevent any post-operation infections. The incision was closed with 4-0 silk suture (Ethicon cutting CE-2, Henry Schein Co., Port Washington, NY) which were individually double tied to prevent loosening of the sutures.

The animals were transferred to a recovery cage which was warmed with a heating pad on low setting. Once recovered ($\sim$1 hour) the animals were placed with the rest of the pups in their respective group.

Drug Administration

Piriprost potassium, 6,9-deepoxy-6,9-(phenylimino)- 6,8 PGI_2 potassium (U-60,257B, lot A 0171-RLA-005C, the Upjohn Company, Kalamazoo, MI) was the drug of choice in this experiment. It was chosen because it is a specific inhibitor of the 5' lipoxygenase pathway and apparently has little or no effect on the 12', 15' lipoxygenase and cyclooxygenase pathways. Because piriprost (mol. wt. 456) is a prostacylin derivative it, like prostaglandins and leukotrienes, must be handled with siliconized glassware or polypropylene containers to prevent adsorption. Piriprost, both in the solid form and in solution, is unstable if exposed to room tem-

perature air and light. Therefore in preparation of the drug care was taken to work quickly and in an ice bath under dark cover. Because the drug is soluble in water it was diluted with normal saline to a final concentration of 3.5 mg/ml solution for injection. Since the free acid tends to come out of solution when the pH drops below 6.5, the pH was adjusted utilizing a micropipet and pH paper (Short Range Alkacid 6.0-8.8, Fisher Scientific) to a pH of 7.0-7.2 with saturated K_2CO_3 and 2 N NCl.

The drug was stored in 1.5 ml snap-cap polypropylene vials (39 x 10 mm, Sarstedt, W. Germany) and stored at -80°C, a temperature at which the drug is reasonably stable. Only enough drug for a series of injections was removed from the freezer. The piriprost was thawed, inspected for solubility and injected at a dose of 10 mg/kg twice daily. The injections were given at the beginning and at the end of the dark cycle of each 24 hour period (8:00 am & 6:00 pm). The animals were weighed prior to the beginning of the dark cycle and the amount of drug administered was adjusted to maintain a dosage of 10 mg/kg (0.15 ml - 0.24 ml per injection). Subcutaneous injections were given by lifting the skin of the neck and administering quickly. The drug appeared to have no deleterious effects on the animals. All pups appeared healthy and normal throughout the experiment.

Sacrifice of the Animals

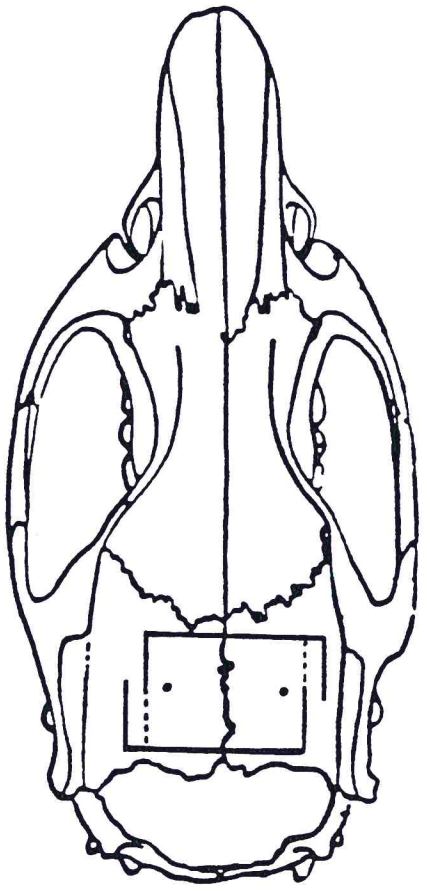
A. Biochemical Assay Sacrifice

Sacrifice of the animals for biochemical assays took place between 8:00 and 10:00 pm on the fifth day of the experiment. This time was chosen because it coincides with peak cortical bone formation during the 24 hour cycle (Simmons, 1979). The levels of leukotrienes would be most effectively measured at peak values, especially if the endogenous levels are generally low. The effects of the inhibitor would also be best measured during this period.

The animals were weighed and then given a 0.2 ml intramuscular gluteal injection of Ketamine/Acepromazine solution. The following procedures were completed as rapidly as possible so as to preserve the metabolites. The surgical area was reopened and the springs were checked for correct and unaltered position. Following this control, the springs were removed with forceps.

Granulation tissue covering the osteotomy was removed with the #15 scalpel. With a #12B scalpel the osteotomy cuts were connected and detached from the underlying connective tissue (Figure 7). Once the bone specimens were removed they were irrigated thoroughly and rapidly with normal saline to remove blood products. The bone was gently scraped with the #15 blade to remove excess granulation tissue. The edges of the bone were scraped to remove any cells accumulated in response to fracture healing caused by the osteotomy cuts. The bone was blotted dry with sterile 2"x2" gauze and then transferred to a 12 mm x 75 mm Pyrex culture tube (Corning Glass

DORSAL VIEW OF RAT CALVARIA



SAMPLES WERE REMOVED BY CONNECTING THE OSTEOTOMY ALONG THE DOTTED LINES.

Works, Corning, NY) which had previously been cooled and filled with liquid nitrogen. When all samples within each group were collected they were immediately placed in a wire basket which was transferred to a 300 ml fast freeze flask (Labconco, Fisher Scientific, Pittsburgh, PA) and lyophilized for 11-12 hours (Virtis, Unitrap Model 10-100, Vistas Co., Gardiner, NY). The rats were killed immediately after calvarial removal with an overdose (0.3 ml) of sodium pentabarbital injected into the heart. A test sample of 1 ml double distilled water and 1 ml toluidine blue dye was added to each sample group to insure proper lyophilization. Total elapsed time from surgical incision to bone removal was approximately 5 minutes. At the end of the lyophilization the samples were stored at -80°C in the assay tubes which were sealed with Parafilm (American Can Company, Greenwich, CT) until ready for pulverization, extraction and assay.

B. Histologic Evaluation Sacrifice

Sacrifice of the animals for histologic evaluation took place between 12:30 and 3:30 pm on the fifth day of the experiment. The animals were weighed and then given a 0.2 ml intramuscular gluteal injection of Ketamine/Acepromazine solution. An intraperitoneal injection of 0.2 ml sodium pentabarbital was given in increments of 0.05 ml until the animal did not respond to tail pinches. The skin of the chest wall was incised and deflected. The rib cage was cut and removed to expose the heart. A 21 gauge hypodermic needle (Becton, Dickinson and Company, Rutherford, NJ) was inserted into

the left ventricle while a small hole in the right auricle was placed to allow for exit of the blood. A solution of one half strength Karnovsky was pumped into the animal using a perfusion pump (Masterflex , Cole Palmer, Chicago, IL) for a total of 4 minutes.

The osteotomy area was reopened and the bone specimen removed in a similar manner as that described for the samples for biochemical assay. The only exception was that instead of irrigating the specimen in saline to remove blood products and other tissues the samples were rinsed in the one half strength Karnovsky fixative. Following irrigation and the scraping of the bone edges the bone samples were hemisectioned perpendicular to the suture. One half (for light microscopy) was placed in 10% formalin and the other half (for a future electron microscopy evaluation) was placed in one half strength Karnovsky solution.

The samples for light microscopy were decalcified in cold (4°C) 10% EDTA and 0.1 M Tris buffer, pH 6.95, and allowed to decalcify for a minimum of 15 hours. Following thorough rinsing in tap water the samples were then transferred to an automatic tissue processor (Autotechnicon Model 2A, The Technicon Company, Chauncey, NY). They were taken through a series of solutions consisting of 10% formaldehyde, 80% alcohol, 95% alcohol, 100% alcohol, Xylene and finally embedded in paraffin (Paraplast Plus, Monojet Scientific, St. Louis, MO). The specimens were than sectioned at 6 μm on a microtome (Spencer #820, American Optical

Corp., Buffalo, NY). The sections were stained with hematoxylin and eosin, mounted and viewed under light microscope for routine descriptive histology.

Extraction of LTB_4, LTC_4 and PGE_2

The extraction procedure was based upon the work of Benson (1983) and Radulovich (1985) which resulted in high recoveries of cAMP, PGE_2 and as developed in my work, high recoveries of LTC_4. Because both leukotrienes and prostaglandins will adhere to the walls of glass containers and certain plastics, the bone extracts were always handled in siliconized glassware (Sigmacote, Sigma Chemical Co.) or polypropylene vessels. The lyophilized bone samples were removed from -80°C storage and brought to room temperature in a desiccator containing Drierite Absorbent (Fisher Scientific). The samples were pulverized in an agate mortor and pestle then weighed to the nearest 0.1 mg on an analytical balance (Sartorius-Werke, West Germany) and placed in 13 mm x 100 mm Nalgene polypropylene centrifuge tubes (Nalge Co., Rochester, NY).

The bone samples were transferred to an ice bath (0-4°C) and allowed to cool for 10 minutes. As mentioned previously, because leukotrienes are sensitive to light the samples were, whenever possible kept under dark cover during the extraction procedure. Cold 1 M perchloric acid (PCA) was added to the bone samples in a ratio of 10 mg bone/1.0 ml PCA and allowed to stand in ice bath for 20 minutes with occasional mixing.

The extracts were then spun with an automatic refrigerated centrifuge (Sorvall Superspeed, RC 2-B, Ivan Sorval Inc., Newton, CT) at 49,500 g (20,000 rpm/SM-24 rotor) for 20 minutes at 2.5°C. The supernatants were removed and placed in 13 mm x 100 mm polypropylene centrifuge tubes. The acid insoluble pellets were returned to the -80°C freezer and stored for protein assay.

The supernatant was neutralized with saturated potassium carbonate (K_2CO_3) to pH 6.0 as determined with pH paper (phydrion 5.2-6.6, Micro Essential Laboratory, B'Kln, NY). The correct neutralizing volume ratios were determined by titrating each batch of PCA and K_2CO_3 at the time the reagents were prepared. The affinity of leukotrienes for the separation column is greater in the acid form. Therefore, the neutralized solution was back titrated with 2 N hydrochloric acid (HCl) to pH 3.5 as determined with phydrion 3.0-5.5.

The back titrated extracts were centrifuged at 49,500 g for 20 minutes (conditions as before). The supernatants were transferred to 1.5 ml polypropylene snap-cap vials and stored in an ice bath until ready for application to the separation columns. Total elapsed time from addition of PCA to placement on the column was 2½ hours for four samples.

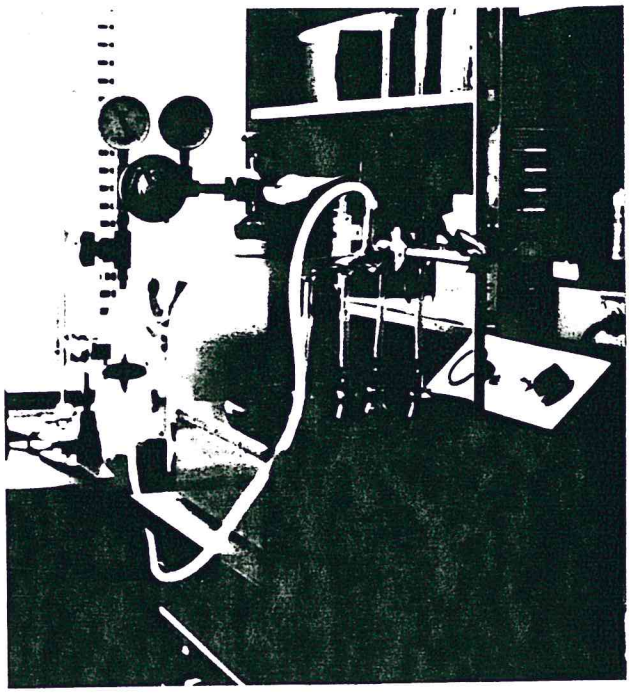
LTB_4, LTC_4 and PGE_2 Column Separation

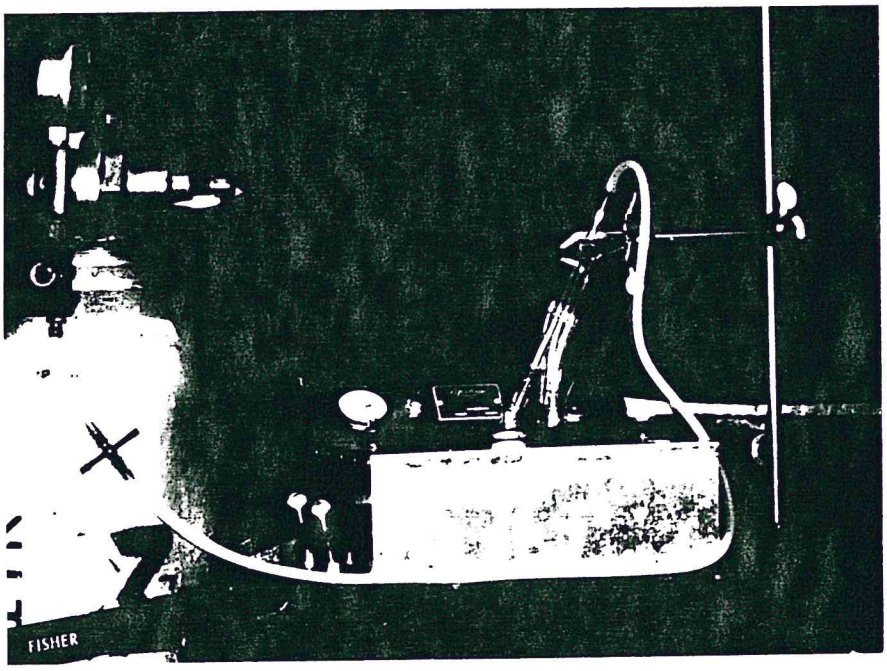
The procedure of column separation was performed by a method similar to that of Powell (1980). The final extracts were drawn into a 1.0 ml tuberculin syringe and added to an octadecylsilyl

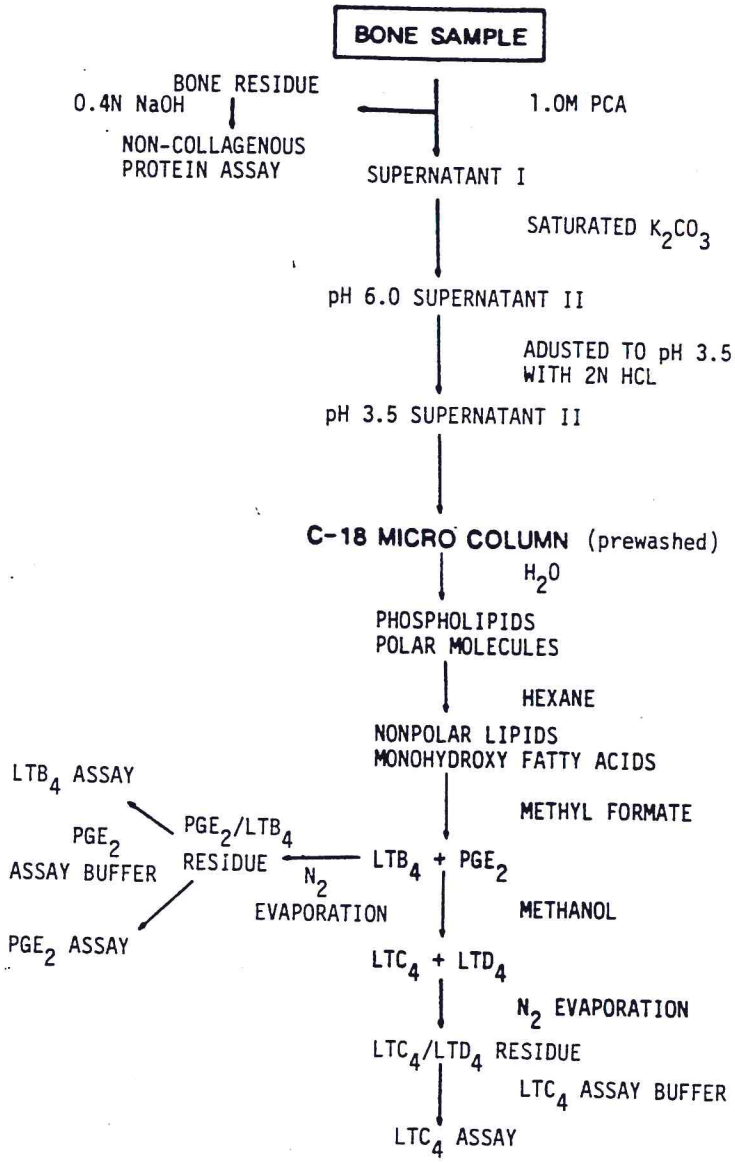
silica column (C_{18} Micro-Cleanup Column, Alltech Associates, Inc., Deerfield, IL). The amounts added to the column ranged from 0.40 ml - 0.72 ml. The columns were prewashed with 20 ml of ethyl alcohol (95% Reagent Quality, AA Per Alcohol and Chemical Co., Shelbyville, KY) followed by 20 ml of double distilled water using a 10 ml syringe with Luer-lok tip (Becton Dickinson, Rutherford, NJ). The syringe was secured to the column utilizing a syringe adaptor (Alltech Associates Inc., Deerfield, IL). The reagents in syringes were forced through the column by hand at a flow rate of approximately 100 drops per minutes.

The columns are designed to preconcentrate the extracts by allowing the compounds with an affinity for the C_{18} phase to be selectively retained by and eluted from the column. Phospholipids, proteins and very polar materials either pass through unretained or are eluted by an application of 10 ml double distilled water. The nonpolar lipids and monohydroxy fatty acids are eluted with the addition of 10 ml of hexane (certified, Fisher Scientific). Elution with a 10 ml fraction of methylformate (Spectrograde, Fisher Scientific) results in a fraction containing both PGE_2 and LTB_4. The methylformate fraction was collected in a siliconized 50 ml Erlenmeyer flask held in an ice bath (0-4°C).

The methylformate fraction was evaporated using a manifold under a stream of highly purified nitrogen to a volume of 1.0 ml in a 30°C water bath (Plates VI & VII). The flasks were rinsed twice with 0.5 ml volumes of methylformate and transferred to 4 ml




FISHER


BONE SAMPLE
BONE RESIDUE
0.4N NaOH
NON-COLLAGENOUS PROTEIN ASSAY
1.0M PCA
SUPERNATANT I
SATURATED K_2CO_3
pH 6.0 SUPERNATANT II
ADUSTED TO pH 3.5 WITH 2N HCL
pH 3.5 SUPERNATANT II
C-18 MICRO COLUMN (prewashed)
H_2O
PHOSPHOLIPIDS POLAR MOLECULES
HEXANE
NONPOLAR LIPIDS MONOHYDROXY FATTY ACIDS
METHYL FORMATE
LTB_4 ASSAY
PGE_2 ASSAY BUFFER
PGE_2/LTB_4 RESIDUE
N_2 EVAPORATION
LTB_4 + PGE_2
PGE_2 ASSAY
METHANOL
LTC_4 + LTD_4
N_2 EVAPORATION
LTC_4/LTD_4 RESIDUE
LTC_4 ASSAY BUFFER
LTC_4 ASSAY

siliconized capped vials (Wheaton Scientific, Millville, NJ). This fraction was evaporated to dryness (∿10 min). The vials flushed with high purity N_2 were sealed with teflon tape, capped and stored at -80°C for future LTB_4 and PGE_2 assay at the earliest possible time. Subsequent elution of the column with 10 ml of methanol (Spectranalyzed , Certified A.C.S., Fisher Scientific) results in a fraction containing LTC_4 + LTD_4 and other peptidoleukotrienes. The same procedure of collection and evaporation was applied for the methanol fraction except that the flasks were rinsed twice with 0.5 ml of methanol prior to evaporation (Figure 8).

Recoveries: LTC_4 and PGE_2

A preliminary study to test the efficiency of the extraction technique using the C_{18} columns was performed. Recovery concentrations of 300 pg PGE_2 prepared from a 100 ng PGE_2/ml standard (PGE_2 ^{125}I RIA Kit, New England Nuclear, Boston, MA) and 8 ng LTC_4 prepared from a 1.6 x 10^3 ng LTC_4/ml standard (LTC_4 ^3H RIA Kit, New England Nuclear) were added to 650 µl PCA and processed similar to the bone sample extracts. In addition to this method of recovery, standards were added to bone samples with similar volumes of PCA. In both cases LTC_4 recoveries ranged from 93-103% and PGE_2 recoveries ranged from 83-89%. In addition, no LTC_4 was detected in the methylformate fraction. No recovery experiments were done for LTB_4.

Assay for LTB_4, LTC_4 and PGE_2

The assays used were radioimmunoassays (RIA). The reason for their use was the specificity and sensitivity needed for the assays. The basic principle of RIA depends on competitive antigen binding. Competition exists between the isotopically labeled antigen (tracer) and unlabeled antigen (standards or samples) for the antibody (antiserum) (Figure 9). When increasing amounts of standard or sample are added to the antibody the amount of tracer-antibody complex is reduced. This results in lower radioactive counts.

The separation of the antibody-antigen complexes from free antigen is achieved differently for the leukotriene (^3H labeled) assays and the prostaglandin (^{125}I labeled) assay. The unbound leukotriene tracer is adsorbed by dextran-coated activated charcoal. After centrifugation the supernatant (bound antigen) is decanted into a 20 ml polyethylene high density scintillation vial (Fisher Scientific) containing a 10 ml scintillation cocktail (ATOMLIGHT , NEF-968, New England Nuclear, Boston, MA) and counted with a spectrometer on the beta channel (Liquimat, Picker Nuclear). The prostaglandin ^{125}I antigen-antibody complex is precipitated with buffered polyethyleneglycol provided in the kit. After centrifugation the unbound antigen is poured off and the remaining pellet is counted using the gamma channel.

LTC_4 + LTD_4 Assay

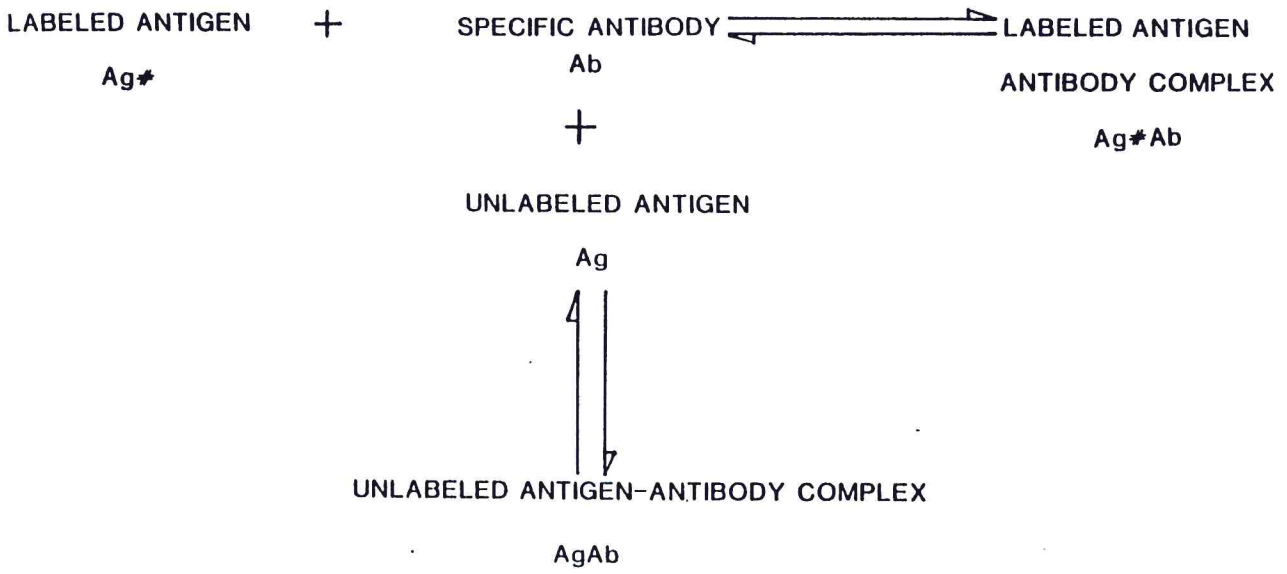
The LTC_4 + LTD_4 was measured with RIA (LTC_4 $^3[H]$ RIA Kit, cat. #NEK-030, NEN, Boston, MA). The kit antibody has a 55% cross-

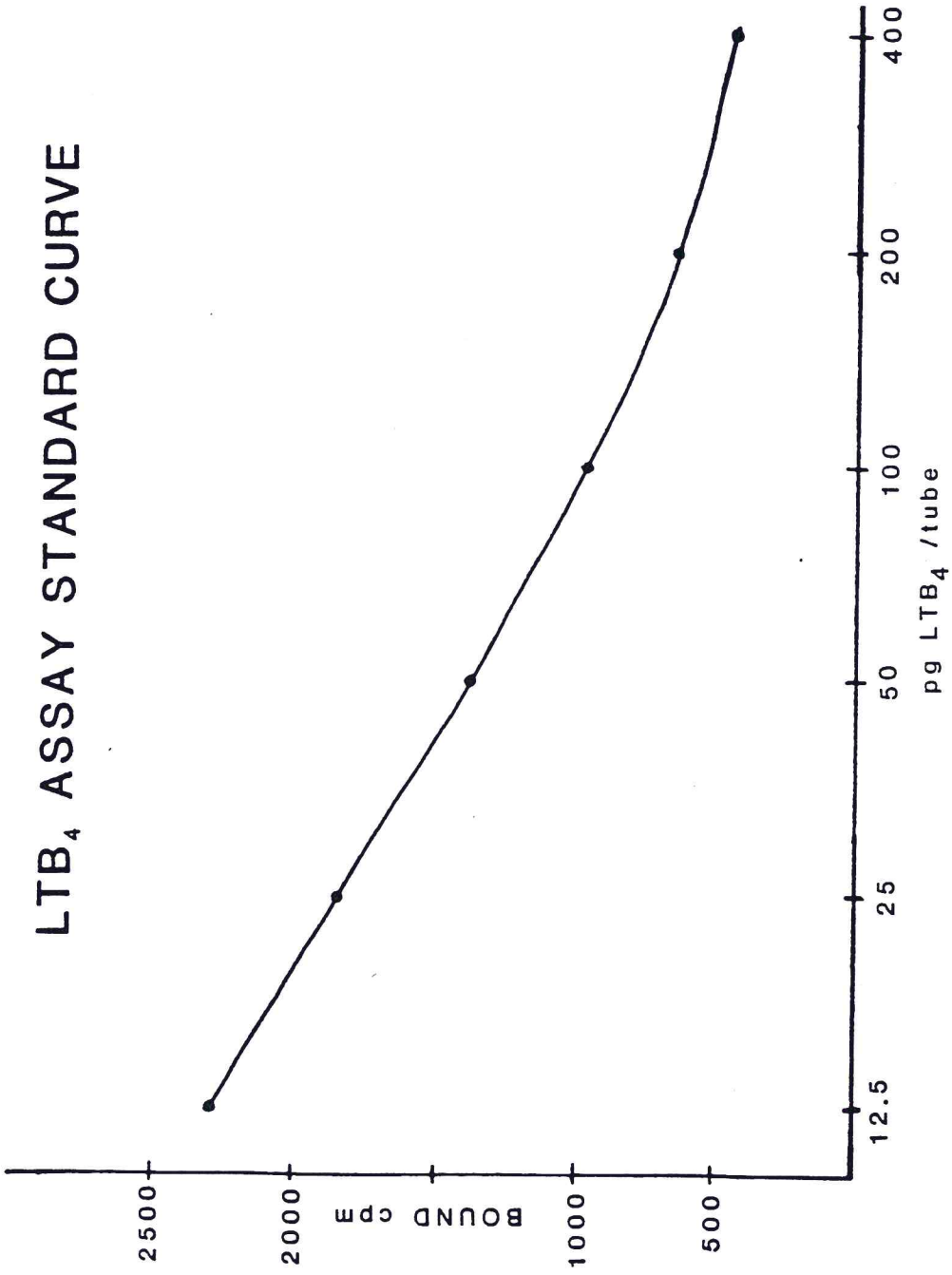
reactivity with LTD_4. The evaporated LTC_4 samples were reconstituted with the LTC_4 RIA kit assay buffer to an equivalent concentration of 9.0 mg bone/ml assay buffer. The protocol from the kit instructions was followed with two modifications. First the 1.6 ng LTC_4 standard on the standard curve was eliminated since this region was not of interest. Secondly a pilot study indicated that if the directions from the kit were followed (2000 g for 15 min/ SM-24 rotor) the total counts were reduced by 15% because charcoal was being poured off and quenching the counts. In the same experiment when the spin was 3730 g for 30 minutes the total counts were reduced less than 5% compared to the control. Thus the centrifuge time was altered to 3730 g for 30 minutes. The radioactivity was counted on the beta channel for 1 minute. A typical LTC_4 standard curve is shown in Figure 11.

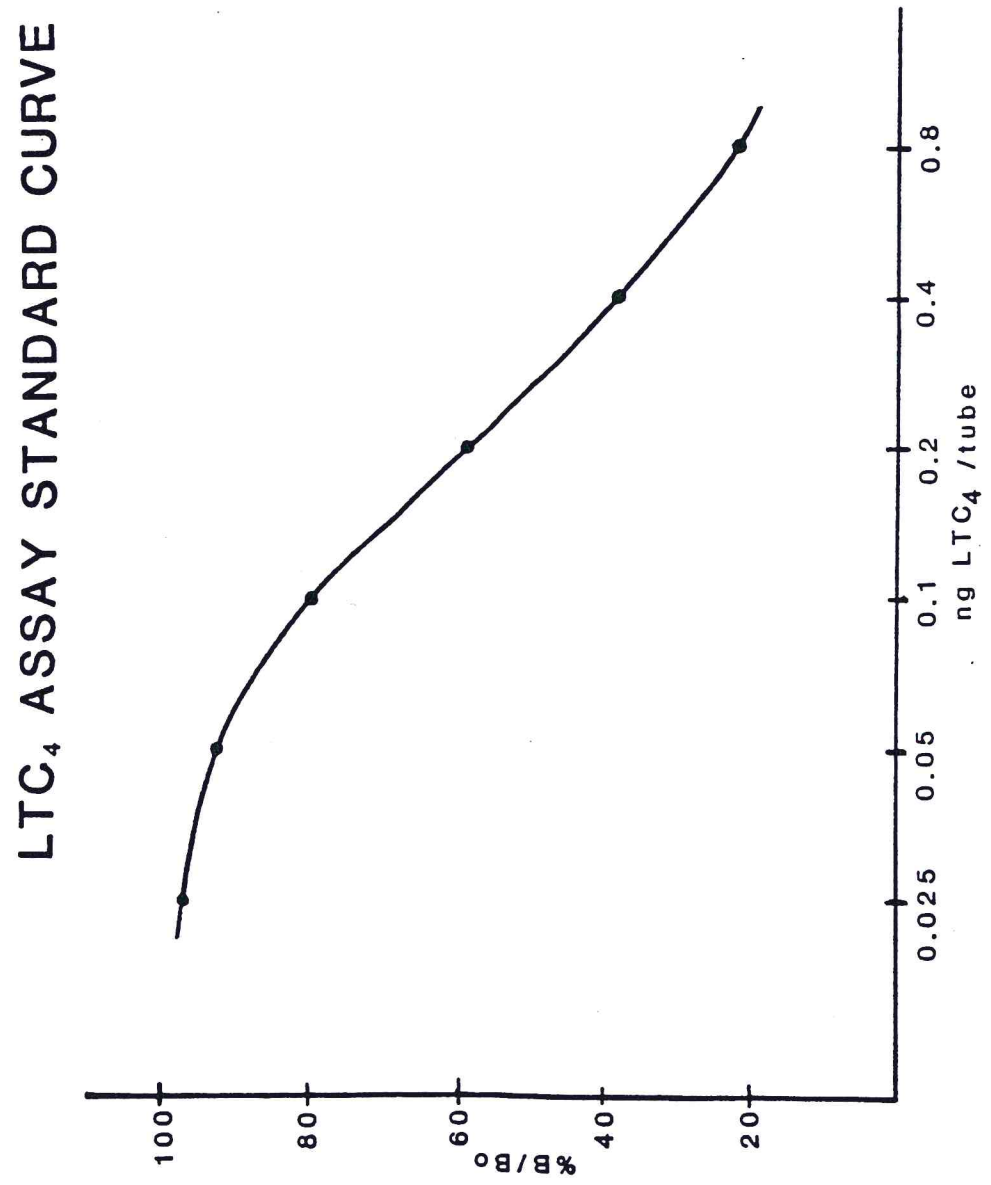
LTB_4 Assay

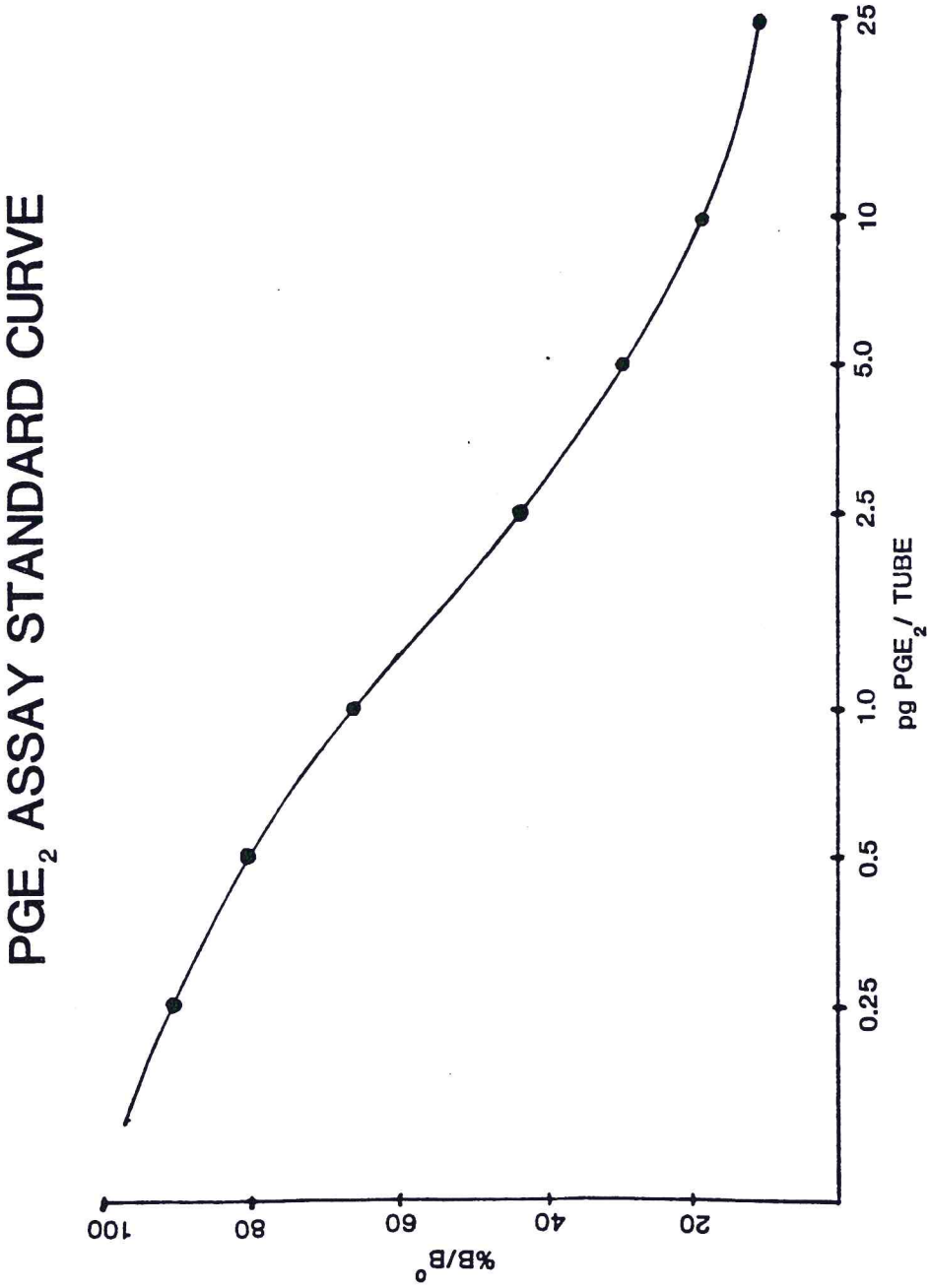
The LTB_4 content was measured by RIA (LTB_4 ^{3}[H] RIA Kit Code TRK 840, Amersham Corporation, Arlington Hts, IL). The LTB_4 and PGE_2 samples are extracted in the same methylformate fraction. The N_2 evaporated samples were reconstituted with PGE_2 assay buffer (pH 6.8 phosphate, part of NEN RIA PGE_2 Kit) to an equivalent concentration of 4.5 mg bone/ml assay buffer (see Radulovich, 1985) to obtain sufficient sensitivity with 100 µl assay volume. The assay technique for LTB_4 is similar to that of LTC_4, except that assay buffer and charcoal separation system are not provided in the kit and need to be prepared prior to assay according to instructions.

PRINCIPLES OF RIA




LTB4 ASSAY STANDARD CURVE
BOUND cpm
2500
2000
1000
500
12.5
25
50
100
200
400
pg LTB4 /tube


LTC4 ASSAY STANDARD CURVE
100
80
60
40
20
%B/Bo
0.025
0.05
0.1
0.2
0.4
0.8
ng LTC4 /tube


PGE2 ASSAY STANDARD CURVE
%B/B°
100
80
60
40
20
0.25
0.5
1.0
2.5
5.0
10
25
pg PGE2 / TUBE

The kit instructions were followed except the centrifugation procedure was altered to 3730 g for 30 minutes. The samples were counted on the beta channel for 4 minutes. See Figure 10 for the LTB_4 standard curve.

PGE_2 Assay

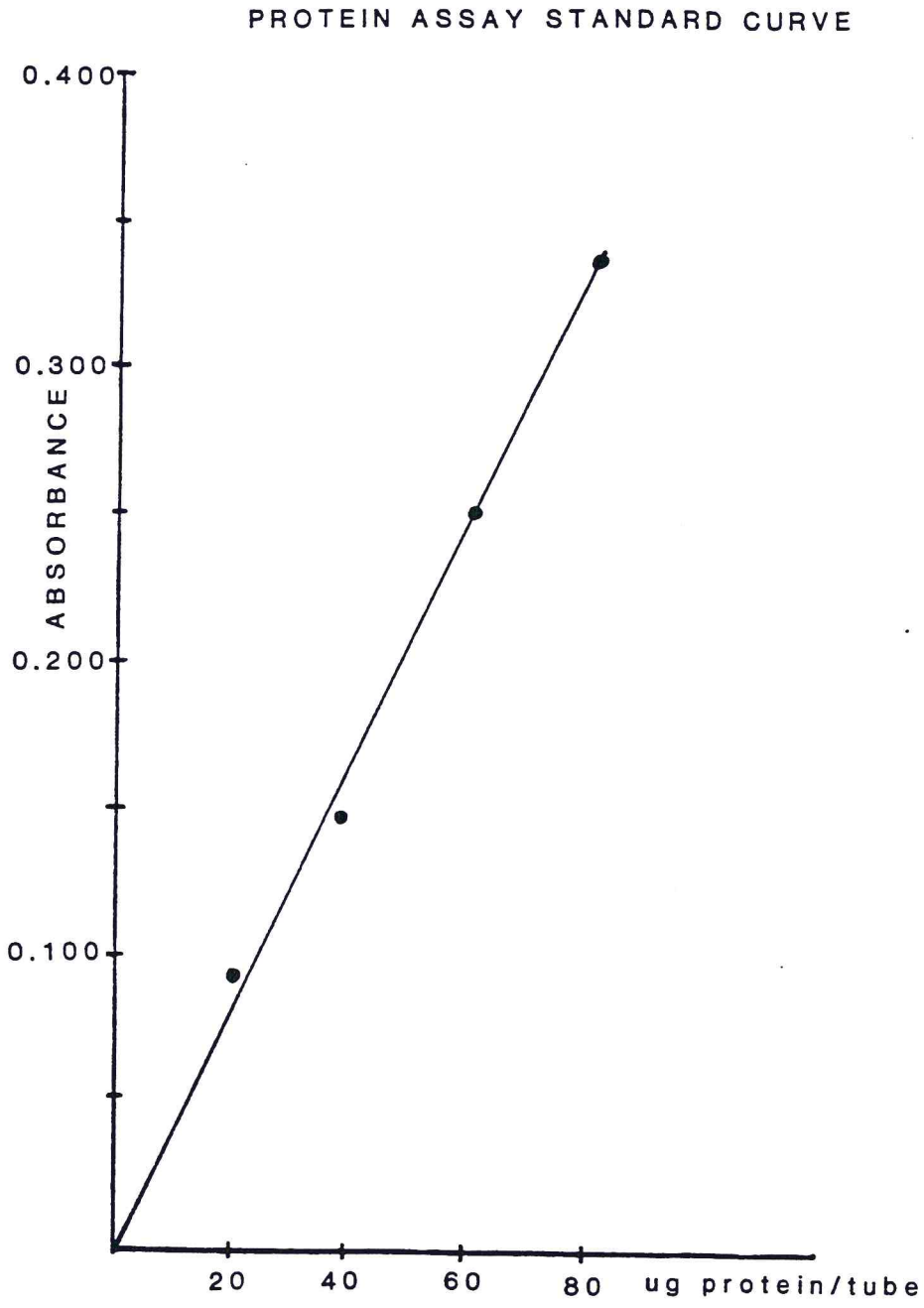
Finally, the PGE_2 content was measured with RIA (PGE_2 ^{125}[I] RIA Kit NEK-020A, NEN, Boston, MA). Because LTB_4 and PGE_2 were evaporated in the same vial, reconstitution was done simultaneously (see LTB_4 assay) with the prostaglandin assay buffer. Again one procedure which was altered from the kit instructions was the spin time. Centrifugation was for 30 minutes at 3730 g under the same conditions as previously described. Each sample was counted at least twice on the gamma channel for 1 minute. A typical standard PGE_2 curve is shown in Figure 12.

Protein Assay

The non-collagenous protein content of the bone samples was determined by a variation of the Folin protein colorimetric method described by Lowry et al. (1951). The PCA insoluble pellet (see metabolite extraction procedure) was extracted with 0.4 N NaOH to make a concentration of 5 mg bone sample/ml of 0.4 N NaOH. The pellets were mixed, allowed to stand at room temperature for 3-4 hours with occasional agitation, tightly sealed and placed in cold storage (0-8°C) for 20-24 hours.

The extracts were spun at 49,500 g for 10 minutes and the supernatant collected and stored in a refrigerator (0-8°C) for

later protein assay. An aliquot of each sample was diluted 1:1 with 0.4 N NaOH (100 µl sample: 100 µl 0.4 NaOH). To each tube was added 5.0 ml of a carbonate-copper-tartrate solution previously prepared (mix 2.0 ml 2.68% NaK tartrate $4H_2O$ + 2.0 ml 1% $CuSO_4$ $5H_2O$ + 100 ml 2% Na_2CO_2 - 0.1 N NaOH). The final reagent added was 0.5 ml of a diluted phenol reagent (1:1 with double distilled water). As the reagent was added it was instantly mixed on a vortex mixer. After a minimum 1 hour color development time each sample was read for absorbance at 750 nm using a Beckman Spectrophotometer (Model 25, Beckman Instruments Inc., Irvine, CA). The protein content of the samples was calculated from a blank corrected standard curve prepared from dilutions of a 1.0 mg/ml bovine serum albumin (Crystallized lyophilized, Fisher Scientific) Figure 13).


PROTEIN ASSAY STANDARD CURVE
0.400
0.300
0.200
0.100
ABSORBANCE
20
40
60
80
ug protein/tube

RESULTS

Response of the Animals to the Experimental Procedure

All animals recovered from the surgical anesthesia rapidly, approximately after an hour they regained normal behavior. The surgical incision healed well without sign of inflammation or infection. There appeared to be no differences between groups upon visual inspection. The behavior of the experimental animals did not deviate from that of the controls.

The weight gain of the experimental rats was not significantly affected by the procedures. Rather, the tension group had a slightly more rapid weight gain compared to controls (Figures 14 & 15). Although this was not a significant difference ($p = 0.1$) it does tend to indicate that the experimental rats were not adversely affected. These weight data are in agreement with those recorded by Radulovich (1985).

Piriprost Toxicity Evaluation

Severe reactions to intravenous injections of piriprost, at 30 mg/kg body weight, in rat tail veins have been reported[1]. Thus, a separate pilot toxicity study was performed. This study consisted of nine animals divided into four groups: (1) 10 mg/kg piriprost injections, (2) 3 mg/kg piriprost injections (3) 1 mg/kg piriprost injection, (4) saline injections. Each group consisted of

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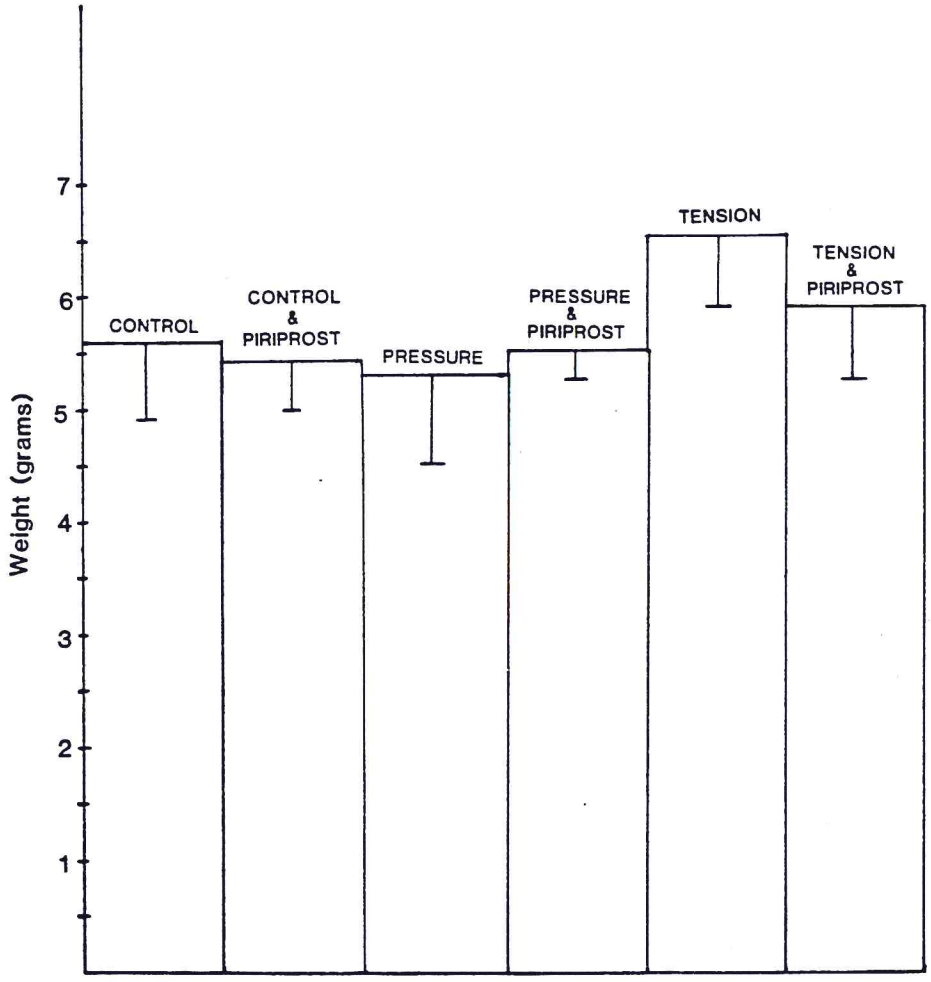
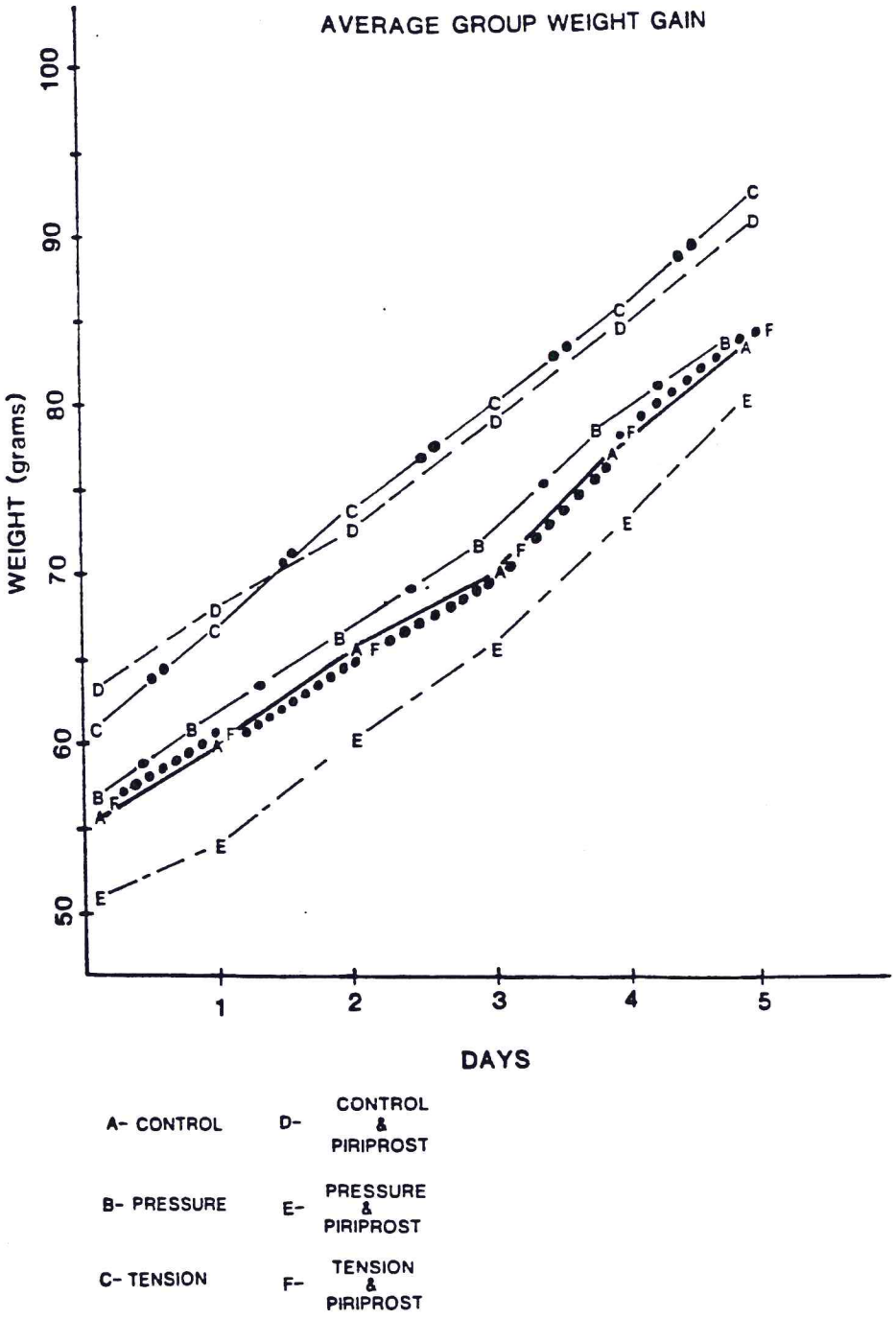
[1] Personal communication with Michael K. Bach (Hypersensitivity Diseases Research Upjohn Company).

AVERAGE DAILY GROUP WEIGHT GAIN




AVERAGE GROUP WEIGHT GAIN
WEIGHT (grams)
100
90
80
70
60
50
1
2
3
4
5
DAYS
A- CONTROL
B- PRESSURE
C- TENSION
D- CONTROL & PIRIPROST
E- PRESSURE & PIRIPROST
F- TENSION & PIRIPROST

animals with either osteotomies only or pressure or tension springs implanted across the midsagittal suture. Administration of the drug was achieved by subcutaneous injections in the back of the neck. This has earlier proved to be a successful route of administration for indomethacin (Radulovich, 1985). At no time during the pilot experimental period did any of the animals show signs of inflammation or toxic reaction, nor was weight gain or behavior altered by drug administration at any of the concentrations.

On closer inspection of the bone surface with a dissecting microscope (7x) the pups with osteotomies and piriprost injections showed a pitting of the bone surface. However, this pitting was not dose-dependent nor was it present when drug administration was combined with either sutural compression or sutural tension.

Because no significant difference in response to the various drug levels was observed, the highest dose (10 mg/kg) was chosen for maximum inhibition in the master experiment. At the end of the main experiment parietal bone segments were histologically examined for evidence of pitting. None of the samples demonstrated any such pitting.

Tissue Reaction to Spring Implantation

Visual inspection of the surgical site at the time of sacrifice revealed an abundant amount of soft tissue covering the bone segments. The springs were totally embedded into this tissue growth. It appeared to be loose granulation tissue which was

easily removed. All springs were still firmly seated in the bony segments at the time of sacrifice.

Examination of the suture with a dissecting microscope before removal of bone segments showed that the animals with pressure springs had an extremely narrow suture compared to controls. The sutures subjected to tensile forces were significantly wider than controls.

Protein and Total Bone Content

The non-collagenous protein content of all bone samples was determined with a modification of the Folin protein assay (Lowry, 1951; Benson, 1983). This provided a means to standardize the bone samples so that the amount of metabolites could be related. As shown in Table II, the protein content of the samples were mostly between 4.2% and 6.5% of bone weight.

Analysis of LTB_4, LTC_4 and PGE_2 Response

Metabolite responses were expressed as ng or pg per mg of bone and per mg of protein. These results are tabulated in Tables III and IV. All data were tested for statistical significance based upon metabolite content expressed per mg of protein. The mean values, with standard deviations, of the metabolite responses are summarized in Table V. For statistical analysis the student t-test was utilized. The 0.05 confidence level was used to test for significance (Tables VI, VII and VIII).

Analysis of the LTB_4 levels reveal relatively high basal values for the control group (Figure 16). However, the animals

treated with applied pressure or tension showed a significant reduction in the LTB_4 response compared to the control group. Also in the control + piriprost group a decrease by a factor of 15 was observed in the levels of LTB_4. On the other hand, no significant differences existed in the level of LTB_4 response between either the pressure and tension groups when compared to when piriprost was given in conjunction with stress application.

LTC_4 levels in Figure 17 are not blank corrected. If so, endogenous levels would be vitually zero. Pressure caused a significant elevation in LTC_4 levels when compared to the controls. Piriprost reduced this stimulated level significantly back to the endogenous levels. There was a slight elevation of the tension group when compared to the control, however it was not statistically significant.

The PGE_2 response is shown in Figure 18. Evaluation of the PGE_2 levels indicates that the basal levels in the controls are clearly measurable. Applied tension resulted in a two fold elevation of the PGE_2 response when compared to control values. Pressure values were slightly elevated above controls yet not statistically significant. The tension + piriprost group had elevated PGE_2 values of similar magnitude as those of the tension no drug group. Thus there was no significant difference between these two groups. However, the tension + piriprost group had significantly higher levels than the drug treated control groups. Also, the

pressure + piriprost group showed increases in PGE_2 levels when compared to the pressure group.

Histological Results

Histologic evaluation utilized thin (6 μm) decalcified sections of the sutural area. Evaluation was based upon (1) overall sutural appearance, (2) evidence of bone formation and/or bone resorption, (3) and evidence of inflammatory response.

The overall sutural appearance in the controls is seen in Plates VIII & IX. The sutural width in the controls (osteotomy only) was used as a comparative baseline. The bone surfaces were approximated in a butt joint united with a dense fibrous connective tissue containing many basophilic cells. The border of the bone surfaces were relatively smooth and lined with ovoid basophilic cells indicating blast cells which is expected for rapidly growing rat calvarial suture.

The animals treated with applied pressure had a narrowed sutural area with overlapping bone surfaces obliterating the sutural continuity (see Hinrichsen and Storey, 1968). This overlapping appeared to have caused areas of tension as well as pressure along the suture and not a pure compressive force. The borders of the bone surface had an extremely scalloped surface indicating areas of resorption (Plate X).

Compared to controls applied tension resulted in a wider suture with the bony segments further apart. The interposing tissues were more eosinophilic when compared to all other groups. Indica-

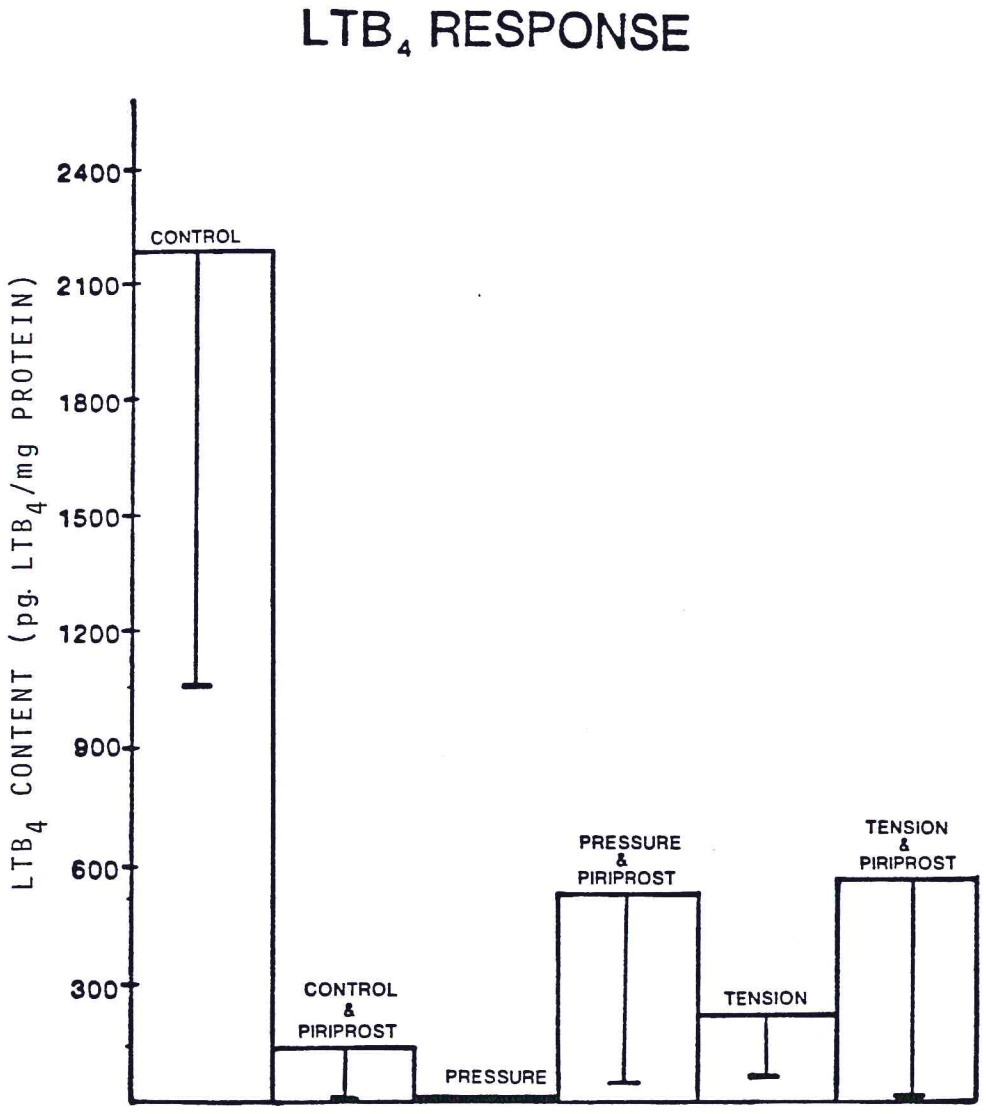
tions that bone bridging was occurring between the sutural margins were seen. In addition, some bone surfaces had a scalloped resorptive border indicating some pressure forces (Plate XI).

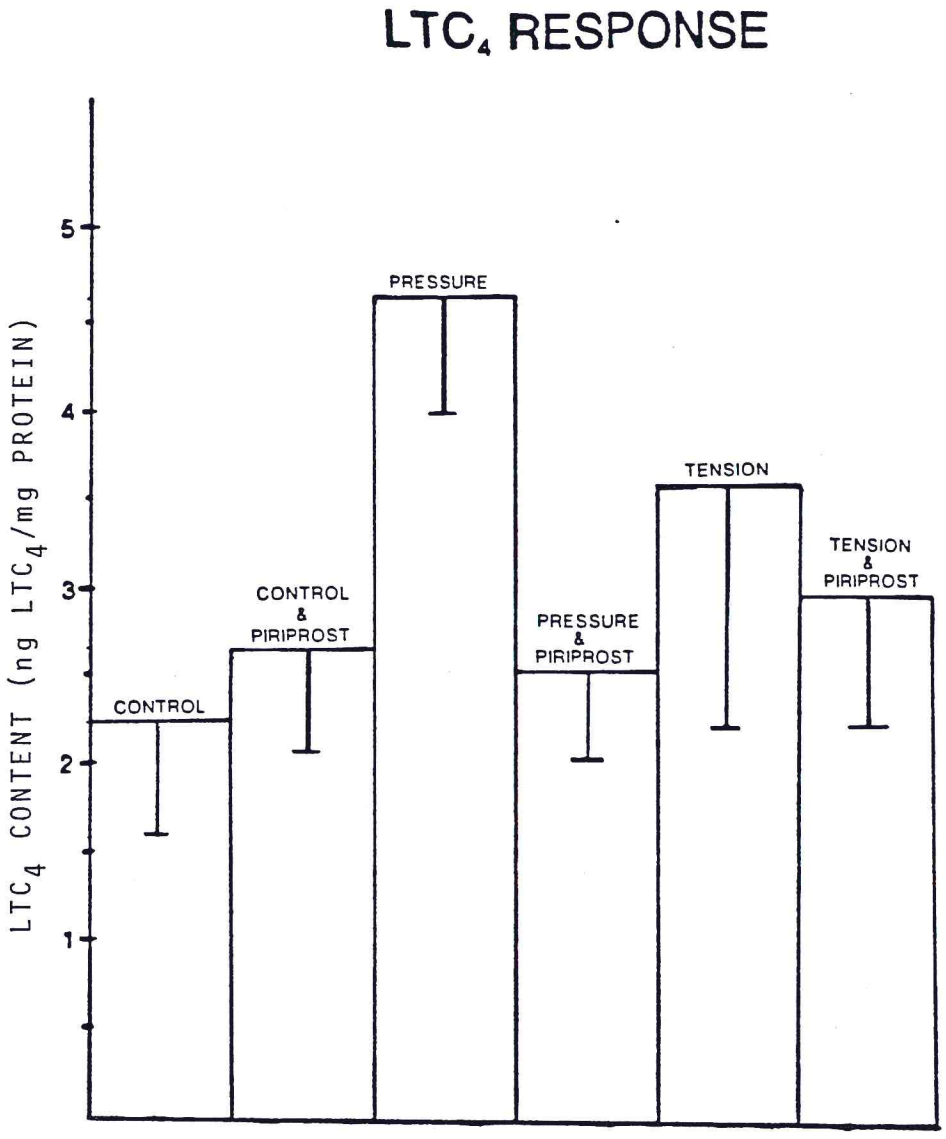
The control + piriprost samples had a similar overall sutural appearance as the controls without drug. However, a more detailed examination revealed areas of scalloped bone surfaces indicating more active bone resorption than the controls (Plate XII).

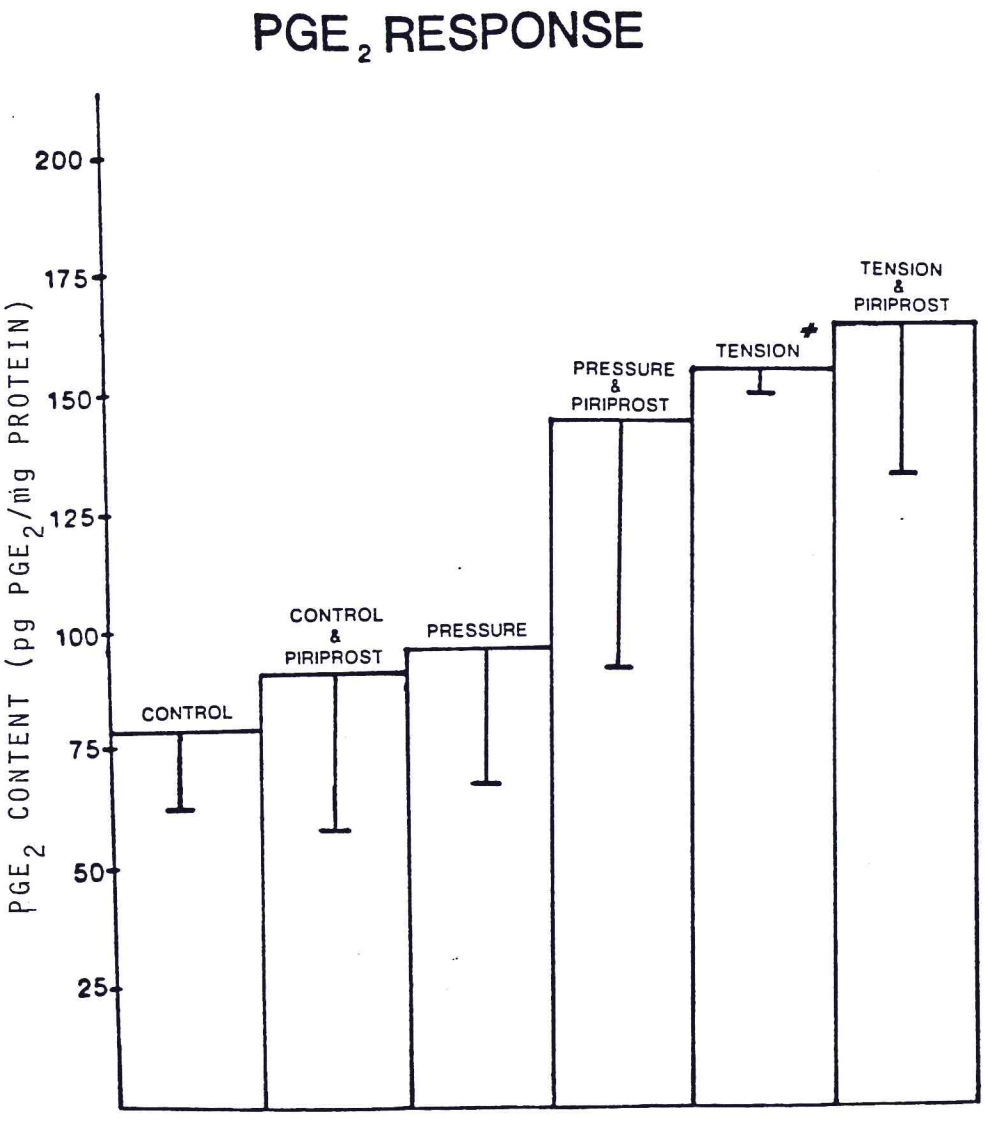
The sections from the pressure + piriprost group were not totally decalcified, thus complete sections showing overall sutural architecture could not be obtained. However, significant observations could still be made. There appeared to be invaginations of the suture causing cortical sinuses lined with basophilic cuboidal cells, apparently osteoblast causing rapid bone formation. These morphological features were unique to this group (Plates XIII & XIV). It must be emphasized that this picture of bone formation took place under pressure conditions.

The tension + piriprost group presented a different picture than did the group with tension alone. The suture was not as wide nor did the sutural matrix show such distinctly eosinophilic staining as it did in the tension group without drug. However, there appeared to be a well developed pattern of basophilic cells presumably osteoblasts lining the bone surface which was unique to this group (Plates XV & XVI).

There was no evidence of an inflammatory reaction or necrosis in any of the groups. All bone and sutural tissues appeared non-pathologic and viable.


LTB4 RESPONSE
LTB4 CONTENT (pg. LTB4/mg PROTEIN)
2400
2100
1800
1500
1200
900
600
300
CONTROL
CONTROL & PIRIPROST
PRESSURE
PRESSURE & PIRIPROST
TENSION
TENSION & PIRIPROST


LTC4 RESPONSE
LTC4 CONTENT (ng LTC4/mg PROTEIN)
5
4
3
2
1
CONTROL
CONTROL & PIRIPROST
PRESSURE
PRESSURE & PIRIPROST
TENSION
TENSION & PIRIPROST


PGE2 RESPONSE
PGE2 CONTENT (pg PGE2/mg PROTEIN)
200
175
150
125
100
75
50
25
CONTROL
CONTROL & PIRIPROST
PRESSURE
PRESSURE & PIRIPROST
TENSION
*
TENSION & PIRIPROST

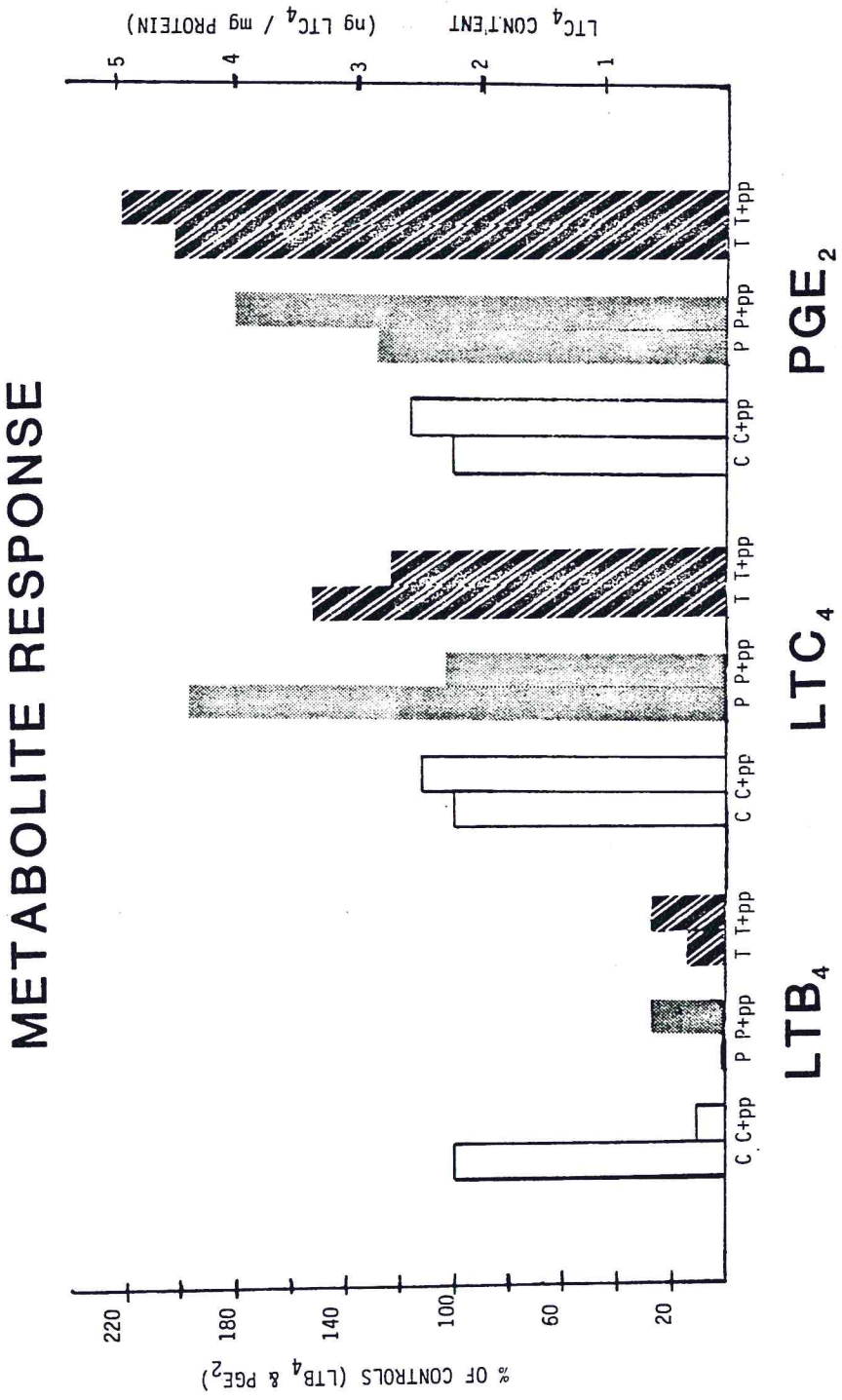

METABOLITE RESPONSE
% OF CONTROLS (LTB4 & PGE2)
220
180
140
100
60
20
LTC4 CONTENT (ng LTC4 / mg PROTEIN)
5
4
3
2
1
C C+pp P P+pp T T+pp
LTB4
LTC4
PGE2

TABLE II

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
Control (No Force)	A1	86.8	7.20	0.301	0.0418
	A2	76.9	7.30	0.323	0.0442
	A3	89.9	8.70	0.400	0.0459
	A4	78.9	5.70	0.264	0.0463
Pressure	B1	86.9	6.60	0.329	0.0498
	B2	85.8	7.30	0.424	0.0582
	B3	82.4	6.20	0.280	0.0452
	B4	78.0	6.10	0.269	0.0441
Tension	C1	87.6	5.70	0.251	0.0440
	C2	100.8	9.20	0.484	0.0526
	C3	89.3	8.50	0.490	0.0576
	C4	95.5	10.00	0.727	0.0727
Control + Piriprost	D1	95.9	6.00	0.290	0.0483
	D2	84.1	6.60	0.273	0.0414
	D3	92.1	7.50	0.300	0.0400
	D4	90.2	7.50	0.351	0.0468
Pressure + Piriprost	E1	81.8	6.70	0.383	0.0571
	E2	78.2	5.80	0.379	0.0653
	E3	78.7	7.40	0.469	0.0634
	E4	80.0	8.00	0.468	0.0585
Tension + Piriprost	F1	87.1	8.00*	0.404	0.0505
	F2	85.1	3.80*	0.291	0.0767
	F3	82.9	7.30	0.420	0.0575
	F4	84.6	6.60	0.271	0.0411

The protein/bone ratio was used to calculate LTB_4, LTC_4 and PGE_2 values to a measure of active bone tissue. The standardized values are presented in Table III.

*Part of bone sample was lost after grinding.

TABLE IIA

LEUKOTRIENE B_4, C_4 AND PROSTAGLANDIN E_2 LEVELS PER SAMPLE

Condition	Sample	Total LtB_4 (pg)	Group Mean LtB_4 (pg)	Total LtC_4 (ng)	Group Mean LtB_4 (ng)	Total PGE_2 (pg)	Group Mean PGE_2 (pg)
Control	A1	1120	689	0.749	0.740	20.2	24.7
	A2	774		0.511		29.2	
	A3	482		0.809		27.0	
	A4	380		0.889		22.2	
Pressure	B1	0	0	1.85	1.54	21.8	30.5
	B2	0		2.10		35.8	
	B3	0		1.12		29.1	
	B4	0		1.10		35.4	
Tension	C1	821	284	1.31	1.56	41.6	(N = 3) 63.6
	C2	0		1.75		71.8	(N = 4) 287
	C2	113		1.47		77.4	
	C4	200		1.70		965	
Control + Piriprost	D1	0	44.4	0.624	0.812	31.8	26.7
	D2	87.8		0.614		33.7	
	D3	0		1.04		18.8	
	D4	90.0		0.968		22.5	
Pressure + Piriprost	E1	223	205	1.04	0.843	80.4	58.4
	E2	425		0.789		50.5	
	E3	67.3		0.123		44.4	
	E4	106		1.42		58.4	
Tension + Piriprost	F1	480	190	1.05	0.432	52.0	58.4
	F2	279		0.676		57.0	
	F3	0		0		81.8	
	F4	0		0		39.6	

Table II-A summarizes the total metabolite per bone sample.

TABLE III

LEUKOTRIENE B_4, C_4 AND PROSTAGLANDIN E_2 LEVELS PER BONE WEIGHT.

Condition	Sample	LTB_4 pg/mg Bone	Group Mean LTB_4 pg/mg Bone	LTC_4 ng/mg Bone	Group Mean LTC_4 ng/mg Bone	PGE_2 pg/mg Bone	Group Mean PGE_2 pg/mg Bone
Control	A1	156	117	0.104	0.106	2.80	3.50
	A2	106		0.070		4.00	
	A3	55.4		0.093		3.10	
	A4	66.7		0.156		3.90	
Pressure	B1	0	0	0.280	0.232	3.30	4.70
	B2	0		0.287		4.90	
	B3	0		0.180		4.70	
	B4	0		0.180		5.80	
Tension	C1	12.0	11.3	0.230	0.191	7.30	(N=3) 8.07
	C2	0		0.190		7.80	(N=4) 29.9
	C3	13.3		0.173		9.10	
	C4	20.0		0.170		95.6	
Control + Piriprost	D1	0	6.33	0.104	0.116	5.30	4.00
	D2	13.3		0.093		5.10	
	D3	0		0.138		2.50	
	D4	12.0		0.129		3.00	
Pressure + Piriprost	E1	33.3	32.3	0.155	0.150	12.0	8.50
	E2	73.3		0.136		8.70	
	E3	9.09		0.129		6.00	
	E4	13.3		0.178		7.30	
Tension + Piriprost	F1	60.0	33.3	0.131	0.160	6.50	9.70
	F2	73.3		0.178		15.0	
	F3	0		0.173		11.2	
	F4	0		0.156		6.00	

These values are presented for comparison with Table IV.

TABLE IV

LEUKOTRIENE B_4, C_4 AND PROSTAGLANDIN E_2 PER PROTEIN WEIGHT.

Condition	Sample	LTB_4 pg/mg Protein	Group Mean LTB_4 pg/mg Protein	LTC_4 ng/mg Protein	Group Mean LTC_4 ng/mg Protein	PGE_2 pg/mg Protein	Group Mean PGE_2 pg/mg Protein
Control	A1	3700	2180	2.47	2.36	66.7	76.9
	A2	2350		1.56		88.9	
	A3	1210		2.02		67.4	
	A4	1450		3.39		84.8	
Pressure	B1	0	0	5.60	4.66	66.0	96.7
	B2	0		4.95		84.5	
	B3	0		4.00		104	
	B4	0		4.10		132	
Tension	C1	273	194	5.23	3.54	166	(N=3) 158
	C2	0		3.65		150	(N=4) 446
	C3	230		2.98		157	
	C4	274		2.33		*1310	
Control + Piriprost	D1	0	146	2.17	2.69	110	90.6
	D2	325		2.27		124	
	D3	0		3.45		62.5	
	D4	261		2.80		65.2	
Pressure + Piriprost	E1	595	524	2.77	2.50	214	142
	E2	1130		2.09		133	
	E3	144		2.05		95.2	
	E4	230		3.09		126	
Tension + Piriprost	F1	1200	538	2.62	2.93	130	166
	F2	952		2.31		195	
	F3	0		2.98		193	
	F4	0		3.80		146	

* Only data expressed in terms of protein weight were used for statistical analyses. Deleted for N=3.

TABLE V

SUMMARY OF LTB_4, LTC_4 AND PGE_2 ANALYSIS

Metabolite Condition	LTB_4 Mean pg/mg Protein ± SD	LTB_4 % of Control	LTC_4 Mean ng/mg Protein ± SD	#	PGE_2 Mean pg/mg Protein ± SD	PGE_2 % of Control
Control	2180 ± 1130	100%	2.36 ± 0.781		76.9 ± 11.6	100%
Control + Piriprost	147 ± 171	7.00%	2.69 ± 0.592		90.6 ± 31.2	118%
Pressure	0 ± 0	0%	4.66 ± 0.755		96.7 ± 28.2	126%
Pressure + Piriprost	524 ± 480	24.0%	2.50 ± 0.509		142 ± 50.8	185%
Tension	194 ± 131	9.00%	3.54 ± 1.24		*446 ± 576 158 ± 8.00	205%
Tension + Piriprost	538 ± 629	25.0%	2.93 ± 0.640		166 ± 32.9	216%

*Table V summarizes the average LTB_4, C_4 and PGE_2 values for each condition plus % of Control. Value n = 3 was included and used in Figure 18.

#Analytical LTC_4 blank - 2.26. All values of LTC_4 were not blank corrected.

TABLE VI

ANALYTICAL STATISTICS - STUDENT T-TEST

LTB_4 (pg/mg Protein)

MEASUREMENT	n_1	n_1	$\bar{x}_1$	$\bar{x}_1$	s_1^2	s_2^2	t	α
Control to Control + Piriprost	4	4	2180	147	1130	171	3.550	0.05
Control to Pressure	4	4	2180	0	1130	0	3.853	0.01
Control to Tension	4	4	2180	194	1130	131	3.486	0.05
Control + Piriprost to Pressure + Piriprost	4	4	147	524	171	480	1.482	NS
Control + Piriprost to Tension + Piriprost	4	4	147	538	171	629	1.201	NS
Pressure to Pressure + Piriprost	4	4	0	524	0	480	2.183	NS
Tension to Tension + Piriprost	4	4	194	538	131	629	1.070	NS

α = Level of significance.

NS = Not significant

TABLE VII

ANALYTICAL STATISTICS - STUDENT T-TEST

LTC_4 (ng/mg Protein)

MEASUREMENT	n_1	n_1	$\bar{x}_1$	$\bar{x}_1$	s_1^2	s_2^2	t	α
Control to Control + Piriprost	4	4	2.36	2.69	0.781	0.592	0.675	NS
Control to Pressure	4	4	2.36	4.66	0.781	0.755	4.227	0.01
Control to Tension	4	4	2.36	3.54	0.781	1.24	1.618	NS
Control + Piriprost to Pressure + Piriprost	4	4	2.69	2.50	0.592	0.509	0.488	NS
Control + Piriprost to Tension + Piriprost	4	4	2.69	2.93	0.592	0.640	0.552	NS
Pressure to Pressure + Piriprost	4	4	4.66	2.50	0.755	0.509	4.726	0.01
Tension to Tension + Piriprost	4	4	3.54	2.93	1.24	0.640	0.878	NS

α = Level of significance.

NS = Not significant

TABLE VIII

ANALYTICAL STATISTICS - STUDENT T-TEST

PGE_2 (pg/mg Protein)

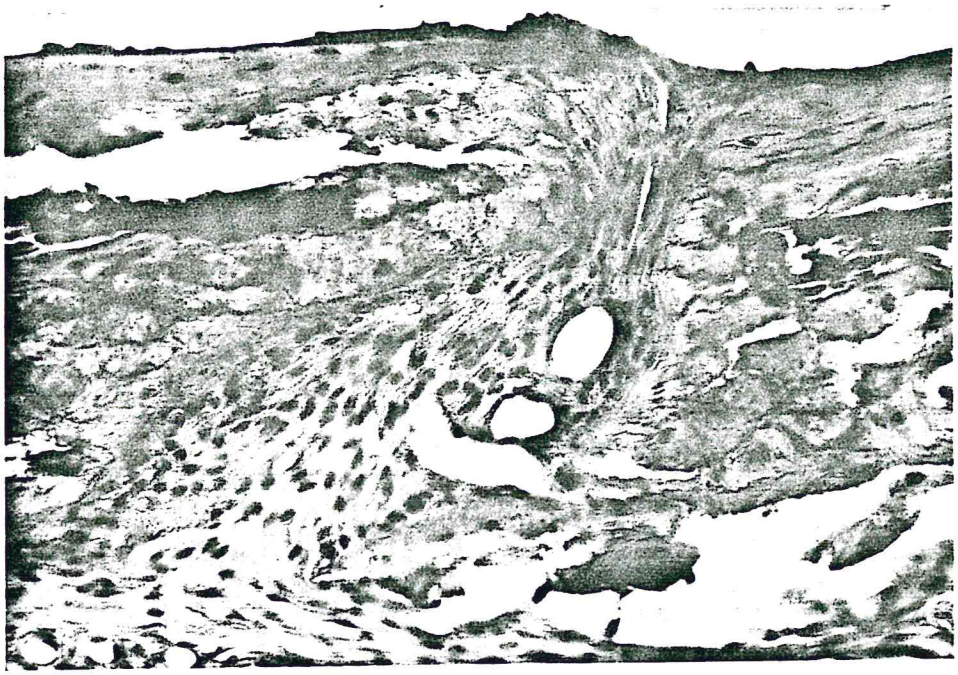
MEASUREMENT	n_1	n_1	$\bar{x}_1$	$\bar{x}_1$	s_1^2	s_2^2	t	α
Control to Control + Piriprost	4	4	76.9	90.6	11.6	31.2	0.824	NS
Control to Pressure	4	4	76.9	96.7	11.6	28.2	1.297	NS
Control to Tension	4	3	76.9	158	11.6	8.00	10.914	0.01
Control + Piriprost to Pressure + Piriprost	4	4	90.6	142	31.2	50.8	2.459	0.05
Control + Piriprost to Tension + Piriprost	4	4	90.6	166	31.2	32.9	3.330	0.05
Pressure to Pressure + Piriprost	4	4	96.7	142	96.7	50.8	1.571	NS
Tension to Tension + Piriprost	3	4	158	166	8.00	32.9	0.496	NS

α = Level of significance.

NS = Not significant

Plate VIII - Control group sutural histology. Sutural histology with no experimental forces resulting in a smooth surface butt joint (160x).

Plate IX - High magnification control group. Shows detailed architecture of control suture (400x).



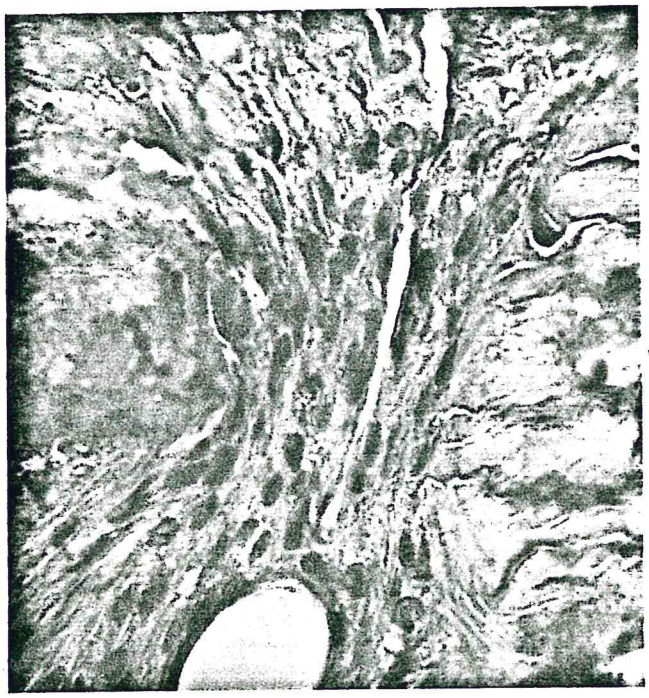
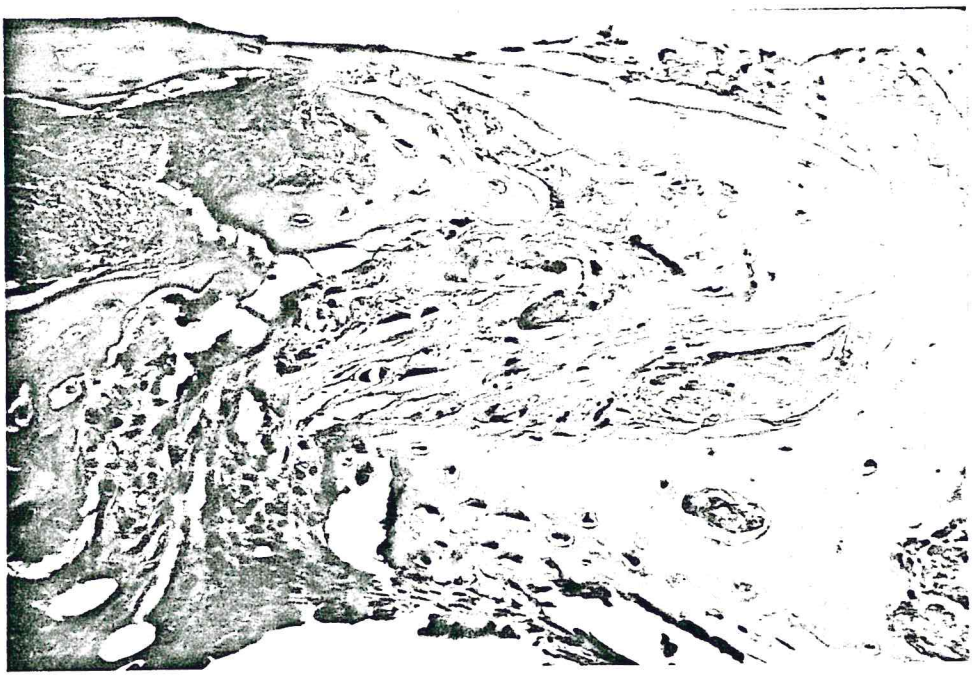


Plate X - Pressure group sutural histology. Sutural pressure resulting in overlapping of bone surfaces and obliteration of sutural continuity (160x).

Plate XI - Tension group sutural histology. Notice wider sutural width, eosinophilic sutural matrix and some areas of resorption (160x).



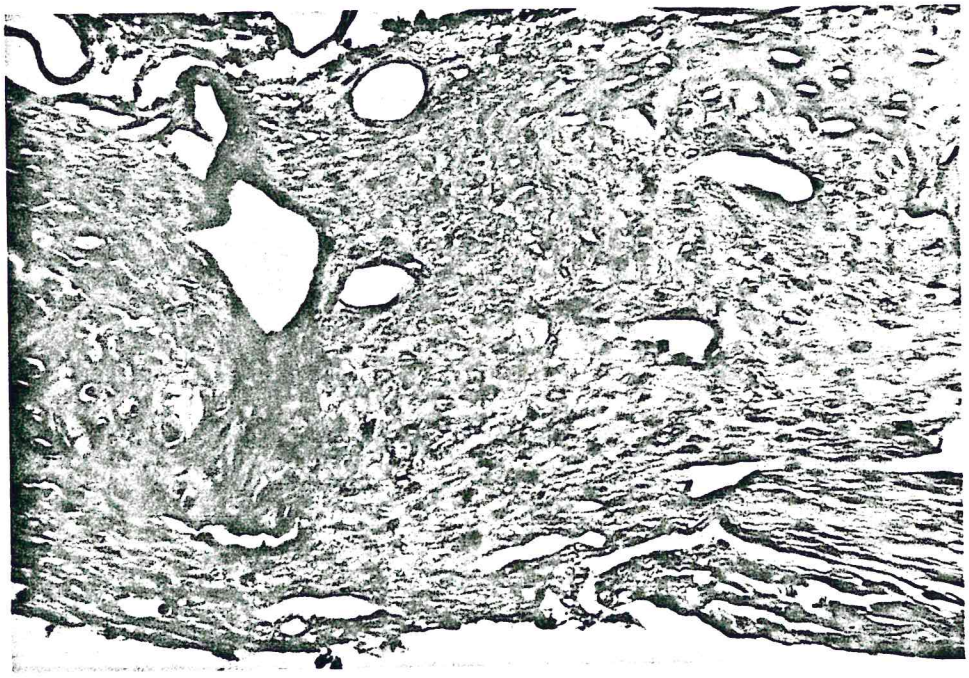


Plate XII - Control + piriprost group sutural histology (160x). The sutural bone surfaces demonstrate a more bone resorptive pattern when compared to the control (Plate VIII).

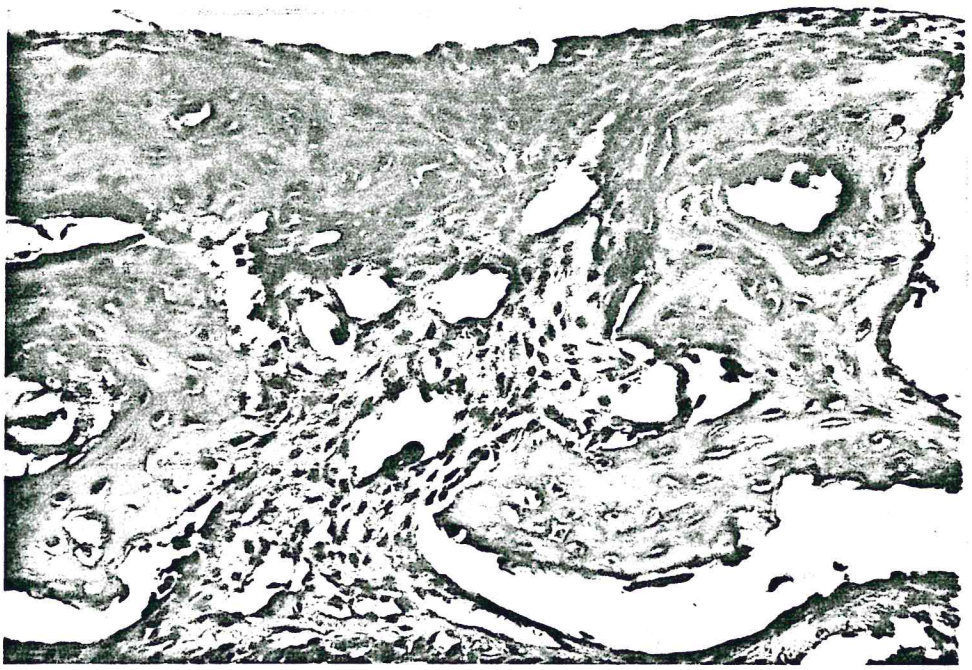
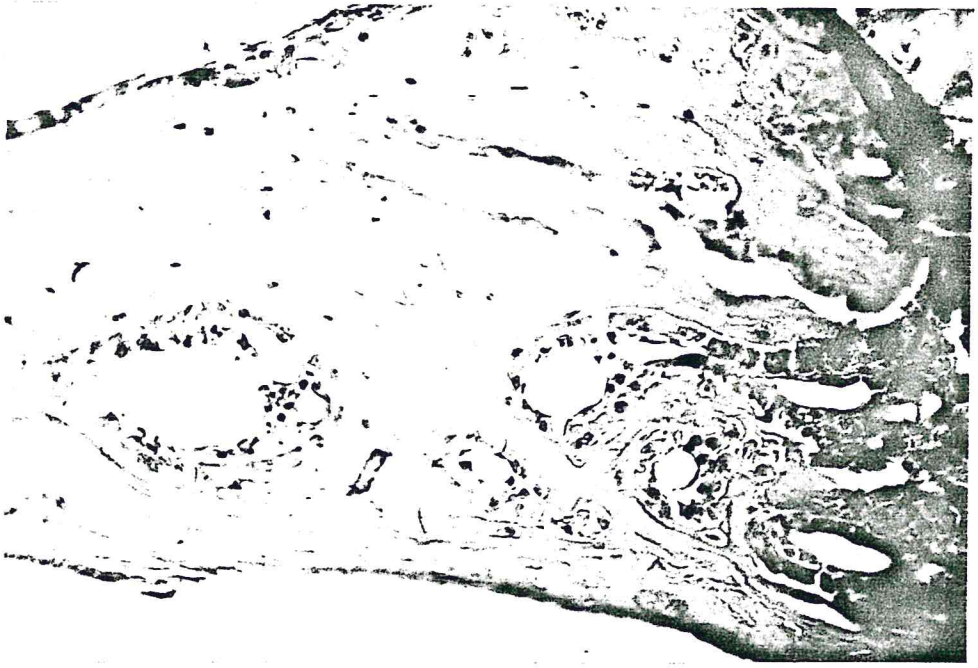


Plate XIII - Pressure + piriprost group sutural histology. Notice invagination of large basophilic cuboidal cells directly from the suture. Only a portion of the suture is shown on the right side of photo (160x).

Plate XIV - Higher magnification of pressure + piriprost group showing cortical invaginations (400x).



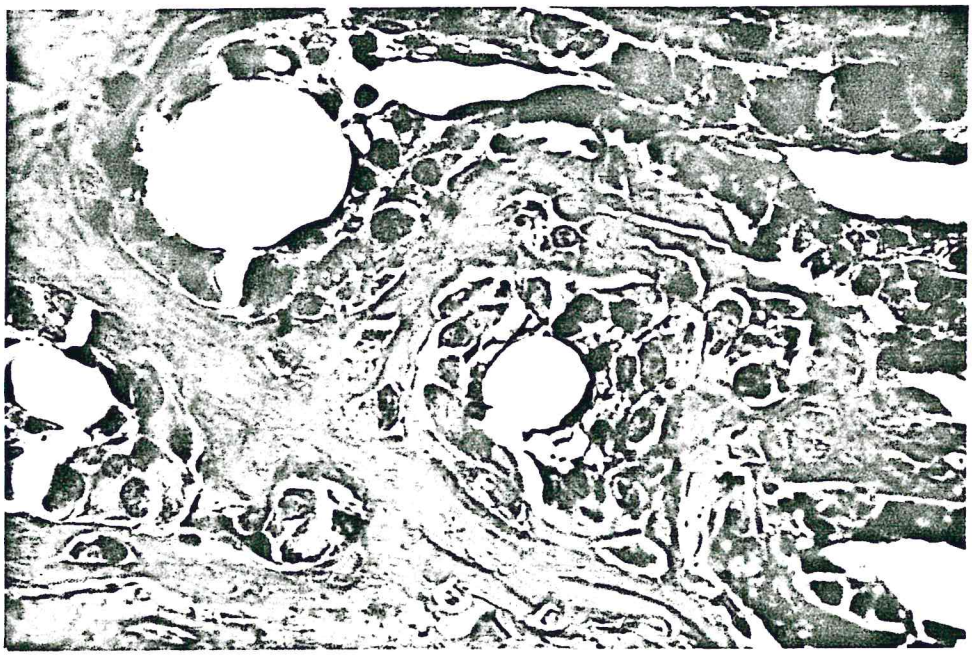
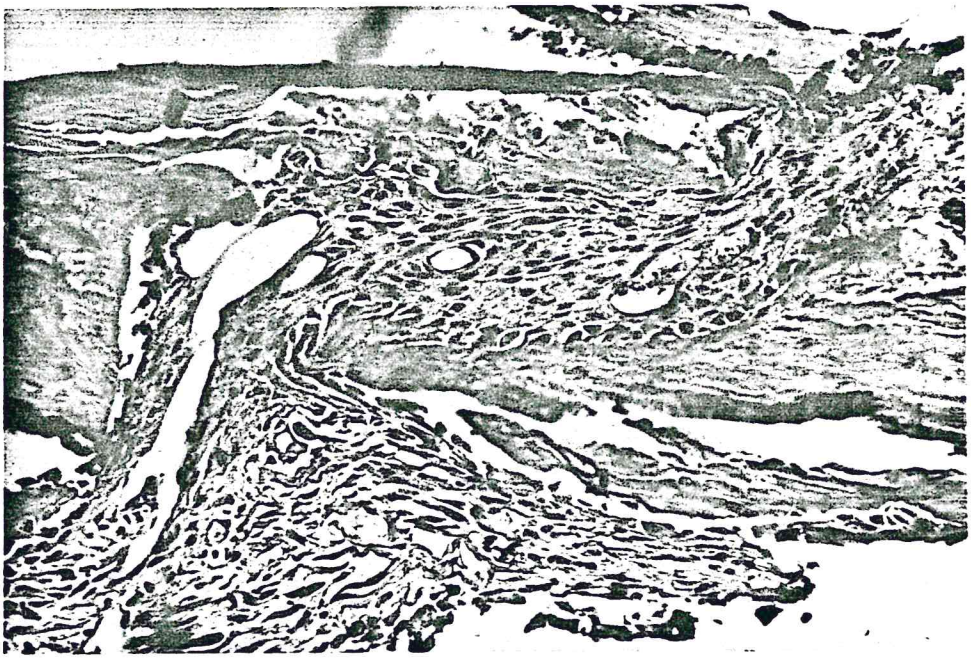
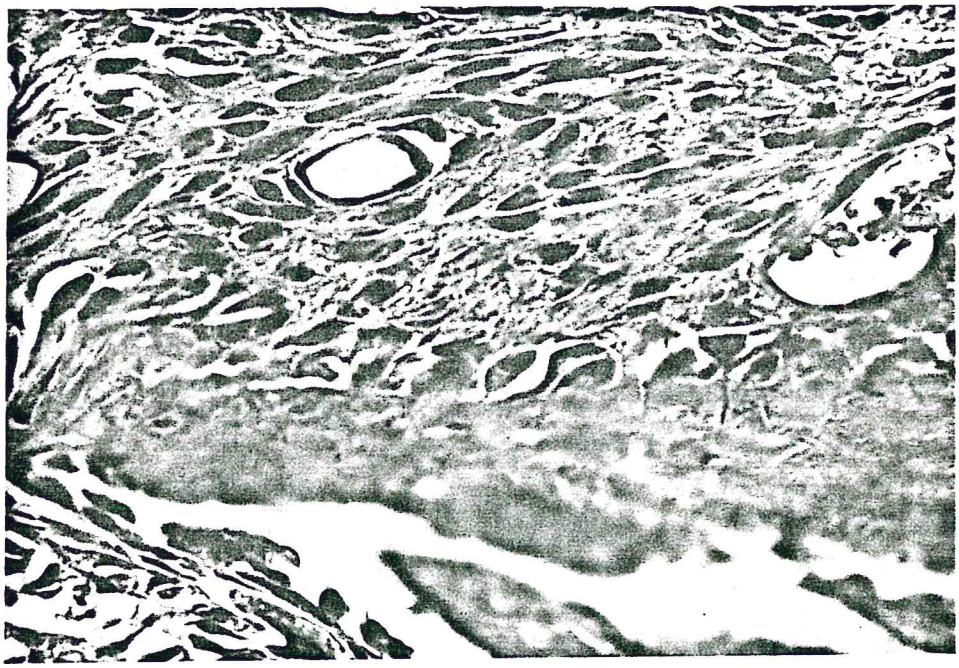


Plate XV - Tension + piriprost group sutural histology. Notice well developed pattern of basophilic cells lining the bone surface (160x).

Plate XVI - Higher magnification of tension + piriprost group showing bone surface lined by presumably active bone forming cells (400x).





DISCUSSION

The presentation of results and the discussion to follow do not imply any particular mode of mechanical stress transmission or primary cellular targets rather, the purpose of this investigation was to evaluate the effect of applied pressure and tension on the content of LTB_4, LTC_4 and PGE_2 in the midsagittal suture of growing rats. An experimental period of five days was chosen because it was estimated that this was a sufficient time to allow for obvious histologic change associated with either bone formation or resorption.

Other investigative groups have used orthodontic tooth movement to study mechanical stress induced bone remodeling (Davidovitch, and Shanfeld, 1980; Chumbley and Tuncay, 1986; Yamasaki et al., 1982, 1984). Because all surfaces 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 tooth model does not allow good discrimination between pressure or tensile forces. To reduce this problem the midsagittal suture of growing rats was chosen as a target tissue for force application (Benson, 1983). In this respect my study also resembled the investigation by Radulovich (1985). In order to isolate the midsagittal suture from surrounding forces, osteotomies were cut into the parietal bones. Upon histologic evaluation of the bone samples it was discovered that the pressure and tension groups did not show a pure resorptive phase nor a pure formative

phase. The bone plates slipped past one another in the pressure group probably causing tension of some surfaces (Plate X).

Perhaps the springs in applied pressure animals was delivering a force in excess of 20 grams or the force was not applied perpendicular to the suture thus allowing slippage of the bone plates. The tension group showed areas of osteoclastic action. Again this may indicate a distortion in the spring which torques the bone segments causing pressure on some surfaces (Plate XI). Although a simple tension/ formation, pressure/resorption point of view is used in this study it is realized that for a number of reasons the relationship between applied force and resulting bone remodeling is often not simple, for example, tissue morphology and compliance, appliance design and implantation. This complexity of applied force indicates the existence of a mixed or mosaic remodeling pattern. However, the predominance of force application was either resorptive or formative.

The experimental model utilized provided a simple, easily tolerated surgical procedure which provided a good model for evaluation of mechanical stress induced bone remodeling. There were no deleterious effects on normal growth or weight gain. There was no sign of inflammation or necrosis due to the experimental conditions. This observation was confirmed by histologic evaluation. Because prostaglandins and leukotrienes are known to be mediators in the inflammatory response (Willoughly, 1972; Samuelsson, 1983), therefore it is important to dissociate the metabolite response as

a possible indicator of a inflammatory reaction rather than caused by mechanical stress.

The non-collagenous protein content of bone samples was used as an estimate of cellular mass to standardize the measurement of LTB_4, LTC_4 and PGE_2 levels. Protein content varied between 4.0% and 7.7% (Table II). Ham (1974) reported the protein content of bone samples to vary from 1-13%. Using a similar experimental model as the one used in my study, Benson (1983) reported a consistent value of 22% and Radulovich (1985) found a range of 9.0-19.4% of total bone weight. The variation in results is probably due to bone sample size and morphology. Benson's bone samples varied between 80 and 100 mg, Radulovich's between 10 and 20 mg, and my bone samples between 5 and 10 mg.

The PGE_2 content can be compared to the values obtained by Radulovich (1985) to evaluate experimental design and results. Radulovich found endogenous levels of PGE_2 (osteotomies only) in the midsagittal suture to be 76.8 pg/mg protein. In my study using a similar animal model and extraction procedure yet a different column separation procedure (XAD-2 versus C-18 micro clean-up), the endogenous level was 76.9 pg/mg protein. Both column separation techniques resulted in a recovery of PGE_2 of 85%. This reproducibility of data illustrates the usefulness of this experimental model for testing PGE_2 endogenous levels.

Applied pressure and tension resulted in PGE_2 content of 141 and 177 pg/mg protein in the Radulovich study. These values were

elevated approximately two-fold above the controls. The results in my study also showed a two-fold elevation in the tension group (158 pg/mg protein). However applied pressure only elevated the PGE_2 response slightly (not significant at 96.7 pg/mg protein). The difference in magnitude of response between Radulovich's study and mine might be explained by a change in experimental design (Table IX). I controlled for the circadian rhythm with regard to sampling time (Simmons, 1979, 1980). Experiments by Simmons et al. (1976) and Rosenberg et al. (1977) seem to indicate peak cortical bone formation and resorption to be the first six hours of the dark cycle. This time period is associated with the peak rat activity rhythm. Radulovich sacrificed his animals at the beginning of the second half of the light cycle (12:00 pm - 2:00 pm) and I sacrificed my animals in the middle of the first half of the dark cycle during peak cortical bone remodeling (8:00 pm - 10:00 pm).

The control data suggest circadian fluctuations are either not measurable or are not associated with non-stressed PGE_2 levels. However, the experimental group data indicate that the stimulated levels of PGE_2 are actually lower during the period of peak cortical bone resorption and formation. This may be attributed to the mechanism of bone remodeling explained by Parfitt (1984). He categorizes bone remodeling of adult bone into five stages: quiescence, activation, resorption, reversal, formation and return to quiescence. In contrast to Parfitt's model, my experimental model consisted of young growing rats in which almost all free bone sur-

faces are either forming or resorbing thus there is no quiescence stage in my experimental model.

Bone remodeling in my experimental groups is operationally defined as that bone formation or resorption caused by mechanical stress. Although bone remodeling was at peak levels (Simmons, 1979) during my sacrifice time it appears that the PGE_2 levels are higher prior to this period, possibly during the activation stage. Activation is responsible for cell recruitment, that is, it is the stage for preparing the machinery for bone resorption.

Examination of the LTB_4 response shows relatively high basal values when compared to the experimental groups at five days. It can not be eliminated from my experimental model that the high LTB_4 levels are caused by the osteotomy alone. Applied pressure and tension caused a marked inhibition of LTB_4 levels. A hypothesis of LTB_4 action in the bone remodeling response is that LTB_4 might be one of the chemotactic agents responsible for movement of cells from the hematopoietic lineage, monocytes from the peripheral circulation, thought to be precursors of osteoclasts (Ford-Hutchinson et al., 1980; Malmsten et al., 1980). Perhaps LTB_4 levels do rise to influence chemotaxis for osteoclasts but at the five day interval all LTB_4 response is inhibited by pressure or tension. Histologic sections showed that the bone surfaces are undergoing marked resorption and formation. Conceivably LTB_4 levels under mechanical stress may be elevated earlier in the experimental period.

It is known that PGE_1 and PGE_2 inhibit LTB_4 release from polymorphonuclear leukocytes (Ham et al., 1983). In my experimental model it appears that increased PGE_2 levels may inhibit the LTB_4 response especially in the applied tension group.

The biologic effects of LTC_4 include contraction of nonvascular smooth muscle (bronchoconstriction) and affecting arteriolar constriction which augments vascular permeability (Samuelsson, 1983). Possible mechanisms of action of LTC_4 in the bone remodeling response could be LTC_4 action in controlling vasculature permeability. Dahlén et al. (1980) showed dose dependent increases in the premeability of the capillary system due to LTC_4 and LTD_4. My experimental data correlate with Dahlén's data. In the pressure group where osteoclast precursors may penetrate through the vasculature, the LTC_4 levels were markedly increased above control levels.

In reviewing the response of the animals injected with piriprost, the results are mostly consistent with those found by Bach et al. (1982). Bach's group found inhibition of LTC_4 and LTD_4 with no inhibition of thromboxane B_2, (TxB_2) a cyclooxygenase derivative. In fact they found a modest increase in TxB_2 levels. The data in my investigation showed inhibition in control levels of LTB_4 and mechanical stressed levels of LTC_4. The control levels of LTC_4 were relatively low when blank corrected and therefore inhibition by piriprost may exist, yet it is not measureable.

The pressure + piriprost group resulted in a significant elevation in the PGE_2 response indicating a possible shunting of the common precursor, arachidonic acid, from one pathway into the other. The tendency for this action was recognized when indomethacin, a cyclooxygenase inhibitor, caused increases in leukotriene levels (Bach et al., 1982).

Histologic data suggest that piriprost has a profound action upon the remodeling pattern. In the control + piriprost group where LTB_4 is markedly reduced the histologic pattern was altered. The overall picture suggested more bone resorption than the control.

The pressure + piriprost group demonstrated a pattern of presumably intense bone formation at five days. Many osteoblast like cells were lining cortical sinuses and apparently depositing bone. Several reversal lines were present indicating a switch from bone resorption followed by bone formation. This histologic picture correlates with a significant elevation in the PGE_2 response and an inhibition of LTB_4 and LTC_4. It must be emphasized that these metabolite changes can be correlated with a change from bone resorption to bone formation.

The tension + piriprost group resulted in sections presenting large, richly staining basophilic cells lining the bone surfaces which represented an enhanced bone formation histologic pattern. Metabolite response in this group had inhibited LTB_4 and LTC_4 levels and elevated PGE_2 levels.

Both histologic and biochemical data suggest that LTB_4, LTC_4 and PGE_2 play a role in stress induced bone remodeling. Because in all experimental groups LTB_4 is reduced it appears to be involved in both bone resorption and bone formation. LTC_4 and PGE_2 seem to function in combination in bone remodeling. When LTC_4 levels are high, bone resorption predominants and when these values are reduced and PGE_2 levels are elevated bone formation predominates. Even in the presence of pressure when LTC_4 levels were reduced with piriprost the histologic data changed from an expected resorptive phase to a formative phase.

Because this was an initial study of leukotriene involvement in bone remodeling there remain many avenues of study. Foremost, histochemical studies which identify osteoblastic activity and histomorphometric descriptive analysis identifying amount of histologic change are needed. The addition of extraneous LTB_4 and LTC_4 to the experimental system would also lead to much needed data to confirm mechanism of action of LTB_4 and LTC_4 as possible mediators of the bone remodeling response.

TABLE IX

EXPERIMENTAL DESIGN DIFFERENCES BETWEEN COLLINS AND RADULOVICH.

Condition	Collins (1986)	Radulovich (1985)
Time of Surgical Procedure	10:00 am - 3:00 pm	9:00 am - 2:00 pm
Time of Sacrifice	8:00 am - 10:00 pm	12:00 am - 2:00 pm
Drug Injected	Piriprost	Indomethacin
Time of Injections	8:00 am & 6:00 pm	8:00 am & 8:00 pm
Template Used for Osteotomy	Yes	No
Total Wt. of Bone Sample	5-10 mg	10-20 mg
Edges of Osteotomy Scraped	Yes	No
Metabolites Analyzed	LTB_4, LTC_4, PGE_2	cAMP, PGE_2
Metabolite column Separation	C-18 Micro-Cleanup	XAD-2
Groupings for Sacrifice	A1; A2; A3; A4	A1; B1; B2; C2
Histologic Evaluation	Yes	No

SUMMARY AND CONCLUSIONS

The mechanisms involved in bone remodeling have attracted a great deal of basic research. Considerable attention has been given to the effects of cAMP and PGE_2 and their interactions. Data indicate that they are potential mediators of the bone remodeling response. Because prostaglandins and leukotrienes are derived from a common precursor, arachidonic acid, and because it has been demonstrated that they interrelate in other biological systems, it is a reasonable working hypothesis that their interaction might be important in the bone remodeling process as well.

The present study was performed to assess possible changes in LTB_4 and LTC_4 in response to induced bone remodeling and to detect any interrelationship between LTB_4, LTC_4 and PGE_2 in regulation of sutural remodeling. A procedure to isolate compressive or tensile forces on the midsagittal suture of growing Sprague-Dawley rats was used. Osteotomies were cut into the parietal bones which partially isolated the midsagittal suture from influences of surrounding areas. Springs calibrated to deliver 20 gms of compressive or tensile forces were implanted across the suture.

This study utilized thirty-six animals that were twenty-one days old at the start of the experiment. The sample was equally divided into three main groups: (1) control, (2) sutural pressure, and (3) sutural tension. Six animals in each group received subcutaneous injections (10 mg/kg) of piriprost, a specific

inhibitor of the 5'-lipoxygenase pathway, thus blocking formation of all major leukotrienes. The other six animals received no injections. Thus, six groups were created with six animals in each: (1) control, (2) control + drug, (3) sutural pressure, (4) sutural pressure + drug, (5) sutural tension, and (6) sutural tension + drug.

At the end of a five day experimental period the bone samples were removed under general anesthesia, quick frozen in liquid nitrogen and lyophilized. Samples were extracted and measured for LTB_4, LTC_4 and PGE_2 content using specific radioimmunoassay procedures. Levels of non-collagenous proteins were determined for each sample by a variation of the Folin protein assay to standarize the bone samples in terms of active bone tissue.

The findings of this investigation were:

(1) The surgical procedure, modified from Radulovich (1985), was well tolerated and provided a good model for evaluation of the bone remodeling response;

(2) An extraction procedure known to produce high recoveries for cAMP, PGE_2 and non-collagenous proteins (Benson, 1983 and Radulovich, 1985) was modified to yield high recoveries of LTC_4 from the same bone sample;

(3) The non-collagenous protein content of the bone samples were mostly between 4.2% and 6.5% of the bone weight;

(4) Measurable endogenous response of LTB_4 and PGE_2, but not of LTC_4, were found in the midsagittal suture of growing Sprague-Dawley rats;

(5) Applied pressure causes strongly reduced levels of LTB_4, a marked increase in LTC_4 levels, and little change in PGE_2 levels when compared to controls;

(6) Applied tension causes reduced levels of LTB_4, a two fold increase in PGE_2 levels, and little change in LTC_4 levels when compared to controls;

(7) Piriprost was an effective leukotriene inhibitor. All LTB_4 responses and the pressure induced LTC_4 responses were inhibited. In contrast, piriprost stimulated the pressure induced response of PGE_2;

(8) The stress application did not result in any histologic evidence of inflammatory reaction or necrosis;

(9) A histologic picture was seen of bone resorption associated with pressure areas and bone formation associated with tension areas;

(10) Histologic evaluation following piriprost administration suggested enhancement of bone formation in the tension group and reversal from bone resorption to bone formation in the pressure group.

Findings from this experimental model suggest that LTB_4 and LTC_4 are potential mediators in sutural remodeling induced by mechanical stress and that these leukotrienes function in

conjunction with PGE_2 in the response mechanism to the applied stress. In this investigation all stress induced remodeling responses were associated with a reduced LTB_4 level. In addition, the data suggest that PGE_2 is a potential mediator of bone formation and that LTC_4 is a potential mediator of bone resorption. It should be emphasized that the metabolite responses may fluctuate with time of day and duration of experimental period and that the findings may be specific for this experimental design.

This is an initial study of leukotriene metabolite involvement in the response mechanism to mechanical stress. The need for future experiments, including histomorphometric descriptive measurements and the addition of extraneous LTC_4 or LTB_4 to the experimental system, are needed.

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