THE ROLE OF PROSTAGLANDINS IN CONNECTIVE TISSUE REMODELLING DURING ORTHODONTIC TOOTH MOVEMENT *IN VITRO*

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ABSTRACT

Histological studies have shown that remodelling of hard and soft connective tissues is an essential process of orthodontic tooth movement. Although the cellular control mechanisms for these remodelling functions are unknown, several potential factors have been proposed, including periodontal pressure and tension changes (Sandstedt, 1904, 1905a, 1905b), oxygen tension changes (Bien, 1966), piezo-electric stimuli (Zengo et al., 1973, 1974), cyclic nucleotides (Davidovitch et al., 1975) and prostaglandins (Yamasaki et al., 1980).

Current evidence suggests that locally produced prostaglandins are involved in tissue reactions analogous to those occurring during orthodontic tooth movement. PGE_2 has been implicated in bone resorption, whether it be of a neoplastic (Tashjian et al., 1972) or inflammatory (Goodson et al., 1974) nature, as well as in the inhibition of collagen synthesis (Raisz and Koolemans-Beynen, 1974) and in the mechanical stretching of cells (Harell et al., 1977). Prostacyclin, which has been identified in blood vessels, appears also to be involved in bone resorption (Raisz et al., 1979). Likewise, a possible involvement of thromboxane A_2, which has also been identified in blood vessels, in bone resorption has been suggested (Heersche and Jez, 1981).

The objective of this study was to investigate the possible role of prostaglandins in connective tissue remodelling during orthodontic

tooth movement. To this end four different experiments were performed.

Firstly, because of the lability of prostaglandins and because of the masking and modulating influences present in the in vivo situation, an in vitro periodontal organ culture system was developed. Previously reported systems had the problems of either an inability for cultured teeth to receive orthodontic type forces or a continuous flow supportive medium which would not allow for the utilization of radiolabelled tracer molecules. The system developed in this study used a 3 molar and surrounding alveolar bone explant taken from mouse mandibles. It was supported in a small volume (800 μl) of Waymouth's 752/1 medium, at 37^0C, in an environment of 95% O_2 and 5% CO_2, for a 24 hour period. Orthodontic forces were applied interproximally with separating springs fabricated from 0.0175 in. multistranded orthodontic wire. Vitality of the explanted tissue was demonstrated histologically and radioautographically. Protein synthesis, as demonstrated by ^{3}H-proline labelling, occurred throughout all explants, with a gradient of labelling intensity from peripheral to central regions. It was suggested that this labelling gradient was related to a gradient of oxygen perfusion throughout the explant. Cell replication, as demonstrated by ^{3}H-thymidine labelling, was evident in the periodontal ligament and endosteal spaces.

The second component of this study involved the utilizatin of the newly developed periodontal organ explant system to examine the

effects of orthodontic forces on collagen synthesis *in vitro*. As collagen is the predominant protein in both hard and soft connective tissues, the effect of orthodontic force upon collagen synthesis was investigated to provide baseline data for cellular responses to force. By extraction of the salt soluble fractions of (^{14}C)-glycine labelled collagens and by using the interrupted gel electrophoresis method of Sykes *et al*. (1976), the synthesis of both type I and type III collagens was demonstrated. Thus the viability of the periodontal organ culture system was further demonstrated. When collagen synthesis in relation to orthodontic forces was examined, there was a significant increase in the ratio of type III collagen synthesized at 1,3 and 5 days of force application. This result supports the involvement of type III collagen in remodelling tissues and in tissues bearing physical stress.

Following this demonstration of the continued vitality of the explant system over a 24 hour period of culture, the third facet of this study examined the effect of orthodontic forces upon prostaglandin synthesis. Utilizing the ethyl acetate extraction of (^{14}C)-arachidonate labelled prostaglandins and their separation on thin layer chromatography plates, synthesis of the prostaglandins 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, TXB_2, PGE_2 and PGD_2 was demonstrated. This once again showed the viability of the system and, for the first time, showed that periodontal tissues were capable of synthesizing a variety of prostaglandins. However, no significant difference was apparent between the relative amounts of prostaglandins synthesized by perio-

dontal tissues stressed for periods ranging from 0 minutes to 14 days. It was suggested that the inability to discern fluctuations in prostaglandin levels during various periods of force application may be due to opposing responses of specific prostaglandins in areas of tension and compression.

Thus the fourth component of this study examined the effects of either compression or tension upon prostaglandin synthesis in the mouse calvaria in vitro. Once again the viability of this system was demonstrated by the synthesis of the prostaglandins 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, TXB_2, PGE_2 and PGD_2. Although evidence was presented that TXB_2 increased significantly in both compressive and tensile treatments after 10 minutes of force application, the wide dispersion of data made only tentative conclusions possible. The reasons for this data dispersion were discussed.

Two in vitro systems capable of receiving orthodontic type forces across the soft tissue ligament have been examined in this study. Although the inherent difficulties in measuring prostaglandins have resulted in only tentative conclusions being possible in relation to the involvement of prostaglandins in the cellular response during mechanical force applications, it is felt that both systems will allow for the study of a variety of hypothesized cellular control mechanisms and the cellular behavior coincident with orthodontic tooth movement. To this end a proposed cascade of events involving force application, cell membrane deformation, prostaglandin synthesis, cyclic nucleotide

synthesis, collagen remodelling and eventual tooth movement has been advanced. The relevance of findings from this study and the proposal of future studies utilizing the reported periodontal organ and calvarial culture systems to test the hypothesized pathway of events are discussed.

DEDICATION

TO JOHN AND OLWYN

WHO THROUGH THEIR CONTINUED SACRIFICE

HAVE PROVIDED ME WITH THE TOOLS OF LIFE

TO MARGARET

WHO HAS GIVEN ME THE REASON TO USE THEM

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CHAPTER 1

REVIEW OF THE LITERATURE

A. Introduction

Hard and soft connective tissue changes occurring within the periodontal ligament during orthodontic tooth movement have been well documented histologically (see Reitan, 1975 for review). These tissue changes involve soft connective tissue remodelling of the periodontal ligament in association with alveolar bone resorption in areas of periodontal ligament compression, with apposition of bone in areas of tension. Bone resorption can be frontal, whereby resorption of the alveolar wall occurs adjacent to the compressed periodontal ligament, or it may be undermining, whereby endosteal bone is removed in conjunction with hyalinization of the compressed periodontal ligament. The term hyalinization describes the loss of cellularity and fiber organization occurring within the compressed periodontal ligament when higher forces are used.

A pathway of cellular events required for periodontal remodelling has been suggested by Melcher (1980) in which the initial stimulus (in this case orthodontic forces) is first transduced to a biological signal or signals whose targets are the progenitor cells that will eventually differentiate into those specialized cells required to remodel the periodontium. The transduction of this mechanical orthodontic force into a biological signal could be one step or a chain of several events of a biochemical and/or biophysical nature. These processes, i.e. the transduction, differentiation, migration and control of activity of the progenitor cells are still to be elucidated.

The problem of transduction is one of greatest clinical importance since this is the step which is influenced by the clinician. The fact that production of finely tuned force levels does not result in predictable tooth movements or lack of root resorption testifies to the lack of knowledge surrounding the transduction step. Greater understanding of the biological mechanisms at this level could lead to improvement in efficiency and long term stability in orthodontic therapy.

The following review of the literature will discuss those concepts relating to cellular control mechanisms involved in orthodontic tooth movement and will identify those areas where crucial questions remain to be answered.

B. Cellular control mechanisms involved in orthodontic tooth movement

1. Pressure-tension theory

The classical hypothesis on the mechanism of tooth movement involving areas of pressure and tension within the periodontal ligament has been open to a variety of interpretations. Originally presented by Sandstedt (1904, 1905a, 1905b), and later supported by Schwartz (1932) and Gianelly (1969), this hypothesis involved the concept of vascular occlusion causing the histologically observable events of hyalinization (apparent cell-free and fibre free periodontal ligament matrix)

and undermining bone resorption (resorption of alveolar lamina dura by osteoclasts on the endosteal surfaces). Cellular activity was assumed to be a direct response to local tissue "pressure" or "tension".

As a simplified explanation of the histological events observed under the light microscope, this hypothesis has remained unchallenged until recently. However, Kardos and Simpson (1980) have introduced the concept of thixotropic behaviour of the collagenous matrix of the periodontal ligament as an alternative interpretation of the pressure-tension theory. Thixotrophy refers to alterations in viscosity of a system in response to applications of force. In relation to the periodontal ligament, applications of pressure would result in a reduction in viscosity of the collagenous matrix. Kardos and Simpson (1980) feel that this alteration in viscosity and, thus, in the physical character of the collagenous matrix would permit rapid cell movement away from the area of compression. These changes would be reflected in alterations of the tissue architecture and staining properties of the matrix, and would thus explain the light microscopic appearance of hyalinization. Substantiation of this theory would require biochemical identification of changes in collagen matrix structure upon applications of force.

Baumrind (1969) has interpreted the pressure-tension theory to imply that alterations in cell replication rates would be expected to differ on the compression and tension sides. However, Baumrind's (1969) prediction of reduction in cell replication rates in areas of

pressure and vascular occlusion and increase in replication in areas of periodontal fibre stretching was found to be incorrect. Autoradiographic studies by Baumrind (1969) demonstrated an increase in cell replication rates in both pressure and tension regions, a factor the author used to challenge the value of perpetuating an hypothesis which had, he felt, outlived its period of heuristic value.

Baumrind's (1969) contention is correct, not because of the cellular behaviour revealed by his investigation, but because the pressure-tension theory does not attempt to relate to any specific cellular control mechanisms. Generalized physical concepts of periodontal ligament pressure and tension are simply related to the histologically observed events occurring during the light microscopic observation of orthodontic tooth movement, without explanation of the necessary cellular pathways which would establish a true cause and effect relationship between pressure or tension and specific cellular activity.

2. Oxygen tension

Bien (1966) hypothesized that capillaries constricted by compression of periodontal ligament fibres form a series of cirsoid aneurysms. Below each site of stenosis, decreased blood pressure results in formation of minute gas bubbles which can diffuse through the vessel wall. The author felt that such oxygen bubbles lodging against bone surfaces would lead to osseous resorption. However, there is no record of attempts to either measure fluctuations in oxygen tension or

to manipulate oxygen tension within the periodontal ligament and observe the effects upon bone resorption.

Nevertheless, Goldhaber and his co-workers (Goldhaber 1958, 1961, 1966;Stern et al., 1966) have shown that it is possible to regulate the extent of resorption of mouse calvaria in vitro by altering the concentration of oxygen in the culture medium. The increased vascularity of the periodontal ligament, which has been observed by Gianelly (1969) and Khouw and Goldhaber (1970) in areas of frontal bone resorption occurring with the appliction of orthodontic force, has further implicated an increased oxygen tension in the stimulation of bone resorption. However, the question of whether the increased vascularity found in areas of active bone resorption is primary or secondary to the resorption has not been resolved.

3. The piezo-electric theory

There is evidence to support the possibility that electric currents are generated within stressed tissues and that these currents are involved with cellular regulatory mechanisms.

Fukada and Yusada (1957) have observed that electric potentials are generated by the application of force to bone. Zengo et al. (1973, 1974) have been able to elicit similar electrical activity upon mechanical deformation of alveolar bone. In addition, tungsten microelectrodes have been used to demonstrate reductions in electrical potential in the compressed periodontal ligament of humans undergoing

orthodontic force application (Smith et al., 1982).

An increase in electrical activity has been used by Basset et al. (1964) and Zengo et al. (1976) to produce an increase in osteogenic activity, as demonstrated by elevations in osteoblast number and quantities of bone formed. Similarly, Davidovitch et al. (1978, 1979a, 1980a, 1980b) have applied direct current electrical stimulation to the dentoalveolar complex of cats undergoing tooth movements. The authors have demonstrated an increase in osteoblast and periodontal ligament cell numbers, and a corresponding increase in intracellular activity, as measured by cAMP content.

Thus a relationship between mechanical stress, piezo-electricity and osteogenesis appears to have been firmly established. However, the regulatory mechanism involved in the transduction of electrical potentials into the observed cellular activity has not been delineated, although hypotheses involving the migration of electrons through biologic membranes (Jahn, 1962), proton charge transport through the cytoplasm (Eigen and DeMaeyer, 1958) and amphoteric ion exchanges (Jahn, 1968) have been advanced.

4. Cyclic AMP

Adenosine 3',5' monophosphate (cAMP) and guanosine 3',5' monophosphate (cGMP) are considered to be intracellular "second messengers" because they mediate the effects of external stimuli on their target cells (Sutherland and Rall, 1960;Hardman and Sutherland,

1969;Sutherland et al., 1965).

The external stimuli can take a variety of forms including systemically acting hormones like calcitonin (Murad et al., 1970;Rodan and Rodan, 1974;Nagala et al., 1975), parathyroid hormone (Chase and Auerbach, 1970;Vaes, 1970;Murad et al., 1970;Peck et al., 1973;Rodan and Rodan, 1974;Nagala et al., 1975), electrical stimulation (Davidovitch et al., 1978a, 1979, 1980 a and b;Rodan et al., 1978) and the mechanical stretch of cells (Binderman and Cox, 1977;Harell et al., 1977;Somjen et al., 1980).

The result of activation of the membrane bound enzymes adenylate cyclase and guanylate cyclase by these external stimuli has been shown to be the conversion of their respective substrates adenosine triphosphate (ATP) and guanosine triphosphate (GTP), to produce cAMP and cGMP (Sutherland et al., 1962;Hardman and Sutherland, 1969). These metabolites have subsequently been demonstrated as serving as cofactors in enzymatic phosphorylation reactions, and hence influence the metabolic activity and functions of the cell (Greengaard, 1978).

Rodan et al. (1975) have applied light pressure to embryonic chick long bones in vitro, and reported a rapid decrease in both cAMP and cGMP concentrations in the preosseous cellular element.

Davidovitch et al. (1975) have measured cAMP levels in periodontal ligament of cats following tooth movement in vivo, observing an initial decrease in cAMP contents both in areas of compression and ten-

sion persisting 24 to 48 hours, followed by a gradual increase over the next two weeks of treatment. Davidovitch et al. (1976a, 1976b, 1977) have further developed a cyclic nucleotide immunohistochemical technique which revealed a decrease in cAMP staining intensity after 3 hours of stress, followed by an increase at 24 hours. Inversely, cGMP staining intensity increased at 3 hours and decreased at 12 and 48 hours.

Fluctuations in cyclic nucleotide levels are useful indicators of cellular activity, although the value of their measurement is limited. This limitation arises from the inability of cyclic nucleotide levels to be related to specific intracellular events. Thus altered levels of cAMP may be related to functions other than osteogenesis and osteoresorption.

Unless mechanical stretching of the cell membrane is directly responsible for cAMP production, an hypothesis invalidated by Somjen et al. (1980), who found that this relationship is PG dependent, then the search for the extracellular stimulus must continue.

5. Other cellular control mechanisms

A variety of possible regulatory mechanisms involved in the transduction of orthodontic forces into cellular activity have appeared in the literature. The ability of parathyroid hormone to enhance orthodontic tooth movement, both micro- and macroscopically, has been recorded by Gianelly and Schnur (1969), Kamata (1972) and Davidovitch

et al. (1972). However it appears that, because of the lack of localized specificity of this hormone, its possible involvment as a regulatory mechanism has been pursued no further.

Roberts et al. (1981) have briefly mentioned that the accumulation of microfractures in bone resulting from mechanical stress, may eventually lead to bone remodelling. As there has been no recorded scientific validation of such a concept, further comment at this time is unwarranted.

Yamasaki et al. (1980) were the first to investigate the possible role of prostaglandins as a mediator of bone resorption during orthodontic tooth movement in rats. This concept appears well founded, because prostaglandins have been implicated in both physiological and pathological bone and collagen metabolism, and also have been shown to be produced in response to the mechanical stretching of cells in vitro. (These topics will be covered in detail in subsequent sections). Unfortunately, the methodology has shortcomings and the conclusions are therefore suspect. Although indomethacin, a known prostaglandin synthetase inhibitor, administered to rats undergoing orthodontic tooth movement resulted in a decrease in the periodontal ligament ostoeclast number, the lack of documented specificity of indomethacin renders these results inconclusive. Furthermore, the attempted extrapolation of osteoclast number to a measure of active bone resorption is unreliable. In addition, attempts to implicate prostaglandins E_1 and E_2 in bone resorption by localized injection of

these substances are inconclusive. It has been well documented that localized injections of prostaglandins will cause bone resorption (Goodson et al., 1974), whether or not adjacent teeth are undergoing orthodontic treatment.

6. Conclusions

Thus cell regulatory mechanisms in connective tissue remodelling during orthodontic tooth movement remain elusive. The pressure-tension theory originates from light microscopic analysis of tooth movement without explaining cellular mechanisms. Oxygen tension fluctuations, although capable of stimulating osteogenic activity in vitro, have never been demonstrated to occur in vivo. The vasculature changes often used to support this concept may in fact be secondary to an increase in bone remodelling behaviour. Piezo-electric potentials have been shown to occur upon mechanical deformation of the dentoalveolar complex, and have also been implicated in an increase in osteogenic cell number and intracellular activity. However, the method of transduction of the electric current into the observed cellular behaviour has not been demonstrated. Cyclic nucleotides, which have been demonstrated to fluctuate in response to the application of orthodontic forces, are intracellular "second messengers" considered indicative of cellular activity. However, the extracellular stimuli responsible for producing alterations in cAMP levels require clarification.

Current evidence suggests that locally produced prostaglandins play an important role in the transduction of mechanical forces into cellu-

lar activity. The involvement of prostaglandins in bone and collagen metabolism is established, although further amplification is required. Mechanical stretching of cells in vitro (Somjen et al., 1980) have resulted in elevated levels of prostaglandins, a factor which adds further support for the involvement in prostaglandins in orthodontic tooth movement.

Because of the relatively recent advent of prostaglandin research, and due to methodology problems, the role of prostaglandins in connective tissue remodelling during orthodontic tooth movement has not, as yet, been documented. This topic will form the basis of this thesis.

C. Prostaglandins

1. Introduction

The biological activity of seminal fluid and prostate gland extracts has been recognized for many years. In ancient China seminal fluid from young adults was recognized to be of therapeutic value in patients with gastric ulceration (Chan, 1972). Amongst some North African tribes, oral ingestion of the father's semen is used to initiate labour when this is delayed (Harley, 1941).

In 1930, two New York gynecologists, Kuzrok and Lieb, observed that the human uterus could react with either strong contractions or relaxation on the introduction of fresh human semen. A few years later von Euler (1934, 1935, 1936, 1939) and Goldblatt (1933, 1935) indepen-

dently observed and studied the strong smooth muscle stimulating activity of human seminal plasma. Lipid extracts of the seminal plasma from a variety of animals led von Euler (1939) to conclude that the active lipid soluble acid fraction was produced by the prostate gland. Thus he named the compound prostaglandin (PG), although Eliasson (1959) was later to prove that the seminal vesicles were the tissues of origin. It is now known that there are a variety of physiologically active prostaglandins and metabolites, which are produced by a variety of tissues.

2. Structure

The PG's are a family of cyclic, oxygenated 20 carbon unsaturated fatty acids which are synthesized from essential fatty acids. The basic 20 carbon skeleton is prostanoic acid, which consists of a cyclopentane ring with two carbon side chains. All the prostaglandins are hydroxylated in the 15 position and contain a 13,14-trans double bond. The subscript numeral after each letter indicates the degree of unsaturation of the side chains. Thus, PG's E_1, F_1, A_1 and B_1 have only the one trans double bond, PG's E_2, F_2, A_2 and B_2 have, in addition, a cis double bond in the 5,6 position while PG's E_3, F_3 and B_3 have an additional cis double bond in the 17,18 position. All the PG's of the E type contain characteristic 11 -hydroxy and 9 keto groups on a 5 membered ring. This structure is easily dehydrated by weak alkali to the 10:11 unsaturated ketone (PGA) that can rearrange to the doubly conjugated ketone (PGB). The F prostaglandins are anal-

ogous to the E compounds but the 9- keto group is reduced to a hydroxyl.

3. Synthesis

The prostaglandins are not stored free in tissues, but are synthesized as a result of membrane perturbations that cause the release of free fatty acids, generally arachidonic acid, from esterified lipid sources (Flower and Blackwell, 1976;Ramwell et al., 1977). The release of arachidonic acid can be brought about by a wide variety of hormones, either directly or indirectly (Hong and Levine, 1976), as well as by inflammatory or immunological stimuli (Humes et al., 1977;Bonney et al., 1979), calcium ionopores (Lapetina and Cuatrecasas, 1979a), ultraviolet light (Plummer et al., 1977), melittin, the membrane active component of bee venom (Hassid and Levine, 1977), tumour promoting agents (Ohuchi and Levine, 1978) and mechanical agitation (Flower and Blackwell, 1976).

The precise mechanism for releasing fatty acid precursors of prostaglandins has not been identified. It is generally agreed, however, that they originate largely from phospholipid reserves in cell membranes. Although phospholipase A_2 has been recognized as an important enzyme in the release of these precursor acids (Flower and Blackwell, 1976), recent studies with platelets implicate a phosphatidylinositol-specific phospholipase C, yielding diacylglycerides and subsequently, arachidonic acid (Rittenhouse-Simmons, 1979;Bell et al., 1979;Lapetina and Cuatrecasas, 1979b). However, the

importance of this new pathway in other cell types has not yet been established.

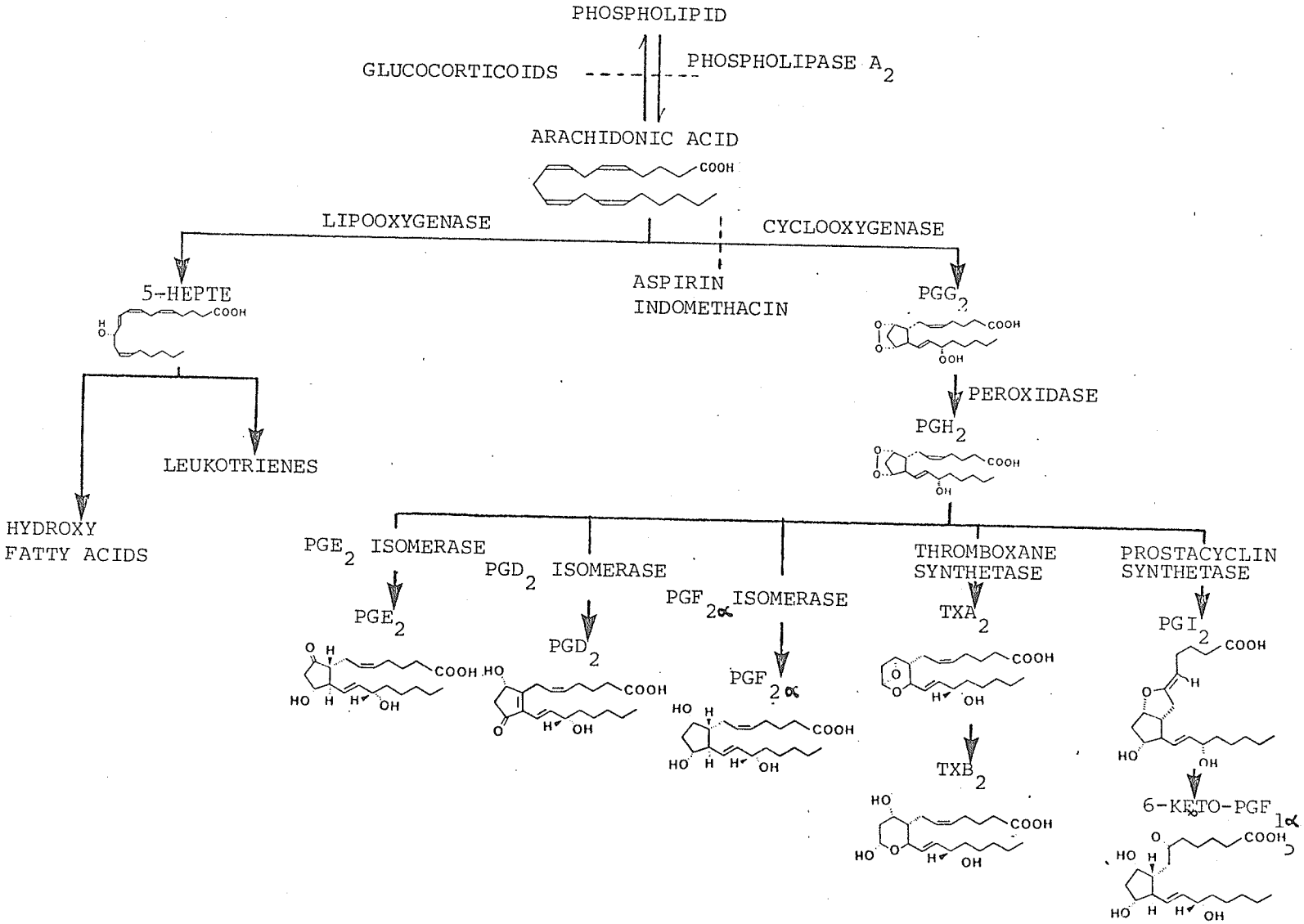
Biosynthetic pathways for oxygenation of the liberated arachidonic acid follow one of two routes (Fig. I-1). The more classical PG's (PGE_2, PGD_2, $PGF_{2\alpha}$) and the recently discovered thromboxanes (Hamberg et al., 1975) and prostacyclins (Moncada et al., 1976;Johnson et al., 1976;Gryglewski et al., 1976) are produced by a series of reactions initiated by cyclooxygenase. The action of lipooxygenase upon arachidonic acid initiates an alternative route, the products and metabolites of which remain to be completely identified, and their involvement in connective tissue remodelling is unknown.

(a). Cyclooxygenase

Arachidonic acid is oxygenated to PGG_2 by a microsomal enzyme designated cyclooxygenase (Samuelsson and Hamberg, 1974). This hydroperoxy acid is largely converted to PGH_2 by a hydroperoxidase. PGH_2 is considered a pivotal compound since it is metabolized by three different reaction pathways. The first pathway yields the classical PG's, PGE_2, PGD_2, $PGF_{2\alpha}$ as a result of PGE_2 isomerase, PGD_2 isomerase and $PGF_{2\alpha}$ reductase respectively (Nugteren and Hazelhof, 1973). The second pathway yields thromboxane A_2 (TXA_2) as a result of thromboxane synthetase (Hamberg et al., 1975). The third pathway involves the formation of prostacyclin (PGI_2) as a result of PGI_2 synthetase activity (Gryglewski et al., 1976;Moncada et al., 1976;Johnson et al., 1976).

FIGURE I-1

STRUCTURE AND SYNTHESIS OF THE MAJOR PROSTAGLANDIN DERIVATIVES OF ARACHIDONIC ACID



An alternative pathway involving the direct conversion of PGG_2 to 15-hydroperoxy PGE_2 (Samuelsson and Hamberg, 1974) and hydroperoxy thromboxane A_2 (Hammarstrom, 1980) has been demonstrated with isolated enzymes, but does not appear to be important *in vivo*.

(b). Lipooxygenases

A competitive route for the oxygenation of free arachidonic acid is provided by the lipooxygenase enzymes (Hamberg and Samuelsson, 1974). The primary products of this system are hydroperoxycicosatetraenoic acids (HPETE's). The hydroperoxides can be metabolized to either the analogous alcohol, or to leukotrienes. The alcohols result from peroxidatic reduction which occurs rapidly with any of the hydroperoxides. However, the leukotrienes are formed only from 5-HPETE by epoxide formation (leukotriene A), with subsequent ring opening either with water to give a diol (leukotriene B) (Borgeat and Samuelsson, 1979) or with glutathione to form leukotriene C (Murphy *et al.*, 1979;Hammerstrom *et al.*, 1979).

4. Inhibition of arachidonic acid oxygenation

An alternative method to measuring specific PG's or adding specific PG's and observing their effects is selective inhibition of enzymes involved in the synthetic pathway. Although little is known in this area, many investigators appear to be currently involved in the examination of various inhibitors (e.g. El Attar and Lin, 1981;Sun *et al.*, 1981;Wilhelm *et al.*, 1981).

Smith and Willis (1971) and Vane (1971) have reported that aspirin and indomethicin block the synthesis of prostaglandins. The cyclooxygenase has been established as a site of action for these drugs because the uptake of molecular oxygen, which had been previously shown by Samuelsson (1965), is blocked (Smith and Lands, 1972).

Glucocorticoids have been shown to result in a decreased release of arachidonic acid from phospholipids (Gryglewski et al., 1975). Since only unesterified fatty acids are used by the cyclooxygenase, this depresses all cyclooxygenase-derived products.

Specific thromboxane synthetase inhibition by imidazole and several imidazole analogues has been reported by Moncada et al. (1977) and Needleman et al. (1977). Thus selective control of the conversion of PGH_2 to thromboxane A_2 is afforded by these compounds. L-1-tosylamido-2-phenylethyl chloromethyl ketone has been shown by Yahn and Feinstein(1981) to inhibit thromboxane synthetase, but a slight inhibitory effect upon the lipooxygenase pathway limits the usefulness of this compound.

Selective inhibition of the lipooxygenase pathway has not, as yet, been reported. Studies involving benoxaprofen (Walker and Dawson, 1979) and 3-amino-0- (m-trifluoro-methyl) phenyl -2-pyrazotone (BN755C) (Higgs et al., 1979) have recorded mixed activities against both the lipooxygenase and cyclooxygenase enzymes, but with a preference for lipooxygenase inhibition. Sun et al. (1981) and Wilhelm et al. (1981) have examined a number of acetylenic fatty acids in rela-

tion to their ability to inhibit the lipooxygenase pathway. Both papers have reported significant inhibition when 4, 7, 10, 13-Eicosatetraynoic acid was employed, although slight effects upon the cyclooxygenase pathway were also apparent.

D. The role of prostaglandins in bone resorption

Evidence for a possible role for prostaglandins in bone resorption was first reported by Klein and Raisz (1970), who showed that PGE_1 and PGE_2 produced similar calcium release and cellular morphological responses to PTH when added to cultured foetal rat bone. Similarly Goodson et al. (1974) had demonstrated rapid bone resorption when PGE_1 was injected under the skin overlying the calvarium of rats. All subsequent studies have been addressed to either the role of prostaglandins in the bone loss associated with neoplastic and inflammatory disease, or to investigations of the specific resorptive activity of the various prostaglandins, their intermediates and metabolites.

The role of prostaglandins in neoplastic bone resorption has been investigated both clinically and in the laboratory. Mice bearing the transplantable HSDM fibrosarcoma or the VX_2 carcinoma have been shown to display increased blood concentrations of PGE_2 and calcium (Tashjian et al., 1972, 1973, 1974, 1977a;Voelkel et al., 1975). Monolayer cultures of these tumour cells produced PGE_2 and the addition of the inhibitor of prostaglandin synthetase, indomethacin, resulted in a reduction in the synthesis of PGE_2 (Tashjian et al.,

1972, 1974;Levine et al., 1972).

Strausser and Humes (1975) have observed tibial pocking and sponginess in mice injected locally with Maloney sarcoma virus, with this effect proportional to tumour prostaglandin content. The authors have reported that these tumours show a normal regression and when this happens the prostaglandin content diminishes and the bone changes disappear. Furthermore, indomethacin treatment prevented the bone damage. Similarly, Lynch et al. (1978) have shown that intramuscularly injected cells of the methylcholanthrene-induced C^3H mouse fibrosarcoma caused hypercalcaemia and substantial destruction of the tibia, both of which were diminished by giving indomethacin.

Galasko and Bennett (1976) have found markedly raised numbers of osteoclasts in rabbits injected with VX_2 tumour into a tibia. The number of osteoclasts was reduced significantly in rabbits given indomethacin, but were still substantially greater than normal. Radiographs indicated that indomethacin markedly reduced, but did not prevent, the bone destruction. Thus data provides evidence for the involvement of both prostaglandins and a non-prostaglandin osteoclast activating substance, possibly osteoclast activating factor (Horton et al., 1972). However, Yoneda and Mundy (1979a,b) have shown that the production of osteoclast activating factor by human peripheral blood leukocytes requires prostaglandins. Therefore, it seems likely that cells other than osteoclasts are also involved in neoplastic bone resorption (Galasko, 1976;Eilon and Mundy, 1978). Investigations in

vitro of human malignancies have further implicated prostaglandins in the pathogenesis of localized bone loss. Powles *et al.* (1973) have shown that Walker carcinoma cells and some human breast tumours *in vitro* cause osteolysis which can be blocked by inhibitors of prostaglandin synthesis. In addition, Powles *et al.* (1976) and Easty *et al.* (1977) have found that the osteolytic activity of human malignant breast tumours *in vitro* correlated with the presence or subsequent development of bone metastases and hypercalcaemia. Although aspirin reduced the osteolysis, *in vitro*, total prevention did not occur, thus once again indicating the possibility of a non-prostaglandin dependent osteoclast-activating factor.

Homogenates of human breast carcinomas have revealed a relationship between the amount of extracted prostaglandin and the incidence of bone metastases as indicated by skeletal scanning near to the time of breast surgery (Bennett *et al.*, 1975, 1976, 1977). Apps and Cater (1973) have reported the production of a prostaglandin-like substance when serum was incubated with finely minced tumour tissue from rat, dog, mouse and man.

Although direct clinical investigations of prostaglandin involvement in bone invading neoplasias are rare, Seyberth *et al.* (1975) and Demers *et al.* (1977) have shown elevated urinary or plasma concentrations of prostaglandins of the E series in patients with bone invading tumours.

The enlargement of dental cysts within the jaws results in a localized loss of bone. Harris and Goldhaber (1973), Harris et al. (1973) and Harris (1978) have demonstrated that dental cysts produce significant quantities of PGE_2 and $PGF_{2\alpha}$ in vitro, as measured by the ability of homogenized cyst material to elicit prostaglandin-like activity when added to rat fundus strip, and that this prostaglandin synthesis is inhibited when the supportive medium contains indomethacin.

The role of prostaglandins in the inflammatory destruction of bone has been principally investigated in relation to periodontal disease. Several investigators have reported the elevation of PGE_2 levels in gingiva taken from patients with periodontal disease (Goodson et al., 1974;El Attar, 1976;Loning et al., 1980). Gomes et al. (1976) have demonstrated that monkey gingival fragments release PGE_1 and PGE_2 in tissue culture. Recently, Rifkin and Tai (1981) have reported elevations in the order of 2-3 times normal in levels of thromboxane B_2, the stable end product of TXA_2, in normal and inflammed gingiva of beagle dogs. Although this reduction is nowhere near the 10-fold increase in PGE_2 levels reported by Goodson et al. (1974), it does illustrate a possible role for prostaglandins other than the E series in bone resorption. El Attar and Lin (1981) have investigated the biosynthesis of PGE_2 and $PGF_{2\alpha}$ in gingival fragments taken from patients with chronic periodontitis. Both types of PG's were synthesized in vitro by these fragments, with a preference for PGE_2 being evident.

Arendorf (1981) has injected PGE_2 locally in an emulsion of Freund's incomplete adjuvant. Although no attempt was made to either test the stability and time release of PGE_2 or to discount the possibility of an immunogenic response to PGE_2, the authors concluded that PGE_2 evokes an inflammatory response and localized bone loss.

Other inflammatory lesions involving bone resorption have been investigated. Synovial tissue from patients with rheumatoid arthritis has been shown to liberate a bone resorbing compound *in vitro* (Robinson *et al.*, 1975;Robinson and Granda, 1974). The resorptive activity, as measured by ^{45}Ca release from mouse calvaria, was diminished when indomethacin was added to the medium. Dayer *et al.* (1976) have demonstrated that isolated rheumatoid synovial cells similarly synthesize PGE_2 *in vitro*.

Aberration of the local immune system is considered to be, in part, responsible for the pathologic tissue destruction in chronic periodontal disease, as well as in other chronic inflammatory reactions. The ability of heterologous rabbit serum to stimulate bone resorption in tissue culture has been demonstrated by Sandberg *et al.* (1977) and Raisz *et al.* (1974). The authors have shown that this resorptive activity is dependent on the presence of complement, and that the activation of complement by immunoglobulins reactive with cell surface antigens stimulates the synthesis of PGE. Absorption of the complement source with rat spleen cells or dilution of the complement abolished the previously observed release of ^{45}Ca from organ cultures of

rat foetal bones, as did additions of indomethacin. Thus, it appears that the immune activation of the complement system, with subsequent bone loss, is mediated by prostaglandin E.

Several studies have been undertaken to investigate the role of different prostaglandin types, their intermediaries, metabolites and precursors upon bone resorption.

Dietrich et al. (1975) have shown that prostaglandins E, F, A and B were able to increase the release of previously incorporated ^{45}Ca from foetal rat bone by 60 to 135 percent. Prostaglandins of the E series were 10 to 100 fold more potent than F, A and B prostaglandins.

Although the bone resorptive activity of PGE_2, and its presence in resorptive lesions of neoplastic or inflammatory organs, has been interpreted as a role for PGE_2 in mediating bone loss in these instances, Tashjian et al. (1977b) have suggested that intermediates in arachidonic acid metabolism or PGE_2 metabolites may have sufficient bone resorbing stimulating activity to be the agent acting on bone cells. However, results from investigations by the authors have shown endoperoxide analogues had very little bone resorption stimulating activity, whilst two PGE_2 metabolites, 13,14-dihydro PGE_2 and 13,14-dihydro-15-keto PGE_2 were only 6.60% and 0.20% respectively as active as PGE_2. However, in a similar investigation, Raisz et al. (1977) have recorded markedly different results. The prostaglandin endoperoxides (PGG_2 and PGH_2) caused a rapid transient increase in ^{45}Ca release, although this release was only short lived. The differ-

ence between these findings and those of Tashjian et al. (1977b) may be attributed to the latter group's use of PGH_2 and PGG_2 analogues, compared with the use of the parent molecules by Raisz et al. (1977). Similar findings for bone resorption were reported by both groups in relation to one $PGF_{2\alpha}$ analogue, 13,14-dihydro-15-keto PGE_2, but a disparity in results occurred in relation to the other PGE_2 analogue, 13,14-dihydro PGE_2. The reason for this is unclear.

Alterations in levels of prostaglandin synthesis precursors have been investigated by Rabadjija and Goldhaber (1974). Increased levels of arachidonic acid, linoleic acid and gamma linoleic acid stimulated bone resorption in cultured mouse calvaria, as did additions of phospholipase A, the initiating enzyme responsible for the cleaving of arachidonic acid from the lipid molecule.

Recent evidence tends to suggest that the latest prostaglandins to be discovered, PGI_2 and TXA_2, may also be involved in bone resorption.

Following the demonstration of elevated levels of 6-keto $PGF_{1\alpha}$, the stable end product of PGI_2, and the reduction of 6-keto $PGF_{1\alpha}$ levels when the serum was inactivated or contained indomethacin, Raisz et al. (1979) have investigated the effects of PGI_2 on bone resorption. Repeated additions of PGI_2 stimulated the release of ^{45}Ca, as did the more stable sulfur analogue of PGI_2, thiaprostacyclin. Since PGI_2 has been shown to be synthesized by blood vessel walls (Bunting et al. 1976), Raisz et al. (1979) have suggested that PGI_2 release may be responsible for the frequently observed association between vascular

invasion and resorption of bone in physiologic remodelling and pathologic osteolysis.

Thromboxane A_2 has been demonstrated by Raisz et al. (1979) to be present in foetal rat bones, although Tashjian et al. (1977b) have shown it is a relatively impotent stimulator of bone resorption in relation to PGE_2. However, Heersche and Jez (1981) have contended that thromboxane A_2 may have a regulatory role in bone remodelling. This contention was based upon the inhibition of calcium release or uptake in mice calvaria in vitro following additions of thromboxane synthesis inhibitors imidazole, 1-methyl-imidazole and benzimidazole.

E. The role of prostaglandins in collagen remodelling

As collagen is the predominant protein in both hard and soft connective tissues (Bornstein and Traub, 1979), a significant role for prostaglandins during connective tissue remodelling would be reflected in a direct relationship with changes in collagen synthesis or degradation. Unfortunately, little is known about the ability of prostaglandins to alter collagen metabolism.

Morphological studies by Kischer (1967) have revealed increased deposition of collagen or collagen related elements in organ cultures of chick embryo skin and developing down feather organs which were supported by a medium rich in PGE_1 and PGB_1. Similarly, Arora et al. (1970) have found fibrous tissue formation around implanted cotton

pellets impregnated with PGE_1 led to increased connective tissue granuloma.

The first study pertaining specifically to collagen synthesis and prostaglandins was reported by Blumenkrantz and Sondergaard (1972). Utilizing 10 day old chick embryo tibiae *in vitro*, total protein synthesis was measured by (^{14}C)-proline and (^{14}C)-lysine uptake, and collagen synthesis was demonstrated by studies of (^{14}C)-hydroxyproline and (^{14}C)-hydroxylysine formation. The authors concluded that PGE_1 and $PGF_{1\alpha}$ increase collagen synthesis.

However, subsequent studies by Goldhaber *et al.* (1973) and Raisz and Koolemans-Beynen (1974) have recorded inhibitory effects of prostaglandins upon collagen synthesis. Goldhaber *et al.* (1973) have reported the inhibitory effects of PGE_2 on the hydroxylation of ^{3}H-proline in mouse calvaria, whilst Raisz and Koolemans-Beynen (1974) have used the same indicator of collagen synthesis in foetal rat calvaria to corroborate this finding. Furthermore, the latter authors have found that PGE_1 also had an inhibitory effect upon collagen sythesis, although it was not as marked as the PGE_2 induced response.

This disparity in findings between the original Blumenkrantz and Sondergaard (1972) study and the more recent investigations of Goldhaber *et al.* (1973) and Raisz and Koolemans-Beynen (1974) does tend to mirror subsequent reports (see following sections) which have appeared in the literature. This may be attributable to the different culture systems or types and concentrations of prostaglandins used.

1. Stimulation of collagen synthesis

Stimulatory effects of prostaglandins upon collagen synthesis have been reported by several authors. Eisenbath et al. (1974) have examined the effects of PGE_1, $PGF_{1\alpha}$ and PGA_1 on total protein synthesis, as measured by ^{3}H-leucine incorporation into synthesized protein of pelvic cartilages of chick embryos in vitro. PGA_1 was found to cause a decrease in protein synthesis, but no changes were reported for PGE_1 and $PGF_{1\alpha}$.

Orthodontic tooth movement has been likened to an inflammatory wound healing response (Storey, 1973). Incisional wounds in rats have been utilized by Lupulescu (1975, 1977) to investigate the effects of PGE_1, PGE_2 and $PGF_{2\alpha}$ on collagen synthesis. Measuring ^{3}H-leucine and ^{3}H-proline incorporation, both total protein and collagen synthesis were significantly stimulated at 1 hour and 24 hours when PGE_1 and PGE_2 were employed. The reverse occurred with $PGF_{2\alpha}$, resulting in a marked decrease in synthetic activity at 1 hour, followed by moderate increases at 5 hrs and 24 hrs.

Ziboh and Hsia (1972) have shown that PGE_2 applied topically to cutaneous lesions induced in rats by an essential fatty acid deficient diet will clear the lesions. Reiser (1950) has shown that the essential fatty acids linoleic acid and gamma-linoleic acid undergo transformations in the animal to give arachidonic acid, and thus PGE_2 may mediate this repair by stimulating collagen synthesis.

2. Inhibition of collagen synthesis

Inhibitory effects of prostaglandins upon collagen synthesis have appeared more frequently in the recent literature.

The possible involvement of prostaglandins in collagenase induced osteolysis has been reported by Dowsett *et al.* (1976). These authors have shown that additions of collagenase to neonatal mouse calvaria *in vitro* increase the release of Ca^{++}, and that this release is inhibited by aspirin or indomethacin. Similar findings of an inhibitory effect of these compounds on bacterial collagenase activity upon achilles tendon collagen have been reported by Brown and Pollock (1970), thus suggesting a direct relationship between collagenase and prostaglandin activity.

Rats deficient in dietary intake of endogenous prostaglandin precursors have been demonstrated by Parnham *et al.* (1977) to exhibit a marked increase in collagen synthesis in granulomata induced by carrageenen impregnated sponges. Such an effect was not observed when non-carrageenen sponges were used. In view of the previously published findings that sponge impregnated with carrageenen produced large amounts of PGE (Higgs *et al.*, 1976), and that normal rats treated with PGE_2 have inhibited granulomata formation, it has been suggested that essential fatty acid deficient treated rats with granulomata produced by carrageenen have an increased collagen production due to a decreased PGE production.

Ohuchi et al. (1977) have examined the effects of $PGF_{2\alpha}$ on collagen synthesis in carrageenen granulomata in rats. The authors have reported inhibition of collagenous and non-collagenous protein synthesis. In a more recent study (Sato et al., 1980) a similar inhibitory effect has been reported, although this inhibitory effect was only short lived. This short period of activity of $PGF_{2\alpha}$, which was less than 3 hours, was found to be a result of desensitization of granulomatous tissue to $PGF_{2\alpha}$, as demonstrated by minimal metabolism of $PGF_{2\alpha}$ in pouch fluid and by the inability of subsequent injections of $PGF_{2\alpha}$ to alter collagen synthesis.

Ducomb et al. (1978) have reported an inhibitory effect of PGE_2 on collagen synthesis in cultured human embryo lung fibroblasts. However, as non-collagenous protein synthesis was also inhibited, the authors have concluded that the action of PGE_2 on collagen synthesis probably reflects its inhibition of total cellular protein synthesis.

Deshmukh and Sawyer (1977) have investigated the role of prostaglandins in phenotypic collagen synthetic expression. In their chondrocyte culture system, PGE_2 and $PGF_{2\alpha}$ had little effect upon phenotypic expression. However, these authors have reported a 20-30% reduction in the amount of type II collagen being synthesized. Rennard et al. (1981) have reported that PGE_1 will cause a shift in collagen phenotypic synthetic expression in a system in vitro involving human embryo lung fibroblasts. Coupled with an overall inhibition of collagen sythesis, the administered PGE_1 caused the type I/type III

ratio to decrease by approximately half.

Thus although there appears to be some disagreement in the literature regarding the effects of prostaglandins upon collagen synthesis, the majority of the evidence tends to suggest an inhibitory effect. Some of the reported differences may be attributable to the different systems employed by the investigators, or they may be due to the types and concentrations of PG's used. Unfortunately, no one particular system has been employed to assess the effects of each of the different prostaglandins upon collagen metabolism.

F. Prostaglandins and mechanical force.

Prostaglandin synthesis by tissues subjected to mechanical stress has received limited attention. In 1973 Kloeck and Jung were able to show that cultured myometrium, obtained from hysterectomy specimens, produced increased levels of PGE and decreased levels of PGF when stretched.

Bone cells obtained from rat calvaria have been used by Harell et al., (1977), Binderman and Cox, (1977) and Somjen et al., (1980) to examine the effects of mechanical force application on prostaglandin synthesis. Plastic petri dishes plated with these cells were subjected to force delivered by orthodontic expansion screws which were attached to the base of the dishes.

Upon application of force, PGE_2 levels increased rapidly, reaching a maximum in 20 minutes. Concurrent increases in cAMP levels have also been observed, with additions of indomethacin blocking the cAMP response. Thus it appears that the production of cAMP is dependent upon the presence of PGE_2. Furthermore, PGE_2 added to non-stressed cells mimicked the effect of physical forces on the production of cAMP. The same physical forces also stimulated the incorporation of thymidine into DNA after 24 hours. This DNA synthetic effect was mimicked on the addition of PGE_2 and was blocked by pretreatment of the stretched cells with indomethacin.

Thus, it appears that there is some evidence relating the physical stretching of cells to prostaglandin synthesis.

G. Future Research Directions

Elucidation of cellular control mechanisms during orthodontic tooth movement will ultimately enhance the efficacy and stability of orthodontic treatment. A possible role for prostaglandins in this process has been implied by their involvement in bone resorption and collagen remodelling, as well as their production in response to the mechanical stretching of cells.

Future research should investigate the presence and fluctuations of levels of prostaglandins in the periodontal tissues in response to orthodontic forces, the effects of the various prostaglandins upon the

behaviour of the various cell populations of the periodontium and the possibility of using certain prostaglandins to stimulate the cellular responses occurring during periods of orthodontic stress, and thus provide a more efficient clinical treatment.

CHAPTER 2

STATEMENT OF THE PROBLEM

The involvement of prostaglandins in bone remodelling, collagen remodelling and the mechanical stretching of cells, tissue reactions analogous to those occurring during orthodontic tooth movement, was discussed in Chapter 1. It was decided to explore the possibility that prostaglandins may be involved in connective tissue remodelling during applications of orthodontic force. To accomplish this the following three major objectives were pursued.

First, because of the masking and modulating influences upon prostaglandin synthesis present in the in vivo situation, and because no static periodontal organ culture system capable of receiving orthodontic forces was available, a new in vitro periodontal explant system had to be developed.

Second, because collagen is the predominant protein in both hard and soft connective tissue, the newly developed culture system would be utilized to examine the effects of orthodontic forces upon collagen synthesis. This collagen data would serve as a baseline for monitoring the effects of prostaglandins on the periodontal tissues in future studies.

Third, as there was no information available on the possible role of prostaglandins in connective tissue remodelling during orthodontic tooth movement, the effects of mechanical stress upon prostaglandins synthesized by periodontal tissues in organ culture would be investigated.

CHAPTER 3

PERIODONTAL ORGAN CULTURE SYSTEM

INTRODUCTION

The development of an organ culture system which can maintain whole mature periodontium undergoing remodelling in response to mechanical stress will provide a model *in vitro* in which conditions comparable to the clinical situation can be imitated. By isolating the tissue *in vitro* one can eliminate those factors *in vivo* which would modulate and mask the presence of possible regulatory mechanisms, and thus facilitate the measurement of such mechanisms.

Previous attempts to culture periodontal tissues have involved either single tooth and surrounding alveolar bone explants from mouse (Melcher *et al.*, 1973; Turnbull and Melcher, 1974; Melcher and Turnbull, 1976), or three teeth and surrounding bone from mouse in a continuous flow system (Yen and Melcher, 1978). Both systems are of limited value for the investigation of orthodontic force effects, the former because of the difficulty of applying mechanical forces to only one tooth, the latter because of the prohibitive cost of any experimentation involving additions of radioactive materials to the large volumes of required medium. A further limiting factor involved in the above systems is the utilization of foetal calf serum in the culture medium. The possible interference of any of the unknown constituents of foetal calf serum with the normal metabolism of any chemical regulatory substance indicates the elimination of this ingredient from the culture medium.

Other culture systems which involve a fibrous joint similar to the periodontal ligament have been developed by Meikle et al. (1979) and Yen and Suga (1980). Meikle et al. (1979) have described the culture of coronal and sagittal cranial suture and adjacent bone from new-born rabbit. Viability was demonstrated by radioautography of ^{3}H-proline treated explants, and by ^{3}H-proline and ^{3}H-leucine incorporation into sutural protein and ^{3}H-proline incorporation into collagen. Mechanical forces of 30 gm magnitude were applied across these sutures by 0.010 in diameter coil springs, resulting in an increase in both protein and collagen synthesis.

Yen and Suga (1980) have developed an in vitro system which utilizes the cranial suture of adult mice. Radioautography of ^{3}H-proline labelled explants and the presence of collagenase-digestible radiolabelled proteins have demonstrated the viability and protein-synthetic capacity of this system.

The objective of this study was to develop a periodontal organ culture system which can be supported by a medium free of foetal calf serum and which would be suitable as a model system for orthodontic stressing of the periodontium in vitro.

METHODS AND MATERIALS

Force Application

Eight week old male white mice, bred in our facility, were treated with orthodontic forces for time periods of 0, 1, 3, 5, 7, 10 and 14 days. The forces were applied by separating springs (Fig. III-1), constructed of multistranded orthodontic wire (0.0175 in. diameter Tri-flex, Rocky Mountain Orthodontics, Denver, Colorado), and inserted into the interproximal area between the first and second mandibular molars of one side of a mouse. The opposite side was used as a control. For insertions of the springs *in vivo*, the mice were positioned on a holding and jaw opening device (Fig. III-2,3).

Two mice were treated for each time period: one for radioautographic evidence of protein synthetic activity and the other for radioautographic evidence of DNA synthetic activity.

Dissection and Culture System

The mice were killed by cervical dislocation and the two mandibles removed. Fine dissection of the first, second and third molar with the surrounding alveolar bone was performed in culture medium under a dissecting microscope. Separation of the explant tissue from the body of the mandible involved a first cut inferior to the apices of the teeth and a second vertical cut distal to the third molar (Fig. III-4). The gingival tissues were removed to maximize perfusion of nutrients. The average explant was 2 mm high and 4 mm long.

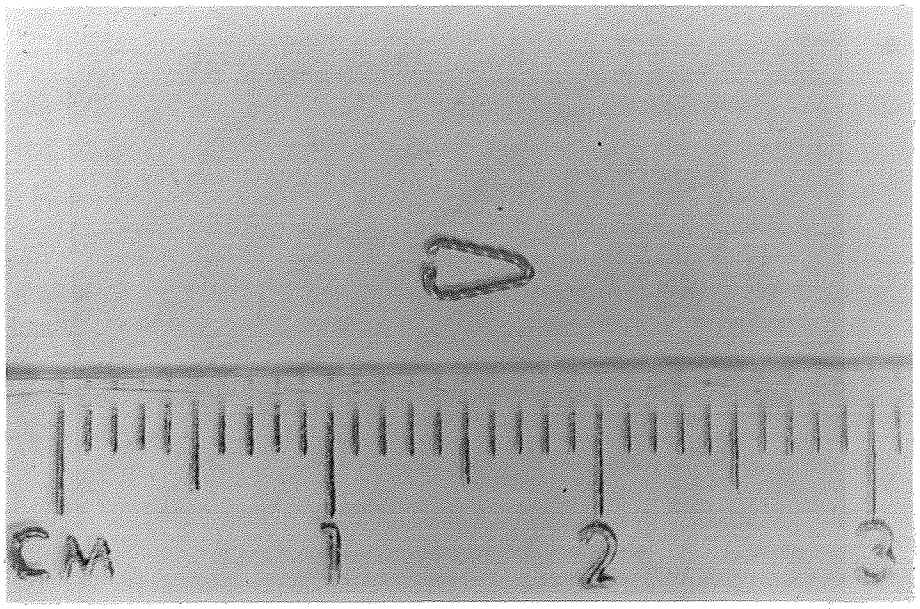


FIGURE III-1. Separating spring fabricated from 0.0175" multistranded orthodontic wire.

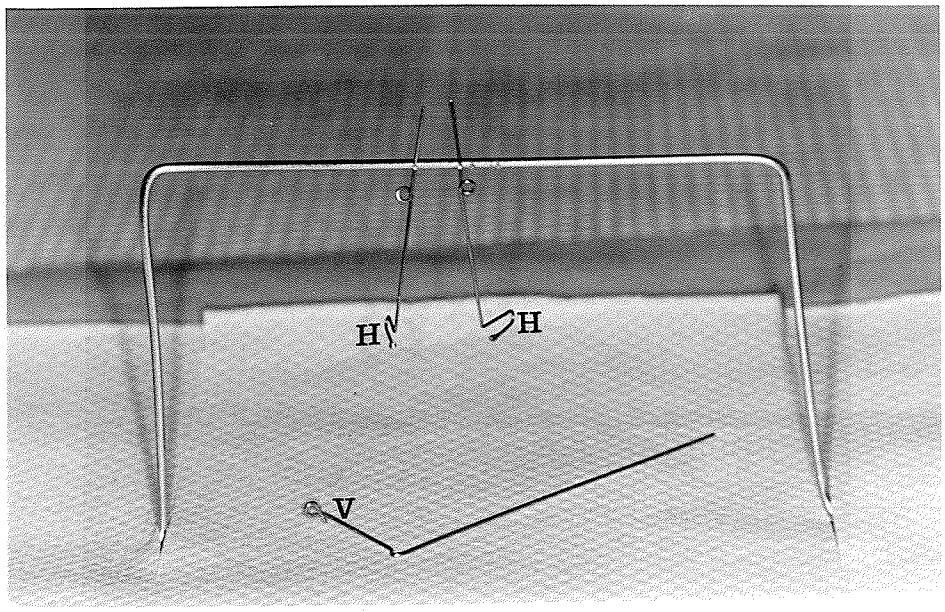


FIGURE III-2. Mouse holding and jaw opening device. Horizontal arms (H) were placed in buccal vestibule for lateral opening of oral cavity. The vertical arm (V) was used to prop the jaws apart.

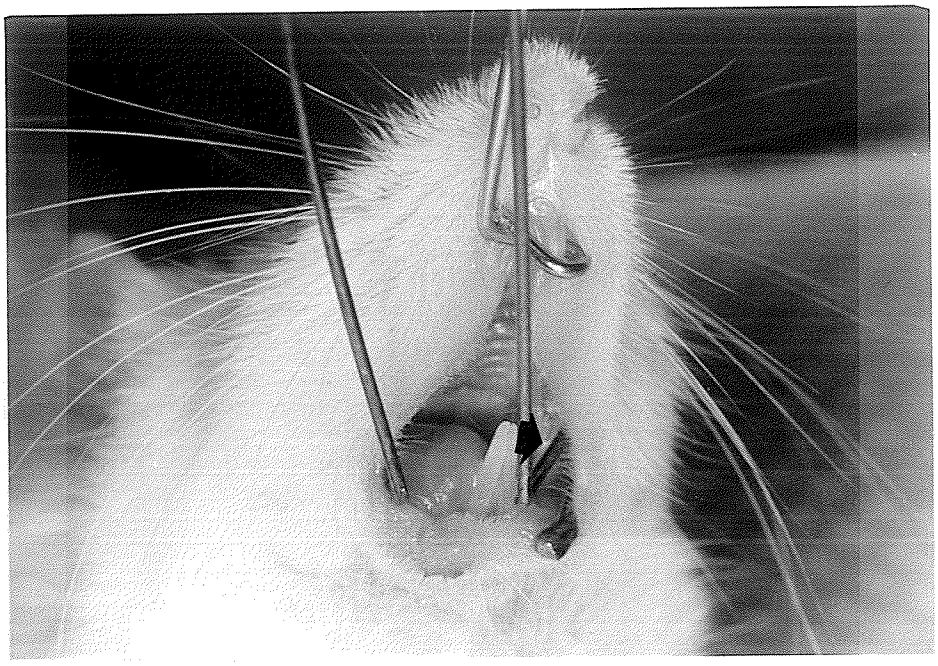


FIGURE III-3. Mouse positioned on holding and jaw opening device. Mandibular left first molar marked with arrow. Separating spring was placed interproximally between the first and second molar.

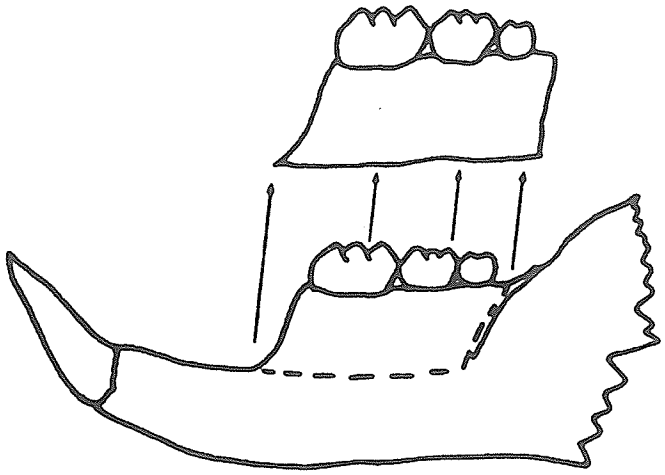


FIGURE III-4. Diagram of periodontal organ explant comprising three mandibular molars and their supporting alveolar tissue.

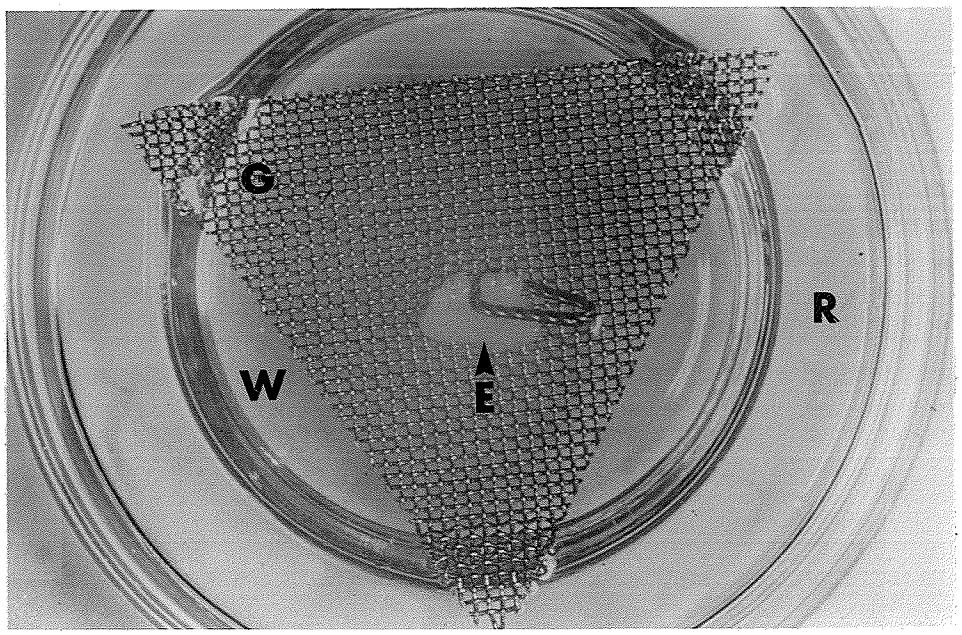


FIGURE III-5. Periodontal organ explant with separating spring *in vitro*. Note separation of first and second molar. (G = stainless steel grid; R = paper ring moistened with sterile distilled water; W = Waymouth's 752/1 culture medium; E = periodontal organ explant).

The explants were cultured in Trowell-type (Trowell, 1959) organ culture dishes (60 x 15 mm, Falcon #3037, Becton, Dickinson and Co., Cockeysville, Maryland) in which the tissue rested on stainless steel grids (Falcon #3014, Becton, Dickinson and Co., Oxnard, California) which were suspended over a centre well in the dish (Fig. III-4). Sufficient medium (800 ul) was introduced to reach the platform of each grid so that the explants stood at the gas-medium interface. The centre well was surrounded by an absorbent filter paper ring which was moistened with sterile distilled water. The dishes were covered and placed in an incubator maintained at 37^{0}C. Explants were cultured for 24 hours.

Medium, Gas, Isotope

The medium used was Waymouth's 752/1 (Grand Island Biological Co., Grand Island, New York) (Waymouth, 1959), supplemented with 300 ug/ml ascorbic acid (Fisher Scientific, Fair Lawn, New Jersey) and an antibiotic-antimycotic mixture (1.5 ml per 100 ml medium) consisting of 10,000 U per ml penicillin, 25 ugm per ml amphotericin B and 10,000 ugm per ml streptomycin (Grand Island Biological Co., Grand Island, New York).

The incubator was flushed continuously with a mixture of 95% O_2 and 5% O_2, humidified by bubbling through water.

Ten uCi per ml ^{3}H-proline (NET 323, L proline 2,3-^{3}H(N), New England Nuclear, Boston, Mass.) with a specific activity of 23.7 Ci

per mmol. was added to the medium of one experimental and one control culture dish per time period 4 hours prior to culture termination. One uCi per ml ^{3}H thymidine (NET-027Z, Thymidine (methyl-^{3}H), New England Nuclear, Boston, Mass.) with a specific activity of 84.8 Ci per mmol. was similarly added to the medium of a control and an experimental dish for each time period 4 hours prior to culture termination. In addition, both isotopes were added for similar time periods to two explants which were killed by freezing and thawing prior to culture, and thus were to serve as background controls.

Radioautography

At the end of the culture period, a control and experimental explant for each time was fixed in Bouin's solution for 48 hours and after extensive washing in 70% alcohol, demineralized for 14 days in 12% EDTA at pH 7.2. After embedding in paraffin, serial sections were cut at approximately 5 u intervals. Every third slide was stained with haemotoxylin and eosin. The intervening slides were prepared for radioautography by dipping in Kodak NTB-2 nuclear tracking emulsion (Eastman Kodak Co., Rochester, New York), stored in the dark at $4^{0}C$ for 2 weeks, subsequently developed in Dektol developer and fixer (Eastman Kodak Co.) and stained through the emulsion with haemotoxylin and eosin.

RESULTS

Visual observation of force stressed explants revealed separation of first and second molars equivalent to the diameter of the separating wire (0.5 mm) (Fig. III-5). Maximum separation occurred between day 3 and day 5 after force application.

On the basis of light microscopic observations, all control and experimental explants appeared to be viable. The periodontal ligament demonstrated normal cellularity when compared with sections taken from live animals (Fig. III-6,7,8). Cellular morphology also appeared normal, with occasional mitotic figures being observed within the periodontal ligament.

Radioautographs showed labelling throughout all ^{3}H-proline treated explants, with occasional evidence of ^{3}H-thymidine incorporation (Figs. III-9,10). Incorporation of ^{3}H-proline appeared to follow a gradient of intensity from peripheral to central regions (Fig. III-9). ^{3}H-proline labelling was extremely heavy in a large peripheral zone which included the cervical, apical and bifurcation regions of the periodontal ligament, peripheral alveolar bone and endosteal spaces (Fig. III-11,12,13,14). Although the more central regions showed a marked decrease in labelling, considerable label was still present (Fig. III-15).

Histological Legends

M = Molar

D = Dentine

B = Alveolar Bone

PL = Periodontal Ligament

H = Hyalinized Periodontal Ligament

P = Pulp

E = Endosteal Space

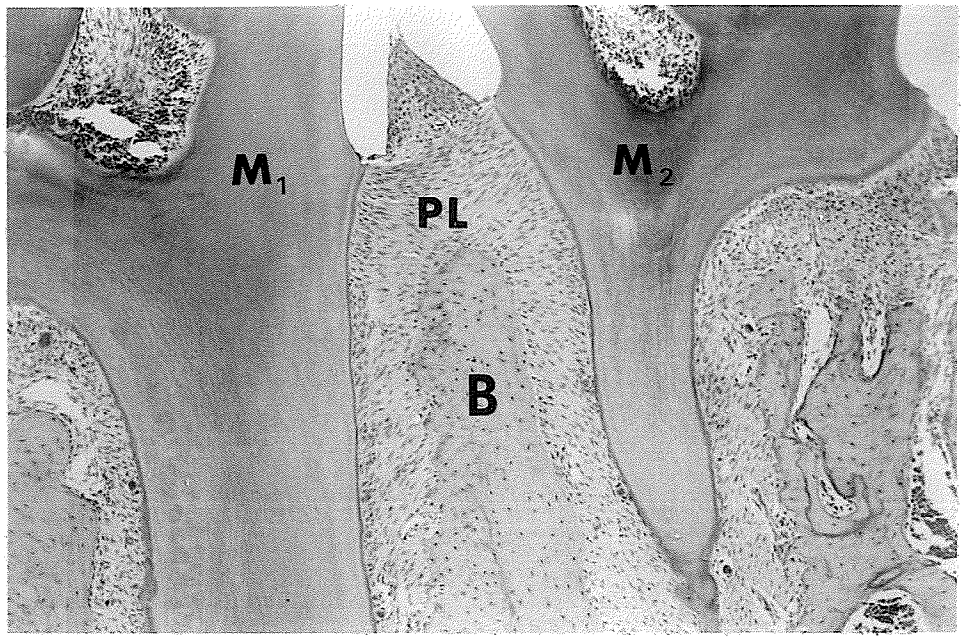


FIGURE III-6. Oblique longitudinal section of first and second mandibular molar after 24 hours <u>in vitro</u>. Haemotoxylin and eosin. X145

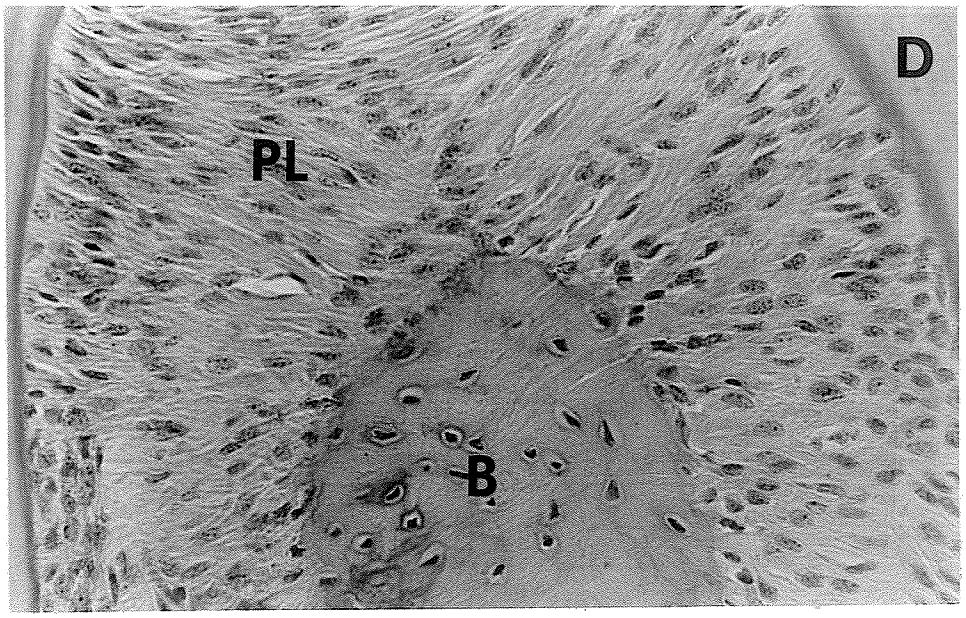


FIGURE III-7. Higher magnification of interseptal area in Fig. III-6. Note appearance of normal cellular morphology. Haemotoxylin and eosin. X600

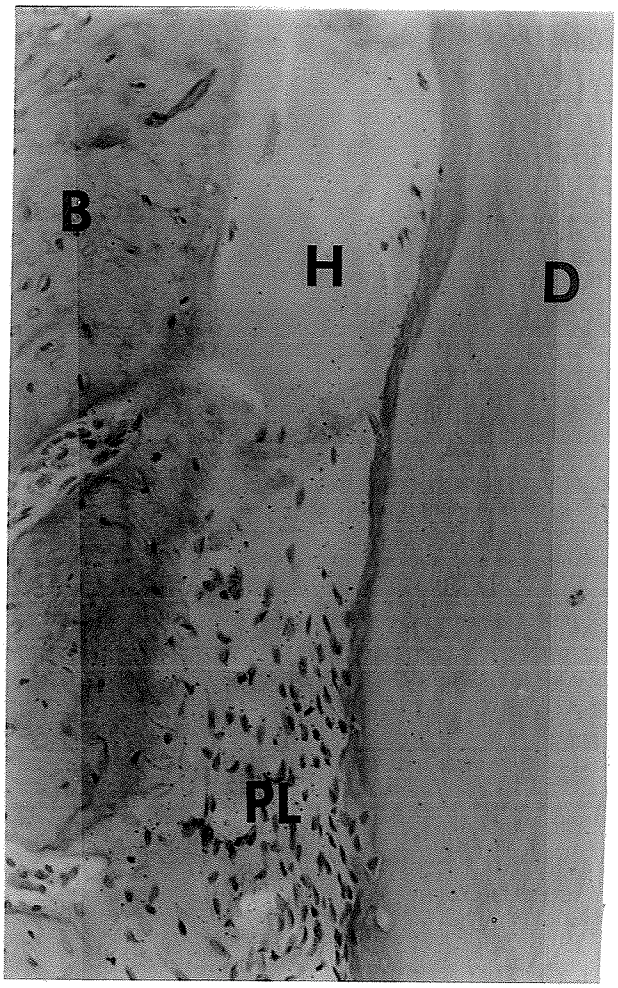


FIGURE III-8. Longitudinal section of cervical area of mesial root of mandibular molar following 3 days of force application and 24 hours in vitro. Note hyalinized periodontal ligament tissue (H). Haemotoxylin and eosin. X400

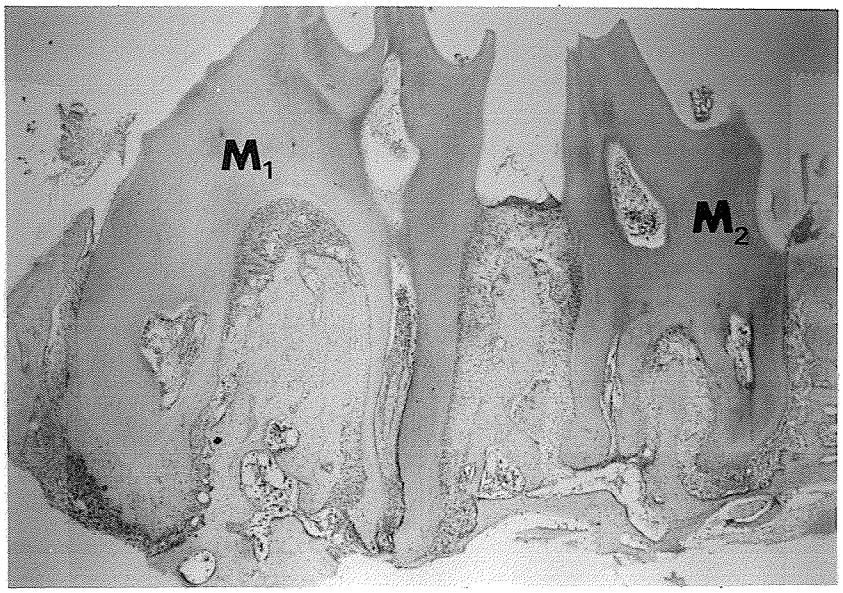


FIGURE III-9. Oblique longitudinal section of ^{3}H-proline labelled radioautograph of first and second mandibular molar after 24 hours <u>in vitro</u>. Note relative density of labelling in peripheral and central regions of the explant. Haemotoxylin and eosin. X60

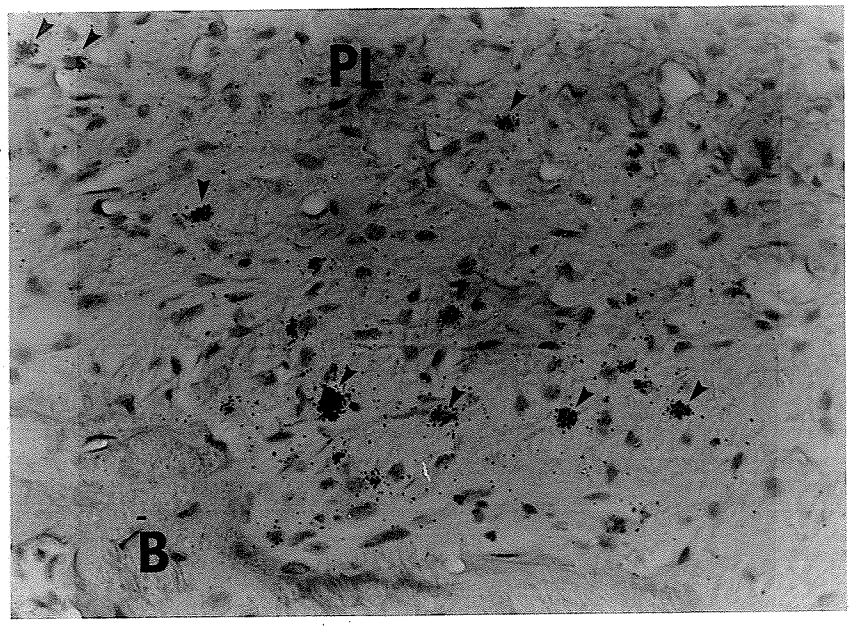


FIGURE III-10. Longitudinal section of ^{3}H-thymidine labelled radioautograph showing labelling of periodontal ligament cell. Haemotoxylin and eosin. X400

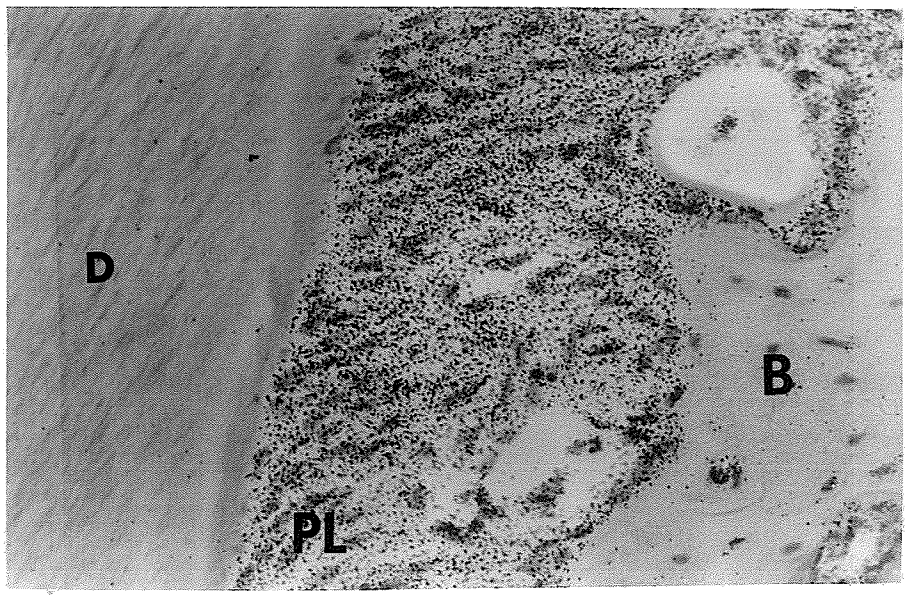


FIGURE III-11. Longitudinal section of cervical region of ^{3}H-proline labelled radioautograph of distal aspect of mandibular first molar after 24 hours in vitro. Note density of labelling. Haemotoxylin and eosin. X400

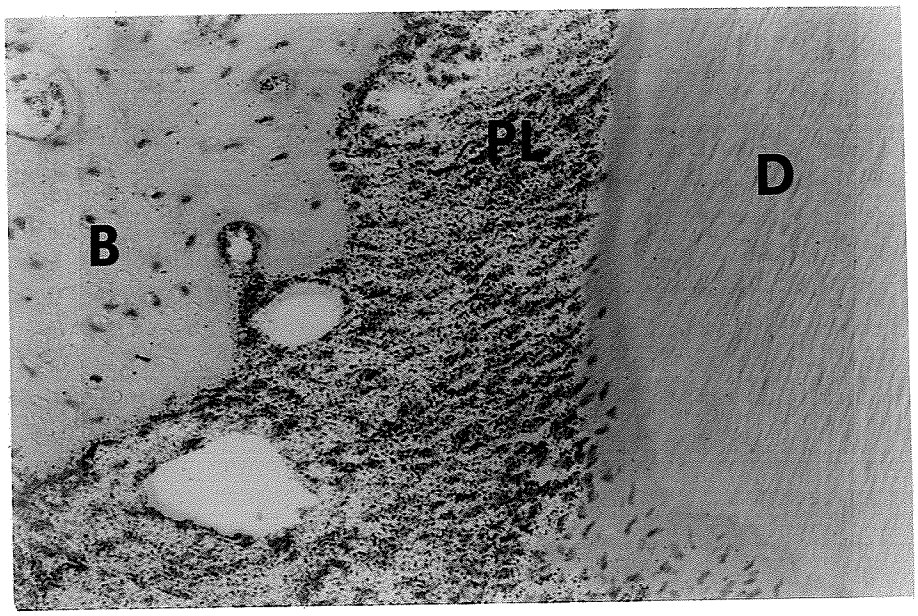


FIGURE III-12. Longitudinal section of apical region of ^{3}H-proline labelled radioautograph of mesial aspect of distal root of mandibular first molar after 24 hours in vitro. Note density of labelling. Haemotoxylin and eosin. X400

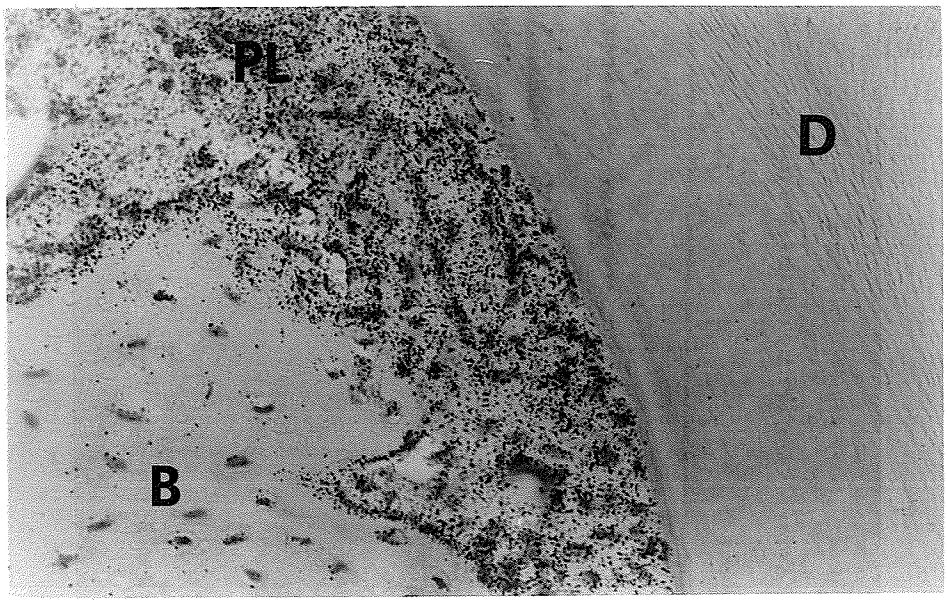


FIGURE III-13. Longitudinal section of bifurcation region of ^{3}H-proline labelled radioautograph of mandibular first molar after 24 hours *in vitro*. Note density of labelling. Haemotoxylin and eosin. X400

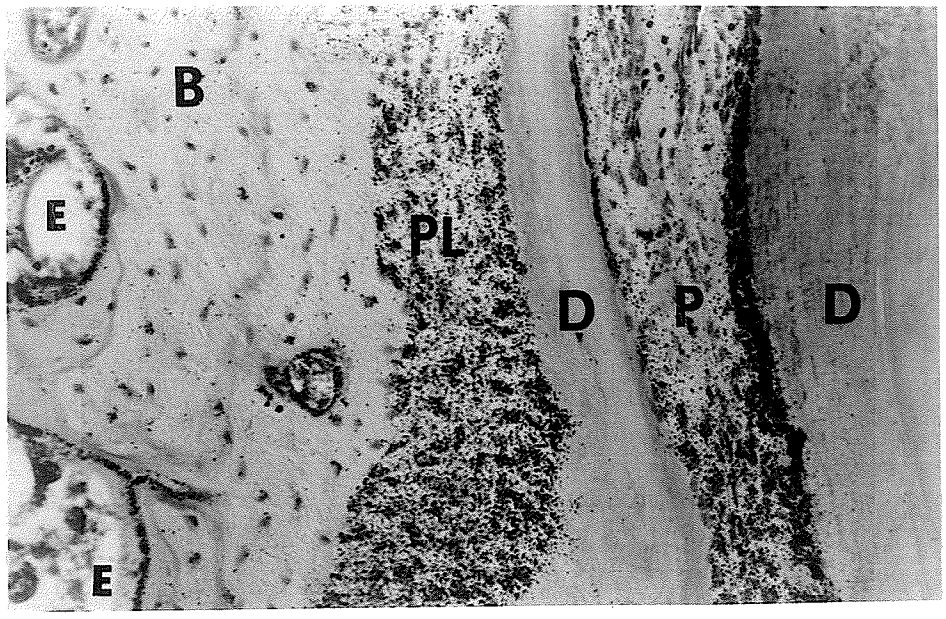


FIGURE III-14. Longitudinal section of apical region of distal root of ^{3}H-proline labelled radioautograph of mandibular first molar after 24 hours *in vitro*. Note labelling of endosteal space (E). Haemotoxylin and eosin. X400

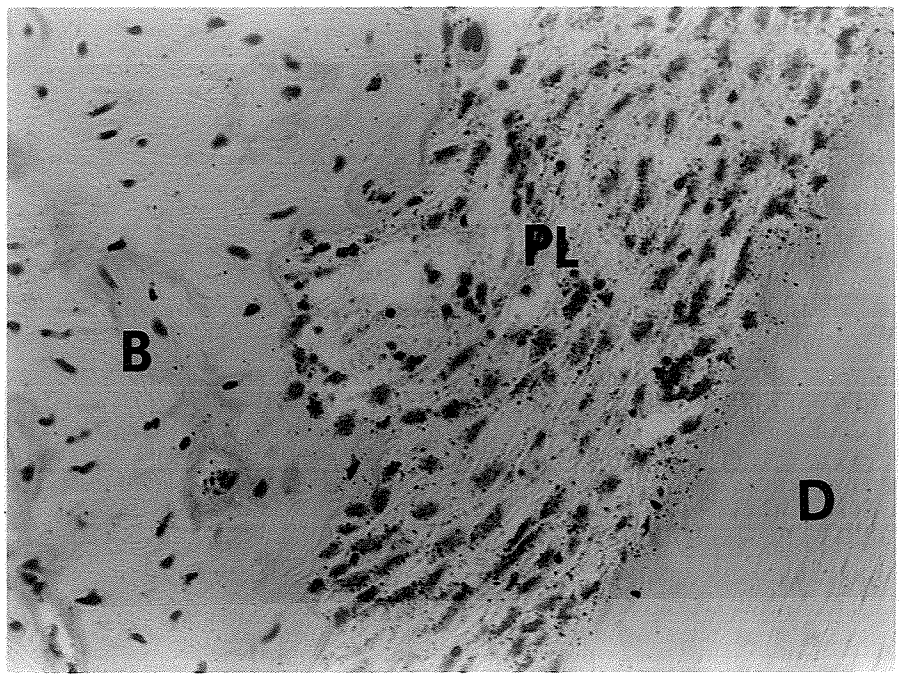


FIGURE III-15. Longitudinal section of middle third of distal root of ^{3}H-proline labelled radioautograph of mandibular first molar after 24 hours *in vitro*. Note reduction in labelling intensity in comparison to more peripheral regions of explant (see Figs. III-11,12,13,14). Haemotoxylin and eosin. X400

Although some differences in labelling did appear apparent between control and experimental explants, especially for the shorter periods of force application corresponding to 1,3 and 5 days, no attempt was made to quantify this difference (Fig. III-16,17).

Examination of radioautographs of the explants that were killed prior to culture showed no incorporation of ^{3}H-proline or ^{3}H-thymidine.

DISCUSSION

The incorporation of ^{3}H-proline and ^{3}H-thymidine into protein and DNA respectively provided a more objective assessment of cellular viability lending further support to the morphological observations. The labelling patterns indicated that connective tissue remodelling in this system would most likely result from activation of already present differentiated cells, as evidenced by the intensity of the ^{3}H-proline labelling response. However, the presence of occasional ^{3}H-thymidine labelled cells indicated that the replication of progenitor cells in this system does occur, although only to a limited degree. The finding that killed explants did not incorporate either label eliminated the possibility of non-specific binding of either substance.

Trowell (1959), MacDougall and Coupland (1967) and Yen et al. (1978) have cultured adult soft tissue organs and reported a similar

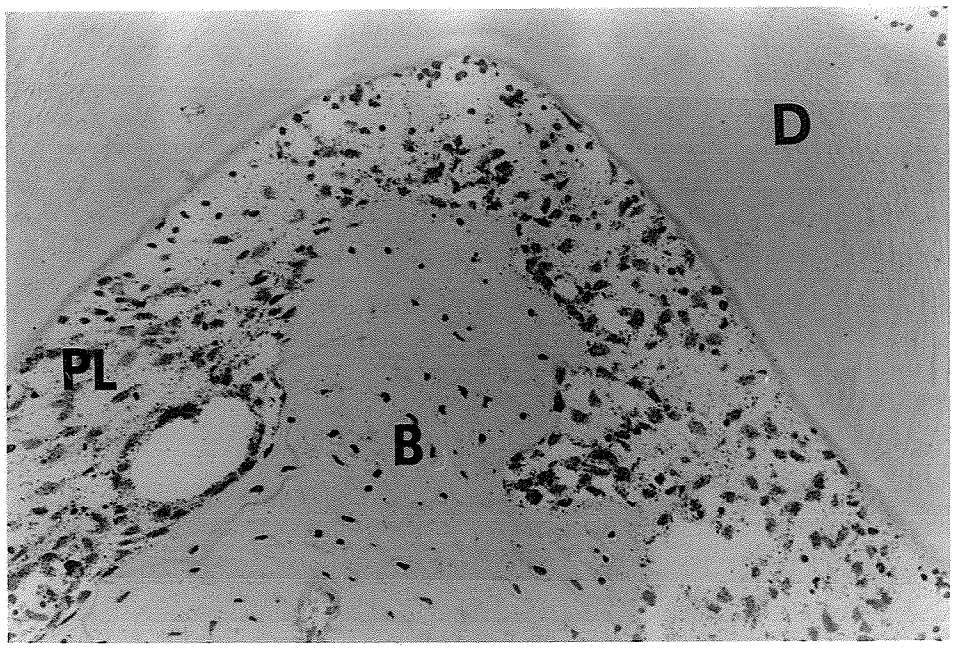


FIGURE III-16. Longitudinal section of bifurcation region of ^{3}H-proline labelled radioautograph of mandibular first molar after 24 hours *in vitro*. No force was applied. Compare with Fig. III-17. Haemotoxylin and eosin. X400

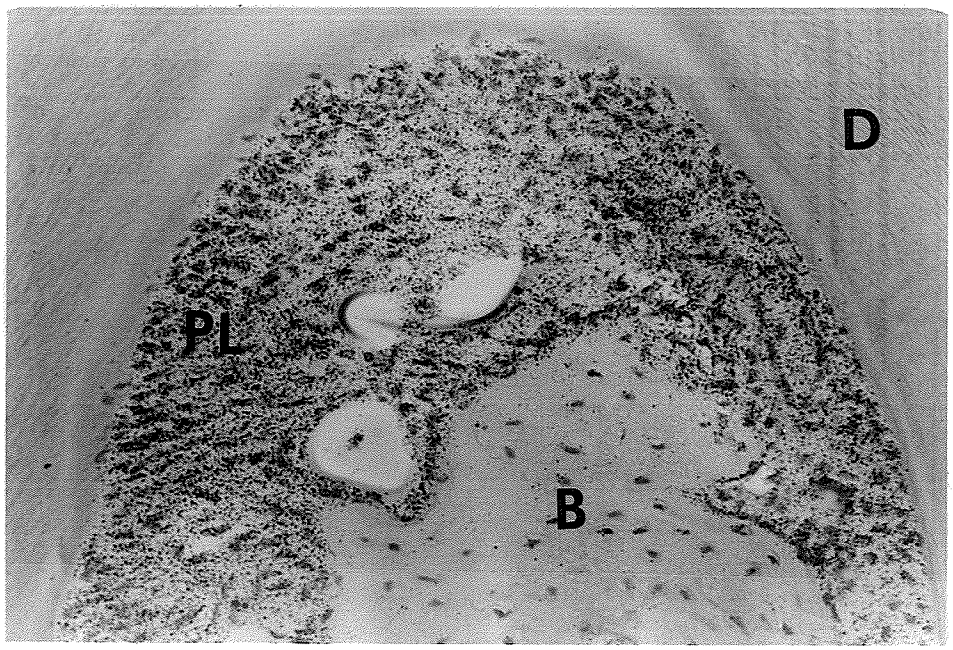


FIGURE III-17. Longitudinal section of bifurcation region of ^{3}H-proline labelled radioautograph of force treated (1 day) mandibular first molar after 24 hours *in vitro*. Haemotoxylin and eosin. X400

gradient in labelling. Trowell (1959) and MacDougall and Coupland (1967) have demonstrated a survival zone located at the periphery of their explants and a central area of necrosis. Yen et al. (1978) have recorded a gradient in ^{3}H-proline labelling intensity in 3 molar explants similar to that reported in this paper.

The relationship of cell survival in the centre of an explant to the diffusion of O_2 has been discussed by each of the above mentioned studies. Trowell (1959) and MacDougall and Coupland (1967) have suggested that diffusion of O_2 is directly related to the size of the explant and the pO_2 of the environment. Similarly, Yen and Melcher (1978) have demonstrated that a widening in a peripheral zone of protein synthesis in three molar tooth explants is related to increases in pO_2 in the culture medium. The authors have suggested that this reflects either a diminishing pattern of oxygen diffusion from peripheral to central regions of the explant, or utilization of oxygen by the more peripheral cells at the expense of more central cells, or both. This agrees with observations by Smith and Han (1968) that the incorporation of ^{3}H-proline by periodontal tissues in vivo is suppressed by anoxia.

The subjective observation of heavier ^{3}H-proline labelling in force stressed explants suggests an increase in protein synthesis in these tissues. This agrees with observations in stressed rabbit cranial suture in vitro (Meikle et al., 1979) and with in vivo ^{3}H-proline radioautography studies in rats (Crumley, 1964;Diaz, 1978) that

labelling may be interpreted as a general increase in cellular activity occurring during active tissue remodelling.

CONCLUSIONS

A three molar periodontal organ culture technique which requires small volumes of supportive medium (800 ul) and does not require foetal calf serum has been described. The viability of this system has been demonstrated by the radioautographic labelling of protein and DNA by ^{3}H-proline and ^{3}H-thymidine respectively. The ability of the newly designed force system to apply orthodontic type forces has been shown by the observation of tooth separation equivalent to the diameter of the separating wire. Initial qualitative assessments have suggested that protein synthesis is markedly greater in explants which have undergone force application for periods of 1,3 and 5 days in comparison to non-stressed control explants.

CHAPTER 4

COLLAGEN SYNTHESIS IN ORTHODONTICALLY STRESSED MOUSE PERIODONTAL TISSUES IN VITRO

INTRODUCTION

The establishment of a culture technique which will allow periodontal organ explants to receive orthodontic-type forces has introduced the possibility of investigating mechanisms regulating periodontal remodelling during tooth movement. Because collagen is the predominant protein in both hard and soft connective tissues (Bornstein and Traub, 1979), it represents the parameter of choice to examine the effects of possible cellular regulators upon connective tissue remodelling.

Within the periodontal ligament there are three main collagen chains, the α_1 and α_2 chains of type I collagen and the α_1 chains of type III collagen (Butler et al., 1975; Limeback et al., 1978; Sodek and Limeback, 1979). In addition to this, current research has indicated the presence of A and B collagen chains which are of a new type V collagen (Burgeson et al., 1976; Chung et al., 1976; Limeback et al., 1978; Brown et al., 1978; Sodek and Limeback, 1979; Sage and Bornstein, 1979) and an α_1 from type I trimer (Limeback et al., 1978).

Type III collagen was originally found in embryonic tissue (Chung and Miller, 1979) and has since been found in a variety of soft connective tissues, including the periodontal ligament (Butler, 1975; Sodek, 1976, 1977, 1978). The possible role of type III collagen in actively growing and remodelling tissues has still to be elucidated.

The effects of mechanical stress upon collagen synthesis in the periodontal ligament are poorly understood. Although some studies have reported radioautographic data on orthodontically stressed teeth (Crumley, 1965; Koumas and Matthews, 1969; Baumrind and Buck, 1970; Diaz, 1978), no investigations of a biochemical nature relating phenotypic expression of collagen synthesis to orthodontic force application have been reported.

Similarly investigations of orthopaedic forces applied to sutures has been principally radioautographic (Murray, 1971). However, Meikle et al. (1979) has shown, in a biochemical study, that ^{3}H-proline incorporation increases two-fold in force stressed cranial sutures of neo-natal rabbit in vitro. However, there was no significant change in the proportion of collagen to total protein synthesized between stressed and non-stressed sutures. Yen et al. (1980) have demonstrated that the phenotypic expression of collagen synthesis in mature mouse cranial suture stressed in vivo is altered in response to orthopaedic force, with the proportion of type III to type I collagen increasing during the initial periods of remodelling.

The objective of this study was two-fold. Firstly to demonstrate the vitality of the previously reported periodontal organ culture system by examining collagen synthesis within the periodontal ligament of the explant. Secondly to examine the effect of orthodontic forces upon collagen synthesis within the periodontal ligament of explants mechanically stressed for periods from 0 days to 14 days.

MATERIALS AND METHODS

The periodontal organ culture technique employed and the method of orthodontic force application have also been described in the previous chapter.

Twenty-seven 8 week old male white mice, bred in our facility, underwent force applications for periods of 0, 4, 16, 24 hours, 3, 5, 7, 10, 14 days. The springs were applied *in vitro* for times corresponding to 24 hours or less of force application and *in vivo* for those times greater than 24 hours. Separating springs were applied between the first and second molars of a randomly selected side of three mice for each time period, with the contralateral side of each mouse serving as a control. The mice were killed by cervical dislocation and the two mandibles removed. Fine dissection of the first, second and third molar with surrounding alveolar bone was performed in culture medium under a dissecting microscope. Separation of the explant tissue from the body of the mandible involved a first cut inferior to the apices of the teeth and a second vertical cut distal to the third molar. The average explant was 2 mm high and 4 mm long.

The explants were cultured in Trowell type (Trowell, 1959) organ culture dishes (60 x 15 mm, Falcon #3037, Becton, Dickinson and Co., Cockeysville, Maryland) in which the tissue rested on stainless steel grids (Falcon #3014, Becton, Dickinson and Co., Oxnard, California) which were suspended over a centre well in the dish. Sufficient

medium (800 ul) was introduced to reach the platform of each grid, so that explants stood at the gas-medium interface. The centre well was surrounded by an absorbent filter paper ring which was moistened with sterile distilled water. The dishes were covered and placed in an incubator maintained at 37^{0}C. Explants were cultured for 24 hours.

The medium used was Waymouth's 752/1 (Grand Island Biological Co., Grand Island, New York) (Waymouth, 1959) supplemented with 300 ugm per ml ascorbic acid (Fisher Scientific, Fairlawn, New Jersey) and an antiobiotic-antimycotic mixture (1.5 ml per 100 ml medium) consisting of 10,000 U per ml penicillin, 25 ugm per ml amphotericin B and 10,000 ugm per ml streptomyocin (Grand Island Biological Co., Grand Island, New York).

The incubator was flushed continuously with a mixture of 95% O_2 and 5% CO_2 humidified by bubbling through sterile distilled water.

20 uCi per ml (^{14}C)-glycine (CFA.30, (1-^{14}C)-glycine, Amersham Corporation, Oakville, Ontario) with a specific activity of 52.2 mCi/mmol was added to the medium of each culture dish 4 hours prior to culture termination.

At the end of the culture period the first, second and third molars of each explant were extracted from the alveolar bone and the teeth and bone were placed in separate 1.5 ml plastic centrifuge tubes which were subsequently frozen for storage.

Collagen Extraction

Following thawing, 1 ml of 50 mM Tris-HCl buffer (Fisher Scientific Co., Fair Lawn, N.J.), pH 7.4, containing 20 mM EDTA (Fisher Scientific Co., Fair Lawn, N.J.), 1 mM p-hydroxymercuribenzoate, 10 uM phenylmethylsulfonyl fluoride and 0.005% bovine serum albumin (Sigma Chemical Co., St. Louis, Missouri) was added to the centrifuge tubes. Extraction of neutral salt soluble collagen was carried out at 4^0C on a rotary platform (model TEK-TATDR V, TEKPRO, American Hospital Supply Corp., Evanston, Illinois) operated at 200 rpm over a 30 hour period. Newly synthesized collagen was extracted three times with 1 ml of the 0.45 sodium chloride solution, followed by a single wash with distilled water. After each extraction the tissues were centrifuged at 10,000 x g for 5 minutes. The more cross-linked forms of collagen remaining in the residue were extracted once with 1 ml of 0.5 N acetic acid for 4 hours followed by suspension in a second 1 ml of 0.5 N acetic acid. The most cross-linked collagen forms were extracted by the addition of 0.1 mg pepsin (2 x crystallized, Sigma Chemical Co., St. Louis, Missouri) with subsequent incubation for 16 hours at 4^0C. The residue was pelleted by centrifugation at 10,000 g and redigested for 12 hours more. The supernatants were freeze dried in preparation for gel electrophoresis.

The supernatants from the neutral salt extractions of each tissue were pooled and then dialized against the extracting buffer before equilibration with 1% acetic acid. The samples were removed from the

dialysis bags and 100 ul of each was placed in mini scintillation vials with 5 mls of scintillation cocktail (Aquasol, New England Nuclear, Boston, Mass.) and counted for ^{14}C in a liquid scintillation counter (Nuclear Chicago Isocap 300, Searle Instrumentation, Toronto, Canada). Undigested and pepsin digested samples were freeze dried in preparation for electrophoresis.

Collagen Separation and Quantification

Collagen α-chains and procollagens were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 20 cm slab gels. The procedure followed for the separation of those collagen components was described by Laemmli (1970) and utilized a 7.5% cross-linked separating gel, a 2.5% stacking gel and Tris/glycine buffers (Bio-Rad Laboratories, Richmond, California). Type I and type III collagen α-chains were separated using the interupted electrophoresis method of Sykes et al. (1976).

Freeze dried samples were dissolved in 70 ul of reservoir buffer containing 2 M urea, 2% sodium dodecyl sulphate and 0.1% bromophenol blue and heated at $60^{0}C$ for 30 minutes to denature the collagen. Electrophoresis was performed for 1 hour at 125 V. The samples were then reduced by the addition of 20% mercaptoethanol to the sample wells. After standing for 45 minutes, the electrophoresis was continued until the tracking dye reached the base of the gel.

For fluorographic visualization of separated radio-labelled colla-

gen bands, gels were washed twice in dimethyl sulfoxide (Fisher Scientific, Fair Lawn, New Jersey) and impregnated with 2,5-diphenyloxazole (New England Nuclear, Boston, Mass.) as described by Bonner and Laskey (1974). The gels were placed on filter paper and dried on a slab drier (Bio-Rad, Richmond, California) and exposed to Kodak XRP-1 x-ray film (Kodak Canada Inc., Toronto, Ontario) at -70^{0}C for 3 weeks.

Individual sample tracks were then scanned at 550 nm and proportions of type III collagen relative to type I were quantified on a spectrophotometer (Beckman DU-8, Toronto, Canada).

RESULTS

The nature of collagens synthesized by the periodontal tissues labelled in vitro for 4 hours during various periods of orthodontic force application is demonstrated in the following photofluorograph and densitometric scan (Fig. IV-1,2). Most of the observable radiolabelled collagen chains were extracted in the salt-soluble fraction, with unquantifiable amounts appearing rarely in the pepsin digested residue (Fig. IV-1). The majority of the collagen chains were extracted from the bone fragments. Inadequate collagen was yielded from the periodontal ligament attached to the teeth of one explant.

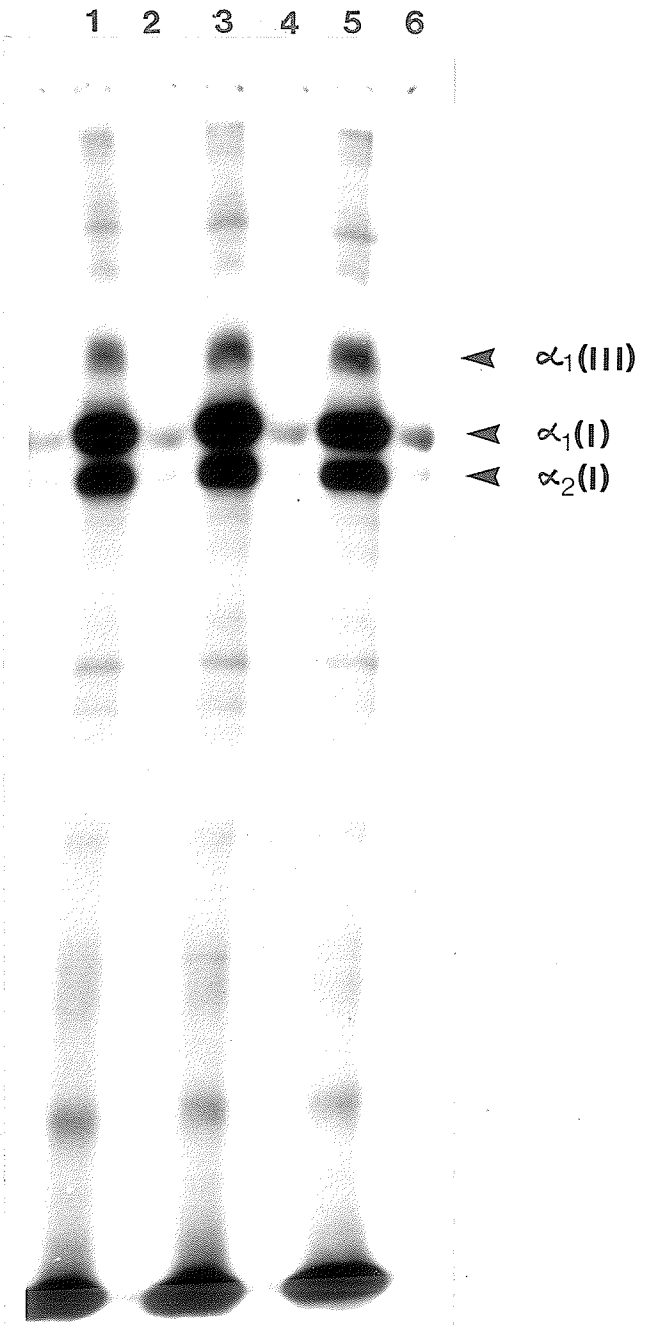
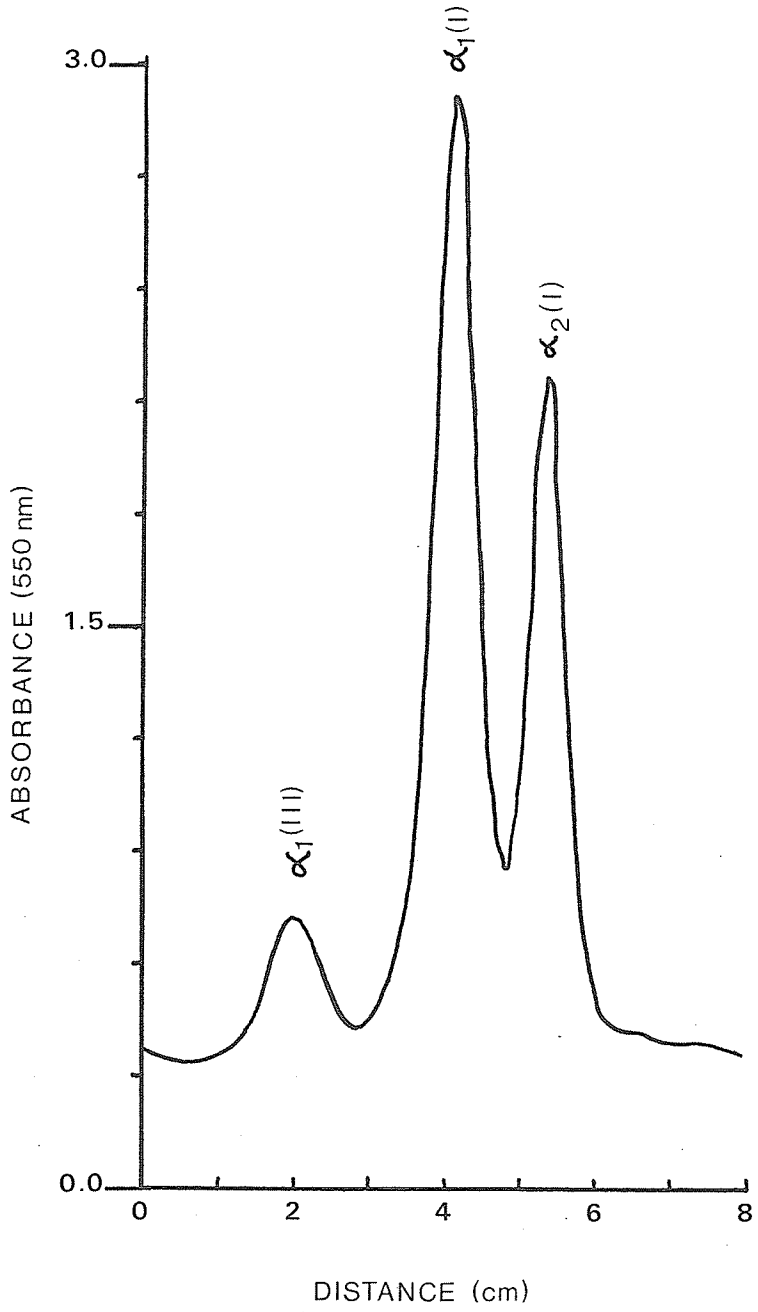


FIGURE IV - 1. Photofluorograph of radiolabelled proteins synthesized by the alveolar bone component of the periodontal ligament explant. Tracks 1, 3 and 5 contain salt soluble fraction; tracks 2, 4 and 6 contain pepsin digested fraction. The positions expected for collagen α chains are shown.

FIG. IV-2

DENSITOMETRIC TRACING OF REPRESENTATIVE FLUOROGRAPHIC BAND PATTERNS PRODUCED BY SALT SOLUBLE PROTEINS OF ALVEOLAR BONE TISSUE LABELLED 4 HOURS *IN VITRO* WITH ^{14}C-GLYCINE



This required the pooling of all the teeth from 3 explants for each time period for collgen extraction from periodontal ligament attached to extracted teeth.

Alpha $_1$(I) and α_2(I) chains were synthesized in relatively large amounts in all bone attached and tooth attached periodontal ligament samples during the 4 hour period of (^{14}C)-glycine labelling. Almost all salt extractions from bone samples produced quantifiable amounts of type III collagen, thus allowing for a comparison of the proportion of type III collagen synthesized in relation to the total type I and type III collagen produced. Table IV-1 summarizes the data from experimental and control bone samples for time periods of 0,4,16,24 hours;0,1,3,5,7,10 and 14 days. Procollagen chains were identified routinely on all bone and tooth photofluorographs.

The proportion of radioactivity associated with type III collagen for alveolar bone tissues was approximately 0-7% in control samples and 3-13% in experimental samples. There was no statistically significant difference between control and experimental situations in the relative amounts of type III synthesized when explants were force stressed for periods of 0,7,10 and 14 days. However, there was a significant difference in type III synthesis at time periods of 1 day ($p < 0.01$), 3 and 5 days ($p < 0.05$) (Fig. IV-3).

TABLE IV-1

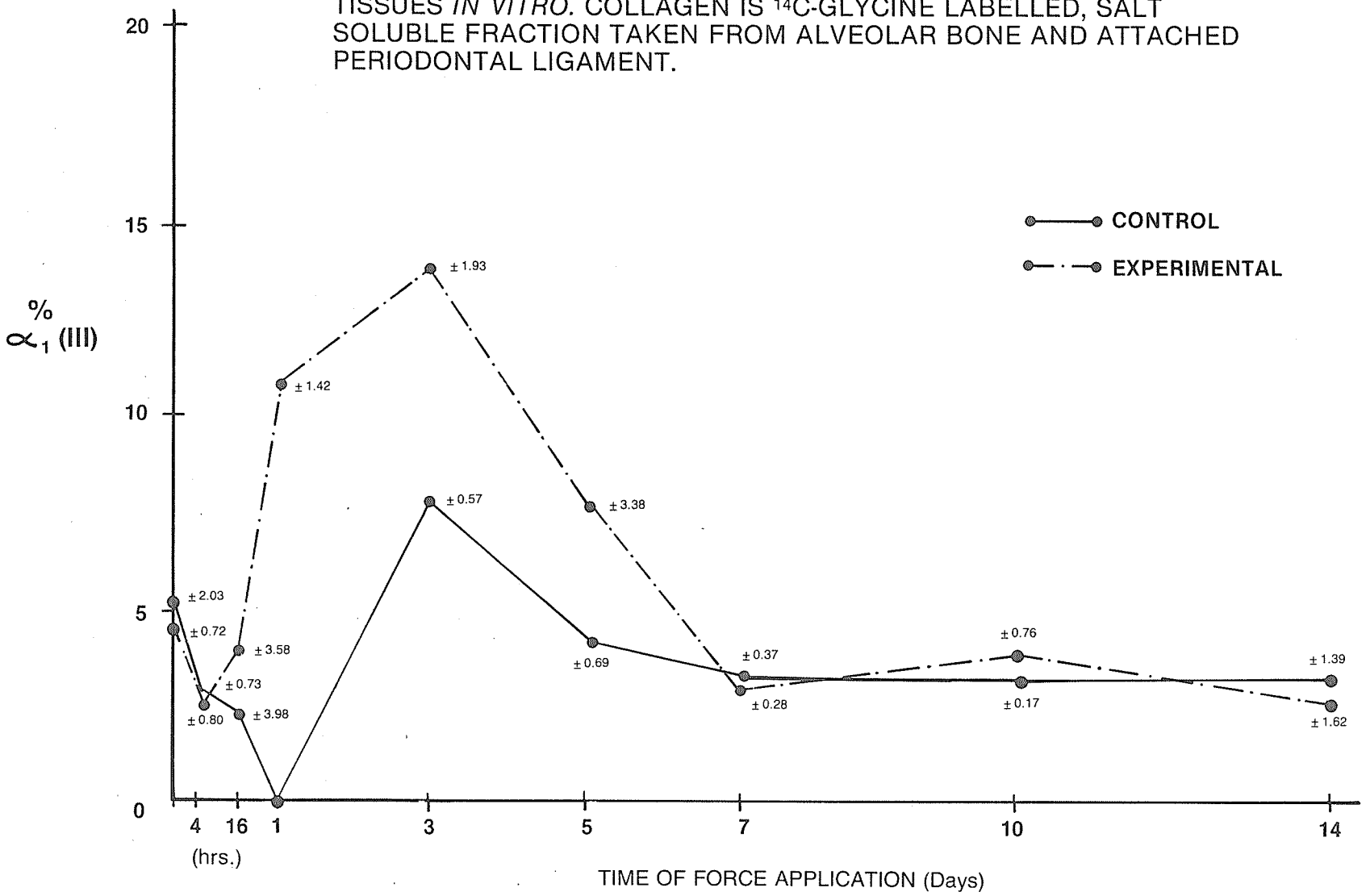
Means and standard deviations of proportions of salt soluble α_1 (III) collagen synthesized by the alveolar bone component of periodontal tissues *in vitro* after 4 hours of labelling with (^{14}C)-glycine.

Time of Force Application (hours)	Difference	Treatment	
		Experimental	Control
0	-0.56	4.73 ± 0.72	5.29 ± 2.03
4	-0.25	2.73 ± 0.80	2.98 ± 0.73
16	1.45	3.75 ± 3.58	2.30 ± 3.98
24 (days)	*10.73	10.73 ± 1.42	0 (unquantifiable)
3	* 6.46	13.88 ± 1.93	7.42 ± 0.57
5	* 3.55	7.25 ± 3.38	3.70 ± 0.69
7	-0.28	2.68 ± 0.28	2.96 ± 0.37
10	0.28	3.49 ± 0.76	3.21 ± 0.17
14	-0.39	2.57 ± 1.62	2.96 ± 1.39

* Denotes difference between control and experimental treatments significant at 0.01 level.

FIG. IV-3

PROPORTION OF α_1 (III) COLLAGEN SYNTHESIZED BY PERIODONTAL TISSUES *IN VITRO*. COLLAGEN IS ^{14}C-GLYCINE LABELLED, SALT SOLUBLE FRACTION TAKEN FROM ALVEOLAR BONE AND ATTACHED PERIODONTAL LIGAMENT.



DISCUSSION

Demonstration of type I and type III α chain synthesis in all explants added further support to the radioautographic evidence presented in the previous chapter that the explants maintained vitality over the 24 hours period of culture.

The lack of appearance of quantifiable collagens in the pepsin digested extract may reflect an insufficient time for enough cross-linking to occur during the 4 hour period of isotope exposure (Sodek, 1977) and it may also reflect an aberration of the *in vitro* system from the *in vivo* situation. The majority of collagens were identified from bone samples because of the greater amount of soft connective tissue associated with the periodontal ligament, its approximating periosteum, and endosteal spaces.

The ability of the explant tissue to synthesize quantifiable amounts of type I and III α chains from a 4 hour period of labelling, was expected in view of the previously reported high turnover rate of collagen within the periodontal ligament (Sodek, 1976, 1977). The relatively low proportion of type III collagen in all explants is in discord with the 15-20% reported in the periodontal ligament of rats *in vivo* (Sodek and Limeback, 1979; Limeback and Sodek, 1979) and cattle (Butler, 1975), but in agreement with the approximate 5% figure reported by Yen (1978) in a small sample size study of mouse periodontal ligament and alveolar bone *in vivo* which utilized ^{14}C-glycine

labelling over a 4 hour period. The low type III rate in both this study and that of Yen (1978) may reflect the longer time required for maturation of type III procollagens, as reported by Sodek and Limeback (1979) and Limeback and Sodek (1979). When the labelling period was increased from 4 hours to 24 hours *in vitro*, the type III ratio increased to 15% (Yen, 1978).

The significance of type III collagen in connective tissues is not clear at this time. Type III collagen has been found to be present in foetal skin and uterine wall (Chung and Miller, 1974); associated with vascularization in the early phases of wound healing (Gay *et al.*, 1978); synthesized by synovial cells from patients with rheumatoid arthritis (Eyre and Muir, 1975) and associated with inflammation (Weiss *et al.*, 1975). It appears that type III collagen serves an important role in rapid remodelling situations, such as wound healing, as well as normal growth and development of connective tissue. Moreover, in view of its presence in tissues such as aorta, uterine wall and periodontal ligament (Chung and Miller, 1974; Butler *et al.*, 1975) type III collagen may have a stress bearing role. Such a concept has received additional support by the findings of Chiang (1981), indicating that type III collagen synthesis increases when the interparietal suture of mice are mechanically stressed.

The role of type III collagen in tissues undergoing active stress and remodelling is further supported by the statistically significant increases in the type III ratio occurring at 1,3 and 5 day periods of

mechanical force application apparent in this study. This period of time may correspond to the period of activity of the separating spring, although macroscopic observation indicated that the spring gained maximum separation of the first and second molars by the 3 day period.

CONCLUSIONS

Synthesis of type I and type III collagen ∝ chains over the last 4 hours of the culture period added further evidence that the periodontal organ explant remained vital during the 24 hours of culture. Significant increases in the ratio of type III to type I collagen synthesized during periods demonstrating the maximum tooth movement and correlating with maximum protein synthetic activity, supports the concept of the role of type III collagen in actively growing and remodelling tissues.

CHAPTER 5

PROSTAGLANDIN SYNTHESIS IN ORTHODONTICALLY STRESSED MOUSE PERIODONTAL TISSUES IN VITRO

INTRODUCTION

Remodelling of hard and soft connective tissues is an essential process of orthodontic tooth movement. The cellular control mechanisms for these remodelling functions are largely unknown, although several potential factors have been proposed, including pressure and tension changes (Sandstedt, 1904, 1905a, 1905b), oxygen tension changes (Bien, 1966), piezo-electric stimuli (Zengo et al., 1973, 1974), cyclic nucleotides (Davidovitch et al., 1975) and prostaglandins (Yamasaki et al., 1980).

There is considerable evidence to support a role for prostaglandins in orthodontic tooth movement. Involvement of prostaglandins in bone resorption (Klein and Raisz, 1970) collagen metabolism (Kischer, 1967;Arora et al., 1970;Blumenkrantz and Sondergaard, 1972;Goldhaber et al., 1973;Raisz and Koolemans-Beynen, 1974) and mechanical stretching of cells (Harell et al., 1977;Binderman and Cox, 1977;Somjen et al., 1980) has been well documented.

The objective of this study is to utilize the periodontal organ culture system described in Chapter 3 in an attempt to identify the presence of prostaglandins and to quantify fluctuations in their levels occurring during orthodontic tooth movement.

METHODS AND MATERIALS

Fifty-one eight week old mice, bred in our facility, were divided into 17 groups of three mice. Orthodontic type separating springs, as described in the previous chapter, were applied to a randomly selected side, interproximally between the first and second mandibular molars, for time periods corresponding to 0, 5, 10, 20, 30, 45, 60 minutes; 2, 4, 8, 16, 24 hours; and 3, 5, 7, 10, 14 days. The periodontium of the contralateral side of each mouse was used as a control. The spring insertions corresponding to 0 minutes to 24 hours were performed *in vitro*, while the longer daily insertions were accomplished *in vivo*. This was to standardize the culture conditions for each explant.

All periodontal organ explants were dissected and cultured for 24 hours as described in chapter 3. The mice were killed by cervical dislocation and the two mandibles removed. Fine dissection of the first, second and third molar with surrounding alveolar bone was performed in culture medium under a dissecting microscope. Separation of the explant tissue from the body of the mandible involved a first cut inferior to the apices of the teeth and a second vertical cut distal to the third molar. The gingival tissue was removed to maximize perfusion of nutrients. The average explant was 2 mm high and 4 mm long.

The explants were cultured in Trowell type (Trowell, 1959) organ culture dishes (60 x 15 mm, Falcon #3037, Becton, Dickinson and Co., Cockeysville, Maryland) in which the tissue rested on stainless steel grids (Falcon #3014, Becton, Dickinson and Co., Oxnard, California)

which were suspended over a centre well in the dish. Sufficient medium (800 ul) was introduced to reach the platform of each grid, so that explants stood at the gas-medium interface. The centre well was surrounded by an absorbent filter paper ring which was moistened with sterile distilled water. The dishes were covered and placed in an incubator maintained at 37^0C. Explants were cultured for 24 hours.

The medium used was Waymouth's 752/1 (Grand Island Biological Co., Grand Island, New York). (Waymouth, 1959) supplemented with 300 ugm per ml ascorbic acid (Fisher Scientific, Fairlawn, New Jersey) and an antiobiotic-antimycotic mixture (1.5 ml per 100 ml medium) consisting of 10,000 U per ml penicillin, 25 ugm per ml amphotericin B and 10,000 ugm per ml streptomyocin (Grand Island Biological Co., Grand Island, New York).

The incubator was flushed continuously with a mixture of 95% O_2 and 5% CO_2 humidified by bubbling through sterile distilled water.

To the culture medium in each dish, 0.5 uCi (^{14}C)-arachidonic acid (NEC-756, (^{14}C(U)) arachidonic acid, (New England Nuclear, Boston, Mass.) of specific activity 160 mCi/mmol was added 2 hours prior to termination. Hydroquinone (0.1 M) was added to the medium, at the same time as (^{14}C)-arachidonic acid, to act as a scavanger of the oxidant released from the hydroperoxy group during the peroxidic reduction of PGG_2 to PGH_2. It has been shown that this oxidizing moiety is capable of deactivating certain enzymes of the prostaglandin biosynthetic pathway (Egan et al., 1979).

Termination of the culture was achieved by pipetting the medium into 12 ml glass centrifuge tubes containing 4 N formic acid (resultant pH 3.0). The radioactive products of arachidonic acid were extracted by the addition of ethyl acetate (Fisher Scientific Co., Fair Lawn, New Jersey), vortex mixing for 10 seconds, centrifuging for 4 minutes at 700 rpm, and removal of the upper layer with disposable glass pipettes (Fisherbrand 13-678-54, Fisher Scientific Co., Fair Lawn, New Jersey). This extract was evaporated under nitrogen and the residue was immediately redissolved with 40 ul distilled ethanol and applied to a silica gel thin layer chromatography plate (Whatman LK5D TLC plate, Whatman Inc., Clifton, New Jersey).

Also included on each plate were standard solutions of PGE_2 (Sigma Chemical Co., St. Louis, Missouri), $PGF_{2\alpha}$ and PGD_2 (gift from Dr. Morris Karmazyne, University of Manitoba) and TXB_2 and 6-K-$PGF_{1\alpha}$ (gift from Dr. Alvin Chan, University of Manitoba). The solvent system for each plate was the organic phase of ethyl acetate/isooctane/acetic acid/water (11:5:2:10, vol/vol) (Fisher Scientific Co., Fair Lawn, New Jersey).

The thin layer chromatography plates were dried and then exposed to Kodak XRP-1 x-ray film (Kodak Canada Inc., Toronto, Ontario) for 14 days. Radioautographs were developed and scanned at 550 nm and the relative percentages of 6-keto-$PGF_{1\alpha}$, PGE_2, PGD_2, TXB_2, $PGF_{2\alpha}$ bands were calculated on a spectophotometer (Beckman DU-8, Beckman Instruments, Toronto, Ontario).

RESULTS

The nature of prostaglandins synthesized by the periodontal tissues labelled *in vitro* for 2 hours during various periods of orthodontic force application is demonstrated in the following photoradioautograph and densitometric scan (Fig. V-1,2). Five different prostaglandin types, or their more stable derivatives, were identified by comparison with standards. These were PGE_2, PGD_2, $PGF_{2\alpha}$, TXB_2 and 6-keto-$PGF_{1\alpha}$.

Tables V-1,2,3,4,5 summarize the data for relative prostaglandin synthesis in experimental and control explants for periods of force application of 0, 5, 10, 20, 30, 45 minutes; 2, 4, 8, 16, 24 hours; 3, 5, 7, 10 and 14 days. PGE_2 was produced in relatively large amounts by all control and experimental explants. 6-keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$ were also easily identifiable on all radioautographs, suggesting a large relative proportion of synthesis of these products. PGD_2 was synthesized in relatively lesser amounts, whilst TXB_2 was produced in very small amounts, with identification of this substance being occasionally impossible.

The differences between control and experimental ratios at each time and over all the time periods were tested statistically using the F factor analysis of variance. No significant differences were found.

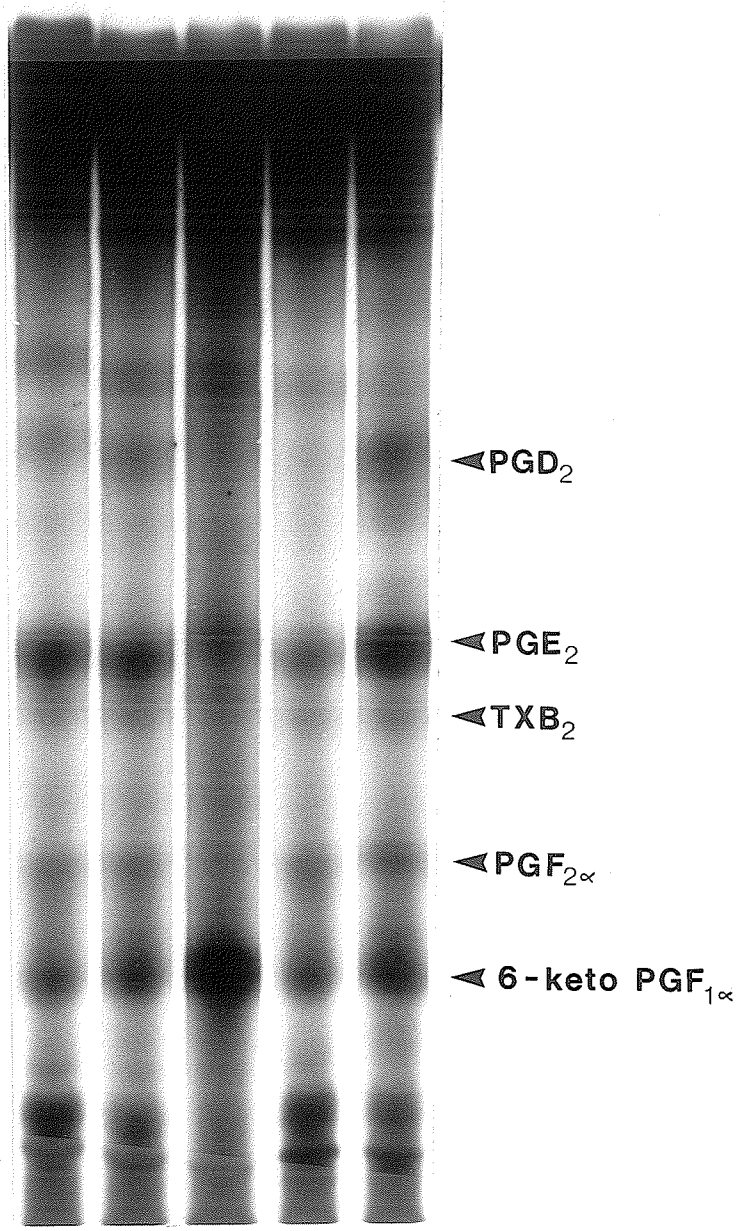


FIGURE V - 1. Photoradioautograph of radiolabelled lipids synthesized by the periodontal organ explant. The positions expected for 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, TXB_2, PGE_2 and PGD_2 are shown.

FIG. V-2

DENSITOMETRIC TRACING OF REPRESENTATIVE RADIOAUTOGRAPHIC BAND PATTERNS PRODUCED BY PERIODONTAL ORGAN EXPLANTS LABELLED 2 HOURS *IN VITRO* WITH ^{14}C-ARACHIDONIC ACID.

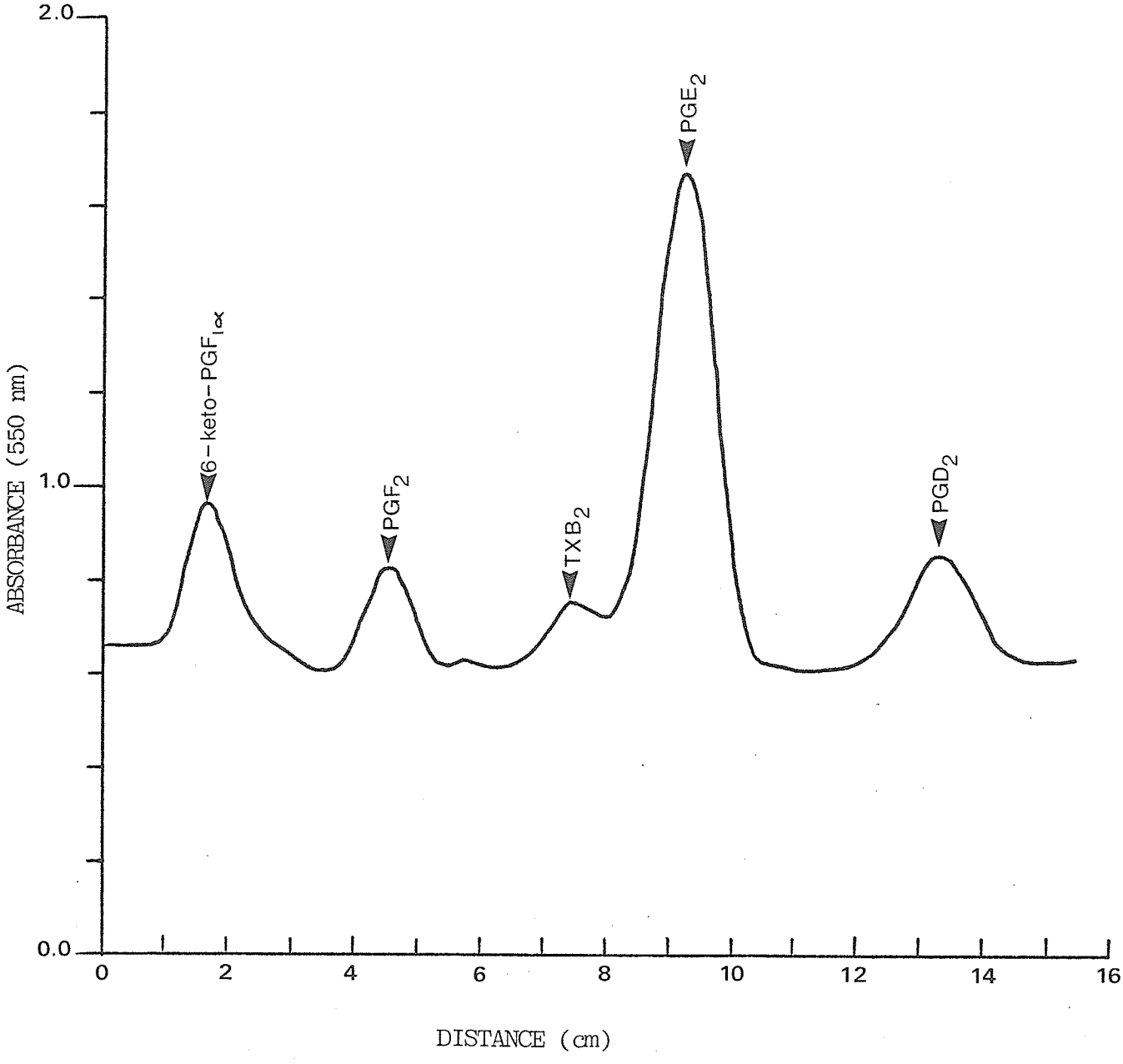


TABLE V-1

Means and standard deviations of proportions of 6-keto-$PGF_{1\alpha}$ synthesized by periodontal tissues *in vitro* after force application from 0 mins. to 14 days.

TIME OF FORCE APPLICATION		TREATMENT: EXPERIMENTAL	TREATMENT: CONTROL
Mins.	0	22.42± 4.70	16.29± 7.02
	5	21.35± 3.82	15.19± 6.51
	10	27.46± 5.42	26.18±10.04
	20	21.83± 3.52	19.59± 4.58
	30	29.10± 3.50	26.32± 8.27
	45	18.73± 6.97	21.90± 6.49
	60	28.75± 8.97	23.83± 2.91
Hours	2	24.68± 8.60	26.34± 3.54
	4	29.10± 7.24	23.18± 3.79
	8	8.87± 5.31	7.38± 1.42
	16	22.50± 4.55	24.11± 6.62
	24	25.13± 3.45	25.65± 7.67
Days	3	17.78± 1.42	32.75± 8.41
	5	16.44± 3.02	27.55± 4.51
	7	17.81± 6.04	20.61± 4.89
	10	34.96± 5.39	33.10± 0.30
	14	34.03± 4.16	18.48± 2.16

(No significant difference between experimental and control treatments)

TABLE V-2

Means and standard deviations of proportions of $PGF_{2\alpha}$ synthesized by periodontal tissues in vitro after force applcation from 0 mins. to 14 days.

TIME OF FORCE APPLICATION		TREATMENT: EXPERIMENTAL	TREATMENT: CONTROL
Mins.	0	12.16± 1.32	11.75± 1.45
	5	14.00± 2.35	14.19± 4.01
	10	15.23± 1.96	10.59± 2.10
	20	12.18± 3.45	13.16± 4.86
	30	8.66± 4.73	10.40± 2.02
	45	13.51± 6.97	15.45± 4.45
	60	7.50± 1.26	9.43± 2.07
Hours	2	9.58± 8.60	8.09± 4.18
	4	15.59± 4.30	12.20± 3.08
	8	14.43± 2.23	13.33± 2.88
	16	8.07± 2.13	7.87± 1.67
	24	10.25± 4.99	5.02± 0.47
Days	3	4.51± 1.35	9.85± 3.28
	5	9.71± 3.86	11.59± 4.06
	7	9.42± 5.19	9.03± 3.14
	10	12.45± 1.89	9.53± 0.30
	14	11.21± 1.45	7.32± 3.01

(No significant difference between experimental and control treatments)

TABLE V-3

Means and standard deviations of proprotions of TXB_2 synthesized by periodontal tissues *in vitro* after force application from 0 mins. to 14 days.

TIME OF FORCE APPLICATION		TREATMENT: EXPERIMENTAL	TREATMENT: CONTROL
Mins.	0	4.14±1.21	4.93±1.01
	5	3.87±1.01	5.13±0.92
	10	2.47±1.33	3.06±1.23
	20	7.40±3.03	8.30±2.44
	30	7.84±0.70	5.63±3.64
	45	3.54±3.10	2.28±0.57
	60	1.59±0.62	1.74±1.09
Hours	2	4.05±1.90	2.80±0.80
	4	2.53±0.63	2.19±0.38
	8	3.05±0.41	2.28±0.66
	16	4.35±1.36	6.42±2.64
	24	4.15±1.57	5.07±1.80
Days	3	7.77±1.61	5.89±1.43
	5	6.41±2.21	4.08±1.52
	7	3.95±1.46	6.09±3.99
	10	2.72±0.77	5.00±3.32
	14	2.91±1.03	4.21±2.14

(No significant difference between experimental and control treatments)

TABLE V-4

Means and standard deviation of proportions of PGE_2 synthesized by periodontal tissues *in vitro* after force application from 0 mins. to 14 days.

TIME OF FORCE APPLICATION		TREATMENT: EXPERIMENTAL	TREATMENT: CONTROL
Mins.	0	46.46± 5.16	43.84± 4.21
	5	51.19± 4.61	54.58± 3.21
	10	48.04± 4.96	52.32± 3.86
	20	45.66± 1.95	45.96± 1.75
	30	43.53± 2.52	47.63± 9.48
	45	54.06± 1.53	52.35± 3.94
	60	51.67± 4.63	51.27± 1.43
Hours	2	44.50± 7.98	37.43± 2.80
	4	40.59± 1.45	50.78± 3.93
	8	59.36± 5.83	61.46± 2.42
	16	39.82± 5.10	42.72± 3.77
	24	42.11± 3.50	42.86± 7.41
Days	3	47.16± 4.83	40.82± 5.61
	5	48.57± 4.97	43.46± 4.09
	7	54.21± 6.42	49.18± 2.54
	10	40.87± 6.09	44.15± 2.57
	14	41.80± 3.61	56.85± 4.31

(No significant difference between experimental and control treatments)

TABLE V-5

Means and standard deviations of proportions of PGD_2 synthesized by peridontal tissues *in vitro* after force application from 0 mins. to 14 days.

TIME OF FORCE APPLICATION		TREATMENT: EXPERIMENTAL	TREATMENT: CONTROL
Mins.	0	9.43± 2.16	10.93± 1.97
	5	8.63± 2.10	9.86± 2.73
	10	6.90± 0.40	7.86± 4.58
	20	12.92± 2.91	12.99± 1.29
	30	10.88± 3.73	10.02± 2.91
	45	10.15± 1.14	8.02± 4.85
	60	10.50± 4.40	13.75± 0.81
Hours	2	17.19± 8.23	25.33± 4.40
	4	12.19± 2.53	11.65± 2.61
	8	14.29± 1.24	15.55± 2.36
	16	25.27± 3.07	18.88±11.40
	24	18.36± 4.60	21.39± 3.25
Days	3	22.79± 5.61	10.70± 4.32
	5	18.86± 5.95	13.32± 1.68
	7	14.61± 4.95	15.10± 5.50
	10	8.99± 1.89	8.21± 1.10
	14	9.06± 2.16	13.15± 3.81

(No significant difference between experimental and control treatments)

DISCUSSION

The vitality of the explanted periodontal organ tissue, as indicated by the radioautographic and collagen synthetic studies, is further supported by the evidence of prostaglandin synthesis occurring in all explants.

The results of this study are also in accordance with other works which have demonstrated the ability of skeletal tissue to synthesize prostaglandins *in vitro*. Foetal rat bones cultured in the presence of complement and antibody to cell surface antigens (Raisz *et al.*, 1974, 1979; Sandberg *et al.*, 1977, 1979), neonatal mouse calvaria treated *in vitro* with collagenase, phorbol esters or mellitin (Dowsett *et al.*, 1976; Tashjian, 1978) and bone cell cultures subjected to mechanical stress (Harell *et al.*, 1977;Binderman and Cox, 1977;Somjen *et al.*, 1980) have all demonstrated the ability to produce prostaglandins.

The role of the various prostaglandin types in normal physiological function remain to be elucidated. However, three prostaglandin types have been implicated in tissue reactions similar to those occurring in orthodontic tooth movement. An increase of PGE_2 during neoplastic (Tashjian *et al.*, 1972) and inflammatory (Goodson *et al.*, 1974) bone resorption, inhibitory effects of PGE_2 upon collagen synthesis in mouse calvaria (Goldhaber, 1973), and foetal rat calvaria (Raisz and Koolemans-Beynen, 1974) and an increase of PGE_2 levels during mechanical stretching of cells (Harell *et al.*, 1977;Binderman and Cox,

1977;Somjen et al., 1980) has been recorded. The recently discovered prostacyclin, the stable derivative of which is 6-keto-$PGF_{1\alpha}$, has been identified in blood vessel walls (Bunting et al., 1976) and appears to be involved in bone resorption (Raisz et al., 1979). Likewise, an involvement of thromboxane A_2 in the stimulation of bone resorption in foetal rat calvaria in vivo has been suggested (Heersche and Jez, 1981).

Although the presence of these prostaglandins or their derivatives has been verified in this study, no proportionate difference in their levels during various periods of force application was apparent.

The ability of arachidonic acid, the prostaglandin precursor to permeate the periodontal ligament of the explant, or similarly of the synthesized prostaglandins of the periodontal ligament to diffuse into the supportive medium is unknown. Although prostaglandins are obviously produced in this system, they cannot be specifically attributed to having been synthesized by the periodontal ligament but may also be a product of the peripheral alveolar cells.

The accuracy of the thin layer chromatography technique for quantifying prostaglandin synthesis is limited by the possibility of contaminating radiolabelled products co-chromatography. In view of this, radioimmunoassay techniques for indentification and quantification of specific prostaglandins may be more useful for delineating fluctuations in levels of a specific prostaglandin during force application.

The orthodontic forces introduced in this system produce a tipping motion on the first and second molars. Such tipping results in areas of compression and tension within different areas of the periodontal ligament associated with each tooth. If the various prostaglandins have specific connective tissue remodelling functions, the increase or decrease of a specific prostaglandin in one localized area of compression may be masked by a concomitant increase in that same prostaglandin in an area of tension, thus creating the appearance of no apparent change in prostaglandin synthesis between experimental and control explants.

Such stresses as compression or tension are impossible to apply independently to the soft connective tissue of this system. In order to pursue the possibility of prostaglandin responses dependent upon specific physical stresses, another in vitro system will be utilized in the following chapter.

CONCLUSIONS

The vitality of the newly developed periodontal organ culture system was further demonstrated by its ability to synthesize prostaglandins. These prostaglandins were 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, TXB_2, PGE_2 and PGD_2.

Although the role of some of these prostaglandins in tissue reactions similar to those occurring during orthodontic tooth movement has

been demonstrated by other studies, no significant fluctuations in their levels during orthodontic tooth movement in this in vitro system were recorded. This may be due to the masking of differences in prostaglandin synthesis due to opposing changes in levels of specific prostaglandins in compressed and tensed periodonal ligament sites. The following chapter deals with the investigation of this possibility by utilizing another in vitro system which allows for individual applications of pressure and tension to a soft connective tissue ligament.

CHAPTER 6

PROSTAGLANDIN SYNTHESIS IN MECHANICALLY STRESSED MOUSE CALVARIAL TISSUES IN VITRO

INTRODUCTION

The application of force to the dentofacial complex by orthodontic appliances has been used by orthodontists for over a century, yet the cellular control mechanisms involved in the remodelling of the craniofacial sutures or the periodontal ligament are unknown. It is unlikely that the direct role of any regulator can be established by experiments *in vivo* because of the difficulty of controlling the numerous variables that are present in the experimental system. An *in vitro* system in the form of an organ culture, therefore, seems likely to provide the most promising experimental model for elucidation of these regulators.

Several potential factors have been proposed in the control of connective tissue remodelling during orthodontic tooth movement, including pressure and tension changes (Sandstedt, 1904, 1905a, 1905b), oxygen tension changes (Bien, 1966), piezo-electric stimuli (Zengo *et al.*, 1973, 1974) cyclic nucleotides (Davidovitch *et al.*, 1975) and prostaglandins (Yamasaki *et al.*, 1980). However, no significant changes in ratio of prostaglandins were found in periodontium stressed orthodontically for various time periods *in vitro* (see Chapter 5). It has been proposed that this may be due to masking of prostaglandin changes in an area of compression by equal and opposite prostaglandin changes in an area of tension. The lack of significant changes in prostaglandin synthesis may also be due to the possible inability of

periodontally produced prostaglandins to diffuse out of the periodontal ligament to the medium.

To overcome these problems, the cranial suture, which is morphologically simpler than the periodontium, will be utilized for force studies *in vitro*. Compressive or tensile forces can be applied across the suture in order to identify changes in prostaglandin synthesis occurring during different periods for one specific type of force application. Because of the more accessible relationship of the sutural soft connective tissue to the culture medium, one would expect a more complete and uniform release of prostaglandins for measurement.

METHODS AND MATERIALS

Dissection and Culture Systems

Male white mice, bred in our facility, were randomly selected to form 6 groups of nine for each of the following six periods of force application - 0, 10, 20, 30, 45, 60 minutes. Following exposure of the calvaria of each mouse, holes (size 1/4 round, Jet carbide burs, Beavers Dental Products Ltd., Morrisburg, Ontario) were carefully drilled into the parietal bones 2 mm either side of the interparietal suture, using a high speed dental drill (Fig. VI-1).

Calvaria were then dissected from the mice and any opposing soft tissue was carefully removed. Using sharp dissection scissors, the calvaria were trimmed to leave only the interparietal suture connecting two adjacent portions of the parietal bones. The explant formed a

FIGURE VI - 1. Mouse calvaria exposed following incision and retraction of skin. Two holes on either side of the interparietal suture are drilled at this stage.

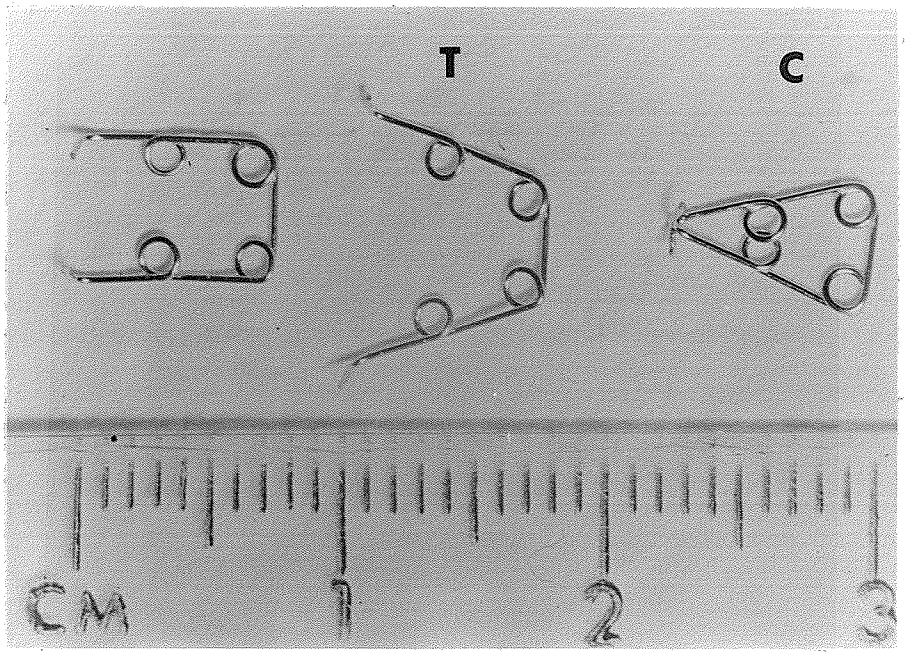


FIGURE VI - 2. Springs for application of tension (T), compression (C). Inactivated spring not marked.

rectangle consistently measuring 8 mm wide and 7 mm long.

The explants were cultured using the method reported by Yen and Suga (1981). They were placed in Trowell-type (Trowell, 1959) organ culture dishes (60 x 15 mm, Falcon #3037, Becton Dickinson and Co., Cockeysville, Maryland) in which the explants rested on stainless steel grids (Falcon #3014, Becton, Dickinson and Co., Oxnard, California) which were suspended over a centre well in the dish (Fig. VI-3). Sufficient medium (800 ul) was introduced to reach the platform of each grid so that the explants stood at the gas-medium interface. The centre well was surrounded by an absorbent filter paper ring which was moistened with sterile distilled water. The dishes were covered and placed in an incubator maintained at 37^{0}C. Explants were cultured for 24 hours.

Medium, Gas and Radioisotope

The medium used was Waymouth's 752/1 (Grand Island Biological Co., Grand Island, New York), supplemented with 300 ugm per ml ascorbic acid (Fisher Scientific, Fairlawn, New Jersey) and an antibiotic-antimycotic mixture (1.5 ml per 100 ml medium) consisting of 10,000 U per ml penicillin, 25 ugm per ml amphotericin B and 10,000 ugm per ml streptomycin (Grand Island Biological Co., Grand Island, New York).

The incubator was flushed continuously with a humidified mixture of 95% O_2 and 5% CO_2. (^{14}C)-arachidonic acid (0.5 uCi/800 ul) (NEC-756, (^{14}C(U)) arachidonic acid (New England Nuclear, Boston, Mass.) of spe-

cific activity 160 mCi/mmol and 0.1 M hydroquinone were added to the culture medium 2 hours prior to termination. The method of prostaglandin extraction and quantification was described in the previous chapter.

Force Application

Either compressive or tensile forces of 10 gm/mm of activation were applied across the interparietal suture by springs fabricated from 0.010" diameter orthodontic wire (Green Elgiboy, Rocky Mountain Orthodontics, Scarborough, Ontario). The springs were activated as shown in Fig. VI-2 and inserted into the holes previously drilled in the calvaria. Three calvaria were prepared with compressive springs, three calvaria were prepared with tension springs and the remaining three calvaria were used as controls, making a total of nine calvaria for each period of force treatment.

RESULTS

The nature of the prostaglandins synthesized by the calvarial tissues labelled in vitro for 2 hours during various periods of compressive or tensile force application is demonstrated in the following photoradioautograph (Fig. VI-4). Five different prostaglandin types, or their more stable derivatives, were identified by comparison with standards. These were 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, TXB_2, PGF_2 and PGD_2. 6-keto-$PGF_{1\alpha}$ and PGE_2 were synthesized in the relatively largest amounts, followed by $PGF_{2\alpha}$, with TXB_2 and PGD_2 being synthesized in

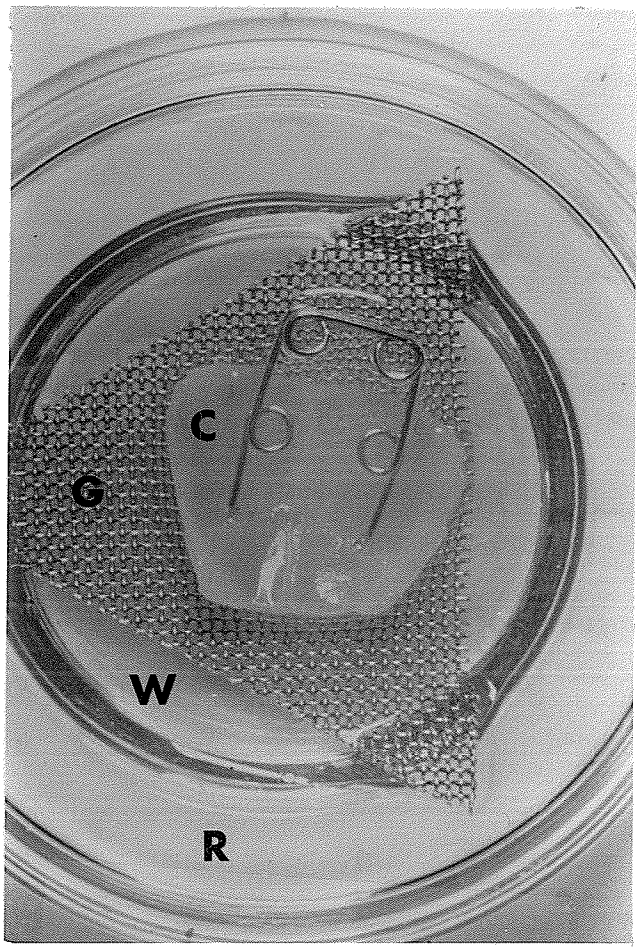


FIGURE VI - 3. Calvarial explant *in vitro*, with spring in place. (G = stainless steel grid; R = paper ring moistened with sterile distilled water; W = Waymouth's 752/1 medium; C = calvaria)

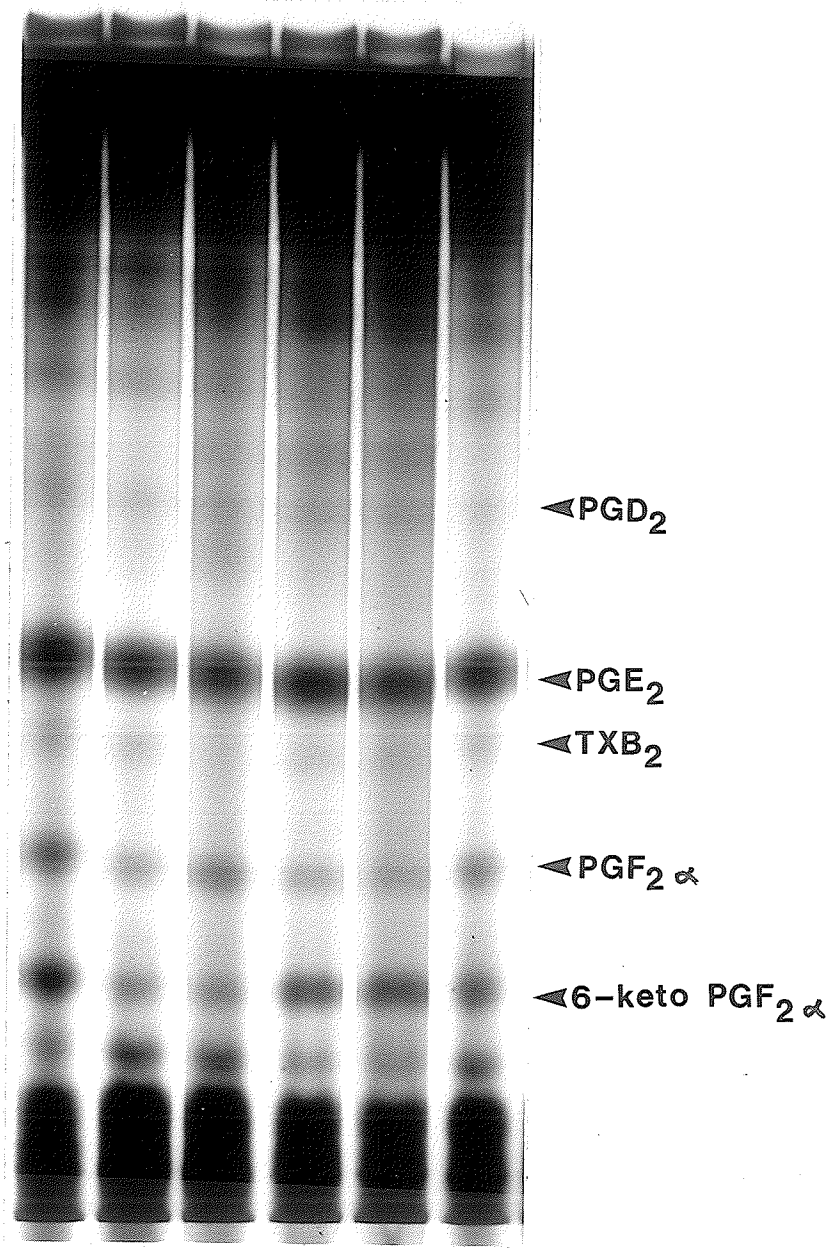


FIGURE VI-4. Photoradioautograph of radiolabelled lipids synthesized by the calvarial explant. The positions expected for 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, TXB_2, PGE_2 and PGD_2 as shown.

the smallest amounts.

Ratios for each prostaglandin type at each time interval for the various experimental and control conditions are summarized in Table VI-1,2,3,4,5. The differences between control and experimental ratios at each time and over all the time periods were tested statistically using the F factor analysis of variance. There was a significant (5%) difference in the ratios of TXB_2 synthesized. Using the lowest standard difference test constructed at the 5% level, there was a significant increase in the ratio of TXB_2 synthesized in response to compression and tension after 10 minutes of force application. Figure VI-5 is a graphical representation of the relative changes occurring in synthesis of TXB_2 during periods of force application corresponding to 0, 10, 20, 30, 45 and 60 minutes.

DISCUSSION

The vitality of the explanted mouse calvaria was demonstrated by the synthesis of prostaglandins by all cultures. This is in accordance with the vitality reported by Yen and Suga (1981) as demonstrated by the ability of the calvaria to synthesize collagen *in vitro*.

This study also agrees with other studies which have demonstrated the ability of skeletal tissue to synthesize prostaglandins *in vitro*.

TABLE VI-1

Means and standard deviations of proportions of 6-keto-$PGF_{1\alpha}$ synthesized by calvaria *in vitro* after force application from 0 mins. to 60 mins.

(No significant difference between experimental and control treatments)

Time of Force Application (minutes)	Type of Force Application		
	Compression	Tension	Control
0	35.31 ± 6.34	29.63 ± 9.21	33.74 ± 10.13
10	46.84 ± 22.18	35.90 ± 6.90	36.14 ± 10.62
20	43.14 ± 9.28	44.11 ± 20.35	30.98 ± 11.50
30	33.73 ± 2.67	23.14 ± 6.26	18.23 ± 5.44
45	40.57 ± 5.27	46.41 ± 12.51	41.14 ± 14.19
60	33.46 ± 2.41	51.06 ± 4.31	42.33 ± 13.06

TABLE VI-2

Mean and standard deviations of proportions of $PGF_{2\alpha}$ synthesized by calvaria *in vitro* after force application from 0 mins. to 60 mins.

(No significant differences between experimental and control treatments)

Time of Force Application	Type of Force Application		
	Compression	Tension	Control
0	16.71 ± 4.76	20.13 ± 8.49	21.46 ± 11.24
10	12.61 ± 10.06	14.79 ± 2.17	10.35 ± 3.39
20	15.53 ± 4.61	12.15 ± 6.27	19.27 ± 16.50
30	28.28 ± 3.51	29.32 ± 5.09	33.11 ± 1.21
45	22.67 ± 1.05	20.48 ± 9.95	20.04 ± 3.91
60	27.49 ± 2.49	16.08 ± 1.72	21.40 ± 7.70

TABLE VI-3

Mean and standard deviations of proportions of TXB_2 synthesized by calvaria in vitro after force application from 0 mins. to 60 mins.

Time of Force Application	Type of Force Application		
	Compression	Tension	Control
0	3.54 ± 1.61	4.23 ± 0.63	4.49 ± 1.10
10	6.39 ± 2.53*	6.33 ± 1.31*	0.73 ± 0.33
20	4.71 ± 1.02	6.35 ± 2.24	5.43 ± 3.48
30	4.08 ± 0.52	6.12 ± 2.85	4.49 ± 0.55
45	1.32 ± 0.11	2.68 ± 1.54	3.26 ± 0.74
60	3.00 ± 1.26	3.76 ± 2.03	3.05 ± 1.32

* Denotes difference between experimental and control treatments significant at 0.05 level.

TABLE VI-4

Means and standard deviations of proportions of PGE_2 synthesized by calvaria *in vitro* after force application from 0 mins. to 60 mins.

(No significant difference between experimental and control treatments).

Time of Force Application (minutes)	Type of Force Application		
	Compression	Tension	Control
0	28.32 ± 6.31	31.64 ± 7.10	25.74 ± 4.16
10	24.40 ± 7.84	28.80 ± 6.37	46.86 ± 6.15
20	27.76 ± 8.08	31.11 ± 13.32	32.97 ± 6.13
30	23.34 ± 4.40	24.55 ± 5.54	25.56 ± 2.16
45	20.81 ± 2.11	20.34 ± 5.49	26.31 ± 3.39
60	24.90 ± 3.74	22.13 ± 6.20	25.11 ± 0.90

TABLE VI-5

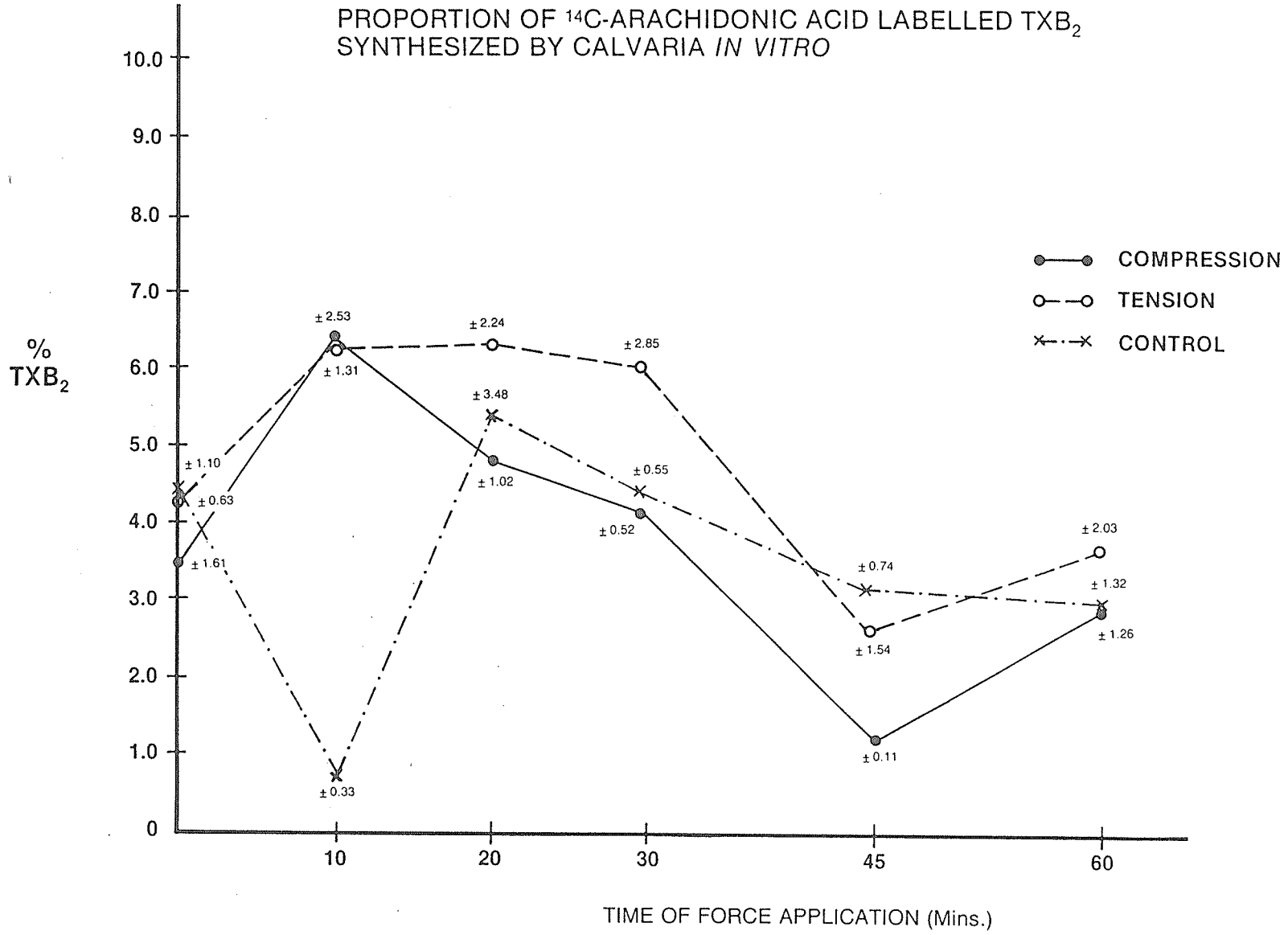
Mean and standard deviations of proportions of PGD_2 synthesized in calvaria *in vitro* after force application from 0 mins. to 60 mins.

(No significant difference between experimental and control treatments).

Time of Force Application (minutes)	Type of Force Application		
	Compression	Tension	Control
0	8.67 ± 4.32	11.17 ± 6.45	8.39 ± 5.63
10	9.62 ± 10.99	10.04 ± 2.25	2.48 ± 0.52
20	14.69 ± 9.19	3.30 ± 1.41	11.67 ± 4.66
30	14.68 ± 9.10	20.05 ± 4.47	17.47 ± 1.15
45	18.18 ± 2.94	14.41 ± 6.31	10.60 ± 8.60
60	17.76 ± 9.04	12.61 ± 6.29	11.99 ± 8.95

FIG. VI-5

PROPORTION OF ^{14}C-ARACHIDONIC ACID LABELLED TXB_2 SYNTHESIZED BY CALVARIA *IN VITRO*



Foetal rat bones cultured in the presence of complement and antibody to cell surface antigens (Raisz et al., 1974, 1979;Sandberg et al., 1977, 1979), neonatal mouse calvaria treated in vitro with collagenase, phorbol esters or mellitin (Dowsett et al., 1976;Tashjian, 1978), bone cell cultures subjected to mechanical stress (Harell et al., 1977;Binderman and Cox, 1977;Somjen et al., 1980), and mouse periodontal organ culture (see Chapter 5) have all demonstrated the ability to produce prostaglandins.

The majority of these papers (Raisz et al., 1974;Sandberg et al., 1977, 1979;Dowsett et al., 1976;Tashjian, 1978;Harell et al., 1977;Binderman and Cox, 1977;Somjen, 1980) have used specific radioimmunoassays to implicate a role for PGE_2 in bone resorption. The thin layer chromatography technique used in this study has only been recently developed (Cottee, 1977). Utilizing a similar technique, Raisz et al. (1979) were able to show a similar prostaglandin synthetic response to the one reported in this paper. The culture of foetal rat calvaria and long bone shafts, in the presence of complement, produced relatively high levels of PGE_2 and 6-keto $PGF_{1\alpha}$, with lesser amounts of $PGF_{2\alpha}$, PGD_2 and TXB_2 being evident (Raisz et al., 1979).

Thus it appears that several skeletal in vitro systems have demonstrated the ability to synthesize PGE_2 under a variety of conditions, and foetal rat calvaria and long bone shafts have been shown to produce comparable prostaglandin types to those observed in this study.

The wide dispersion of data for the relative amounts of each prostaglandin synthesized in response to each of two different types of force application at various periods have made meaningful analysis of the data difficult. The size of the deviations may be a result of one or more factors. These factors may be experimental design related, where an increase in sample size would increase confidence in data interpretation, or they may be related to experimental methodology, where measurement sensitivity is of critical importance. This sensitivity is related to the percentage recovery of total added label and the linear recovery of any label present in the medium, but more importantly it is dependent on the efficient recovery of individual labelled prostaglandins. A differential in recovery of one prostaglandin type over another prostaglandin type would invalidate conclusions related to relative fluctuations in prostaglandin synthesis.

The use of expensive radiolabelled standard prostaglandins would enable the recovery of the various prostaglandins to be tested. Percentage recovery of total label was measured in this study and was found to be an average of 70%, with a range from 60-80%.

The role of the various prostaglandin types in normal physiological function remain to be elucidated. However, three prostaglandin types have been implicated in tissue reactions analogous to those occurring during orthodontic tooth movement. An increase of PGE_2 during neoplastic (Tashjian et al., 1972) and inflammatory (Goodson et al., 1974) bone resorption, inhibitory effects of PGE_2 upon collagen

synthesis in mouse calvaria (Goldhaber, 1973), and foetal rat calvaria (Raisz and Koolemans-Beynen, 1974) and an increase of PGE_2 levels during mechanical stretching of cells (Harell et al., 1977;Binderman and Cox, 1977;Somjen et al., 1980) has been recorded. The recently discovered prostacyclin, the stable derivative of which is 6-keto-$PGF_{1\alpha}$, has been identified in blood vessel walls (Bunting et al., 1976) and appears to be involved in bone resorption (Raisz et al., 1979). Likewise, an involvement of thromboxane A_2 in the stimulation of bone resorption in foetal rat calvaria in vivo has been suggested (Heersche and Jez, 1981).

The apparent increase in relative amounts and TXB_2 at the 10 minute period of both compressive and tensile force applications illustrates a possible role of this substance in initial stages of connective tissue remodelling during mechanical stress. However, the difference between the force treated and control synthesis of TXB_2 is partly attributable to a decrease in the control level of TXB_2. The reason for this reduction in the ratio of TXB_2 in the control explants is unclear. The lack of difference in TXB_2 synthesis between compressive and tensile treatments suggests that the specific prostaglandin synthetic response is independent of the type of force applications.

The increase in the relative level of TXB_2 must be related to the response of the total organ in vitro. This response includes the remodelling of the fibrous suture, the remodelling of the opposing pieces of calvarial bone, and the response to the trauma resulting

from explant dissection and drilling of the holes which eventually receive the springs.

In addition, it must be remembered that the level of TXB_2, like all of the prostaglandins measured, is purely relative. Thus the measurements are not representative of individual specific fluctuations in amounts of prostaglandins synthesized. To obtain this information, a radioimmunoassay analysis of specific prostaglandin types would be necessary.

CONCLUSIONS

Synthesis of five different prostaglandins or their more stable end products has been demonstrated in the mouse calvarial suture *in vitro*. The use of this model enabled the effects of compressive or tensile forces upon prostaglandin synthesis to be examined.

An increase in the relative proportion of TXB_2 synthesized after 10 minutes of either compressive or tensile force application was recorded. In view of the previously suggested involvement of TXA_2 in bone remodelling, the results from this experiment may support the involvement of this substance in connective tissue remodelling during orthodontic tooth movement.

However, the response in TXB_2 synthesis may be an artifact resulting from experimental methodology problems. These problems include the unknown efficiency of the extraction procedure, where factors like

the non-linear recovery of specific prostaglandins are of concern, or they may be related to the sensitivity of the prostaglandin synthetic response to a variety of external stimuli. Synthesis of prostaglandins in response to non-force application related factors, such as the trauma associated with calvarial dissection and the drilling of holes, is almost certain to occur. The effect of this upon data analysis is uncertain.

However, the development of this in vitro technique and the periodontal organ culture technique reported in Chapter 3 will allow for examination of prostaglandins and other possible cellular regulatory mechanisms acting during orthodontic tooth movement at a molecular level.

CHAPTER 7

DISCUSSION

Celsus (25 BC - A.D. 50) was the first to record a method of treatment for a developing malocclusion (Weinberger, 1942). He stated

> "If a second tooth should happen to grow in children before the first has fallen out, that which ought to be shed is to be drawn out and the new one daily pushed toward its place by means of the finger until it arrives at its just proportions."

In retrospect, even with the advent of today's sophisticated appliances, the principle of much of present day orthodontic treatment remains basically the same as it was in Celsus' day. That is, tooth movement in orthodontic treatment today involves application of a force to the crown of a tooth. Although we know somewhat more today about the tissue behaviour occurring during tooth movement, just as in Celsus' day, nothing is really known about the control mechanisms responsible for transduction of the application of force to produce the resultant cellular response.

Histological studies (see Rietan, 1975, for review) have demonstrated that remodelling of hard and soft connective tissues is an essential process of orthodontic tooth movement. Although the cellular control mechanisms for these remodelling functions are unknown, several potential factors have been proposed, including periodontal pressure and tension changes (Sandstedt, 1904, 1905a, 1905b), oxygen tension changes (Bien, 1966), piezo-electric stimuli (Zengo et al., 1973, 1974), cyclic nucleotides (Davidovitch et al., 1975) and prostaglandins (Yamasaki et al., 1980).

The pressure and tension theory (Sandstedt, 1904, 1905) originated from light microscopic analysis of tooth movement without explaining cellular mechanisms. Oxygen tension, although capable of stimulating osteogenic activity *in vitro* (Goldhaber, 1958, 1961, 1966; Stern *et al.*, 1966;Khouw and Goldhaber, 1970) have never been demonstrated to occur *in vivo*. The vasculature changes often used to support this concept may in fact be secondary to an increase in bone remodelling behaviour. Piezo-electric potentials have been shown to occur upon mechanical deformation of the dento-alveolar complex (Zengo *et al.*, 1973, 1974) and have also been implicated in an increase in osteogenic cell number and intracellular cAMP activity (Davidovitch *et al.*, 1978a, 1978b, 1979, 1980a, 1980b). However, the mechanism of transduction of the electric current into the observed cellular behaviour has not been demonstrated. Cyclic nucleotides, which have been shown to fluctuate in response to the application of orthodontic forces (Davidovitch *et al.*, 1975, 1976, 1977), are intracellular "second messengers" considered indicative of cellular activity. However, the extracellular stimuli responsible for producing alterations in cAMP levels require clarification.

Current evidence suggests that locally produced prostaglandins may play an important role in the transduction of mechanical forces into cellular activity. A possible role for prostaglandins during neoplastic (Tashjian *et al.*, 1972) and inflammatory (Goodson *et al.*, 1974) bone resorption, collagen remodelling (Goldhaber, 1973; Raisz and Koolemans-Beynen, 1974) and the mechanical stretching of cells (Harell

et al., 1977; Binderman and Cox, 1977; Somjen et al., 1980) has been reported.

In view of this, the objective of this study was to investigate the possible involvement of prostaglandins in connective tissue remodelling during orthodontic tooth movement. In order to accomplish this, it was necessary to develop an organ culture system which could maintain whole periodontium undergoing remodelling in response to mechanical stress. By isolating the tissue in vitro one can eliminate those factors in vivo which would modulate and mask the presence of prostaglandins, and thus complicate their measurement.

The periodontal organ culture system which was developed consisted of the fine dissection of three mandibular molars and surrounding alveolar bone from mouse, followed by the placement of this explant at the gas-medium interface in Trowell type culture dishes containing Waymouth's medium 752/1, but without foetal calf serum. Foetal calf serum is a commonly used ingredient in mediums for organ culture, but many of its unknown constituents may interfere with prostaglandin metabolism and measurement. The cultures were flushed with 95% O_2 and 5% CO_2 and were maintained in a humidifier at 37^0C.

Initial histological and radioautographical examination of the periodontal organ explant was carried out in order to verify the vitality of explants following 24 hours of culture. Light microscopic cell morphology revealed a normal periodontium in comparison to specimens taken from live animals. Radioautographical evidence using

^{3}H-thymidine and ^{3}H-proline label demonstrated cell proliferation and protein synthetic activity. ^{3}H-proline labelling was heavier at the periphery of the explants, with a decreasing gradient of labelling occurring towards the central area of the explants.

The labelling gradient was felt to result from a pattern of diminishing oxygen diffusion, which had been discussed by earlier workers in organ explants (Trowell, 1959; MacDougall and Couplant, 1967; Yen and Melcher, 1978) or perhaps from the utilization of oxygen by more peripheral cells, as had been suggested by Yen and Melcher (1978). Similarly, a diffusion gradient for the labelled precursors may explain the resultant label gradient, although the direct relationship between label gradient and oxygen tension (Yen and Melcher, 1978) would argue against this.

However, despite the presence of a labelling intensity gradient, the presence of reasonably heavy labelling in central regions of the organ cultures, in association with the appearance of normal cellular histology, represented significant evidence supporting the overall vitality of the explants after 24 hours of in vitro support.

Of interest was the subjective evaluation that ^{3}H-proline labelling appeared heavier in explants which were undergoing active orthodontic stress.

The development of a periodontal organ culture system which was capable of receiving orthodontic type forces had introduced, for the

first time, an opportunity to examine a variety of cellular mechanisms functioning during orthodontic tooth movement *in vitro*. As collagen is the predominant protein in both hard and soft connective tissues (Bornstein and Traub, 1979), the effect of orthodontic force upon collagen synthesis was examined to provide baseline data for cellular response to forces.

Newly synthesized collagen, labelled with (^{14}C)-glycine, were salt solubilized and separated using the interupted electrophoresis method of Sykes *et al*. (1976). Examination of collagen synthesized during periods of force application ranging from 0 hours to 14 days revealed a relative increase in type III collagen in comparison to type I collagen at 1, 3 and 5 days at force application. These time periods coincided with the observed period of activity of the orthodontic separating springs.

The significance of type III collagen synthesis is not clear at this time. Type III collagen has been found to be present in foetal skin and uterine wall (Chung and Miller, 1974), associated with vascularization in early phases of wound healing (Gay *et al*., 1978), synthesized by synovial cells from patients with rheumatoid arthritis (Eyre and Muir, 1975) and associated with inflammation (Weiss *et al*., 1975). Thus it appears that type III collagen serves an important role in rapid remodelling situations, such as wound healing, as well as normal growth and development of connective tissue. Moreover, in view of its presence in tissues such as aorta, uterine wall and perio-

dontal ligament (Chung and Miller, 1974; Butler et al., 1975) type III collagen may have a stress bearing role.

A role for type III collagen in either rapid connective tissue remodelling, wound healing or the bearing of physical stress has gained further support from our experimental data. Each of these three functions occur during orthodontic tooth movement, and a significant relative increase in type III collagen was evident during periods of active force application and rapid remodelling.

Following this further demonstration of the value of the organ explant technique, as evidenced by the collagen synthesis data, the possible role of prostaglandins in orthodontic tooth movement was investigated.

Orthodontic forces were applied to the periodontal organ in vitro for time periods ranging from 0 minutes to 14 days. Prostaglandins, labelled with (^{14}C)-arachidonic acid, were extracted from the supportive medium and placed on thin layer chromatography plates along with various prostaglandin standards.

The vitality of the explanted periodontal organ tissue, as indicated by the radioautographic and collagen synthetic studies, was further supported by the evidence of prostaglandin synthesis. Five different prostaglandin types, or their more stable end products, were routinely identified - 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, TXB_2, PGE_2 and PGD_2.

This study is also in accordance with other works which have demonstrated the ability of skeletal tissue to synthesize prostaglandins in vitro. Foetal rat bones cultured in the presence of complement and antibody to cell surface antigens (Raisz et al., 1974, 1979; Sandberg et al., 1977, 1979), neonatal mouse calvaria treated in vitro with collagenase, phorbol esters or mellitin (Dowsett et al., 1976; Tashjian, 1978) and bone cell cultures subjected to mechanical stress (Harell et al., 1977;Binderman and Cox, 1977;Somjen et al., 1980) have all demonstrated the ability to produce prostaglandins.

The majority of these papers (Raisz et al., 1974;Sandberg et al., 1977, 1979;Dowsett et al., 1976;Tashjian, 1978;Harell et al., 1977;Binderman and Cox, 1977;Somjen, 1980) have used specific radioimmunoassays to implicate a role for PGE_2 in bone resorption. The thin layer chromatography technique used in this study has only been recently developed (Cottee, 1977). Utilizing a similar technique, Raisz et al. (1979) were able to show a similar prostaglandin synthetic response to the one reported in this paper. The culture of foetal rat calvaria and long bone shafts, in the presence of complement, produced relatively high levels of PGE_2 and 6-keto $PGF_{1\alpha}$, with lesser amounts of $PGF_{2\alpha}$, PGD_2 and TXB_2 being evident (Raisz et al., 1979).

Thus it appears that several skeletal in vitro systems have demonstrated the ability to synthesize PGE_2 under a variety of conditions, and foetal rat calvaria and long bone shafts have been shown to pro-

duce comparable prostaglandin types to those observed in this study.

The role of the various prostaglandin types in normal physiological function remain to be elucidated. However, the involvement of prostaglandins in tissue reactions similar to those occurring during orthodontic tooth movement has been demonstrated by several studies. An increase of PGE_2 during neoplastic (Tashjian et al., 1972) and inflammatory (Goodson et al., 1974) bone resorption, inhibitory effects of PGE_2 upon collagen synthesis in mouse calvaria (Goldhaber, 1973), and foetal rat calvaria (Raisz and Koolemans-Beynen, 1974) and an increase of PGE_2 levels during mechanical stretching of cells (Harell et al., 1977;Binderman and Cox, 1977;Somjen et al., 1980) has been recorded. The recently discovered prostacyclin, the stable derivative of which is 6-keto-$PGF_{1\alpha}$, has been identified in blood vessel walls (Bunting et al., 1976) and appears to be involved in bone resorption (Raisz et al., 1979). Likewise, an involvement of thromboxane A_2 in the stimulation of bone resorption in foetal rat calvaria in vivo has been suggested (Heersche and Jez, 1981).

Although the presence of these prostaglandins or their derivatives has been verified, no proportionate difference in their levels during various periods of force application was apparent. This may be due to a variety of reasons, including experimental design and methodology problems.

The most significant factor contributing to the inconclusive results from an experimental design viewpoint was the small sample

size. Increases in sample size may have reduced the standard deviations for the various proportionate prostaglandin levels, thereby making data analysis more meaningful.

Methodology problems involve a variety of sources of error. The ability of either arachidonic acid or synthesized prostaglandins to diffuse throughout the periodontal ligament is unknown, and therefore those prostaglandins measured may only be synthesized by alveolar bone cells, as has been demonstrated by other studies involving skeletal tissues (Raisz et al., 1979). As peripheral alveolar bone cells are not all likely to be effected by applications of orthodontic force, no fluctuations between control and experimental situations would become apparent.

Another important source of methodological problems may be related to the sensitivity of the prostaglandin measurement. Problems pertaining to the percentage recovery of added radioactive label, and the non-linear extraction of different prostaglandins would seriously jeopardize the accuracy of the recovery procedure and thus the accuracy of any quantification.

The final methodological problem may involve the organ system itself. The orthodontic forces introduced in this system produce a tipping motion on the first and second molars. Such a tipping results in areas of compression and tension within different areas of the periodontal ligament associated with each tooth. If the various prostaglandins have specific connective tissue remodelling functions, then

whilst the overall level of a prostaglandin reaching the medium may be unchanged, localized area specific differences may occur. This would depend on the type of physical stimulus applied to the cells of compressed or tensed ligament.

Because such stresses as compression or tension are impossible to apply independently to the soft connective tissue of the periodontal system, another in vitro system utilizing the calvarial suture of mice was used. In this system, calvaria were dissected from eight week old mice and placed at the gas-medium interface in Trowell type culture dishes containing Waymouth's medium. As with the periodontal organ system, no foetal calf serum was used. The cultures were flushed with 95% O_2 and 5% CO_2 and were maintained in a humidifier at 37^0C.

Compressive or tensile forces were applied across the interparietal suture with springs fabricated from orthodontic wire for periods from 0 to 60 minutes. Using the same labelling and prostaglandin extraction technique described for the periodontal organ system, the effects of the two force treatments upon prostaglandin synthesis were investigated.

The five different prostaglandin types which were identified in the periodontal organ culture system, were similarly identified in the calvaria in vitro system. Although only tentative conclusions could be reached because of the wide dispersion of data when fluctuations in prostaglandin levels were examined, significant increases in the relative amount of TXB_2 were observed following 10 minutes of both com-

pressive and tensile force applications. As discussed previously, this is of interest because of possible role for TXA_2 in stimulation of bone resorption in foetal rat calvaria in vitro (Heersche and Jez, 1981).

However, the lack of difference in prostaglandin synthesis between compressive and tensile treatments suggests that the specific prostaglandins synthetic response is independent of the type of force applications.

As with the periodontal organ culture technique, experimental design and methodology problems may have been responsible for the wide dispersion of data. A small sample size, inadequate diffusion of labelled arachidonate and/or prostaglandins throughout the soft connective tissue of the suture, insufficient recovery of added radioactive label and a non-linear extraction of specific prostaglandins may have individually, or in conjunction with each other, contributed to an inaccurate result. Also the synthesis of prostaglandins due to non-force application related stimuli, for example the dissection and preparation of the calvaria, may have masked meaningful data. Most of these problems are specifically related to prostaglandins, and may be alleviated by alternative methodology. For example, radioimmunoassay techniques could be used to quantify specific alterations in individual prostaglandin synthesis. However, because of the lability of individual prostaglandins, and the sensitivity of the prostaglandin response to a variety of external stimuli, some of the methodology

problems are intrinsic to any study investigating prostaglandin behaviour.

Of critical significance in this study is the development of two *in vitro* systems which will allow for investigation of cellular regulation and behaviour during orthodontic-type mechanical stress. Both techniques offer an environment for study which diminishes the masking and modulatory factors present in the *in vivo* situation and concomitantly offers significant improvement from cellular culture techniques, which are so far removed from the norm.

Melcher (1980) has suggested that a pathway of events required for the transduction of mechanical force into the resultant cellular behaviour occurring during orthodontic tooth movement involves a series of biological signals. The mechanical stretching of cells, which most likely occurs during orthodontic force applications, has been shown to cause an increase in the amount of PGE_2 synthesized *in vitro* (Harell *et al.*, 1977; Binderman and Cox, 1977; Somjen *et al.*, 1980). Prostaglandins have been implicated in the elicitation of a cAMP synthetic response (Yu *et al.*, 1976). Somjen *et al.*,(1980) have shown that the synthesis of cAMP coincident with the stretching of cells is prostaglandin dependent. Remodelling of hard and soft connective tissues involves the metabolism of collagen. Regulation of collagen synthesis is dependent on the regulation of both the intracellular synthesis of collagen and the intracellular degradation of newly synthesized collagen (Bienkowski *et al.*, 1978). Increased cAMP

production has been shown to be related to an increase in intracellular collagen degradation, and thus it has been suggested that cAMP may be involved in the regulation of collagen synthesis (Baum et al., 1980).

Thus it appears that possible sequence of events involved in the transduction of mechanical force into the observed collagen synthetic response can be advanced. The application of force would result in the physical deformation of cell membranes, with resultant prostaglandin synthesis. This prostaglandin synthetic response would result in activation of the membrane bound enzymes adenylate cyclase and guanylate cyclase, which would be responsible for converting their respective substrates to cAMP and cGMP. These two substances would have an effect upon the eventual amount of collagen synthesized and degraded intracellularly, and perhaps also upon the phagocytosis of extracellular collagens.

Thus a possible chain of events (Fig. VII-1) functioning during orthodontic tooth movement may involve force application, cell membrane deformation, prostaglandin synthesis, cyclic nucleotide synthesis, intracellular synthesis and degradation and extracellular degradation of collagen and thus eventual tooth movement.

The development of the periodontal organ and calvarial culture systems will allow for the investigation of this proposed pathway of events. For example, evidence from this thesis suggests that thromboxane A_2 may be involved at the prostaglandin synthesis step.

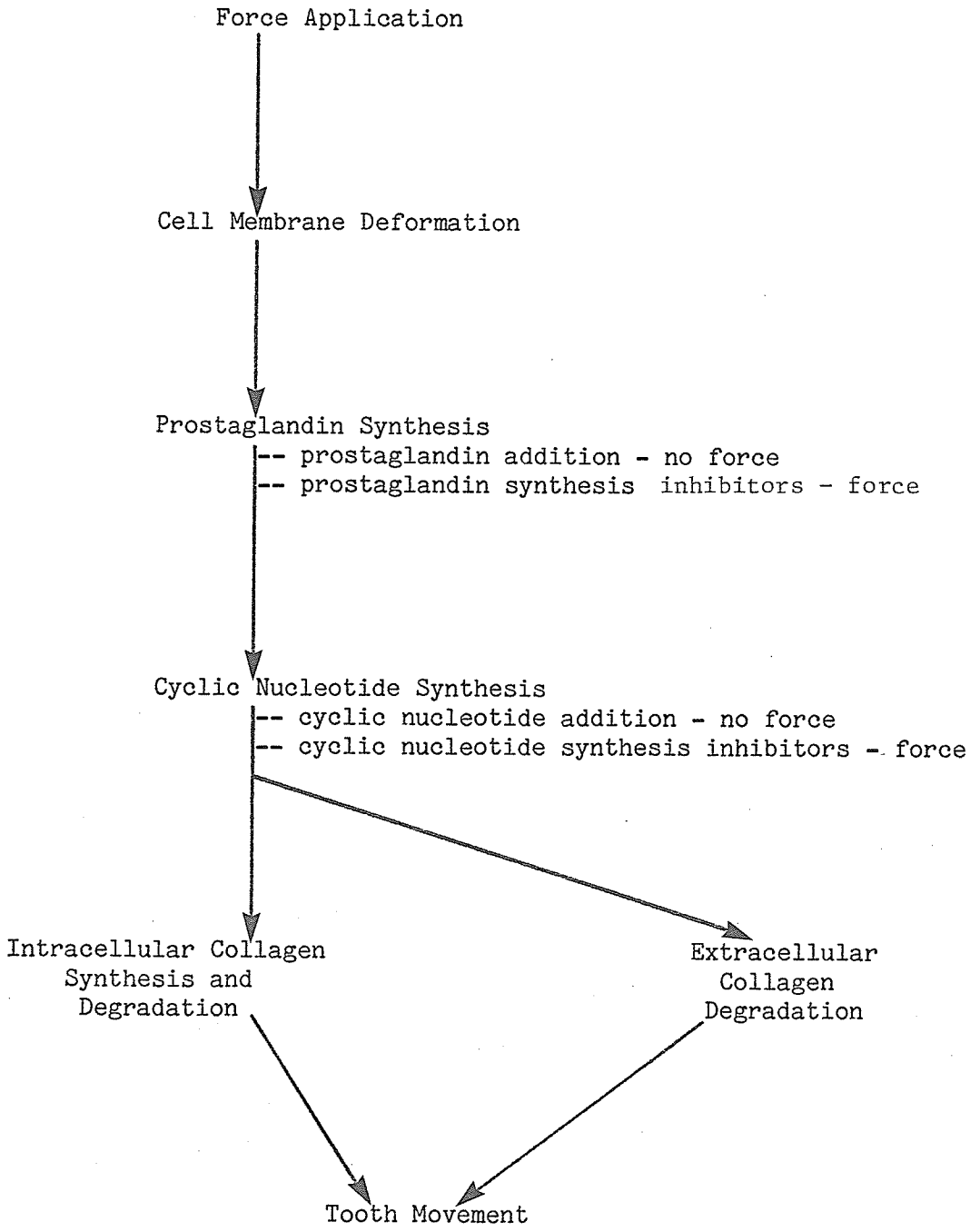
Similarly the collagen synthetic data reported in Chapter 4 represents baseline information against which the hypothesized preceding steps can be tested.

For example, referring to Figure VII-1, the ability of prostaglandins added to non-force stressed explants to mimic the collagen synthetic response during force applications could be investigated. Alternatively, or in addition, the effect of prostaglandin synthesis inhibitors of a general or specific nature could be examined in relation to their effect upon collagen synthesis in force stressed explants. Both of these studies would offer significant information on the possible role of prostaglandins in the proposed cascade of events.

Similarly, once again referring to Figure VII-1, the addition of cyclic nucleotides or their derivatives to non-force stressed explants, or the addition of cyclic nucleotide synthesis inhibitors to force stressed explants, and the effects of these substances upon collagen synthesis, would offer information relating cyclic nucleotides to the proposed sequence of events.

Factors other than prostaglandins have been proposed in cellular regulation during orthodontic tooth movement, for example, piezoelectricity and oxygen tension. Once again experimentation could be designed to examine the role of these parameters in collagen synthetic activity during orthodontic tooth movement.

FIGURE VII-1. Hypothesized cascade of events involved in the transduction of orthodontic force into tooth movement. Proposed areas of future experimentation are marked --



In many of these proposed studies, as with a variety of the biochemical and histological techniques in use today, the use of expensive radiolabelled tracer molecules is indicated. In the past it has been prohibitively expensive for many researchers to become involved in studies necessary in the in vivo situation. The development of the reported culture systems, with their small volume of supportive medium (800 µl), will now allow a larger number of research institutions to become involved in the exciting and important field of cellular regulation in orthodontic tooth movement.

CHAPTER 8

SUMMARY AND CONCLUSIONS

(1). A three molar tooth and surrounding alveolar bone periodontal organ culture system which is capable of receiving orthodontic type forces, and which does not require foetal calf serum in the supportive medium, has been developed.

(2). The vitality of the periodontal explants after 24 hours of culture was demonstrated by radioautographical evidence of protein and DNA synthesis and by biochemical studies indicating collagen and prostaglandin synthesis.

(3). Collagen synthesis in the periodontal organ explant <u>in vitro</u> during periods of force application of 0,4,16,24 hours; 3,5,7,10 and 14 days showed a significant increase in the relative amount of type III collagen synthesized at 1,3 and 5 day periods. As these times correspond to the period of activation of the orthodontic separating spring, this study supports the concept of type III collagen playing a role in rapidly remodelling tissues and in those tissues undergoing active stress.

(4). Prostaglandin synthesis in the periodontal organ explant system demonstrated the presence of five different prostaglandins or their stable end products. These were 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, TXB_2, PGE_2 and PGD_2. No statistically significant fluctuations in the relative amounts of each prostaglandin during periods of force application corresponding to 0, 5, 10, 20, 30, 45, 60 minutes; 2, 4, 8, 16, 24 hours; 3, 5, 7, 10 and 14 days, were found. It was hypothesized that the inability of this study to

reveal discernable alterations in relative prostaglandin levels may be due to the mixture of specific connective tissue remodelling functions which are related to specific stimuli. Thus the increase or decrease of a specific prostaglandin in one localized area of compression may be masked by a concomitant change in that same prostaglandin in an area of tension.

(5). In order to pursue the possibility of prostaglandin responses being dependent upon specific physical stresses, a mouse calvaria in vitro system which is capable of receiving either compressive or tensile forces was utilized. Similar prostaglandin synthetic patterns to those observed in the periodontal organ explant system were described. The wide dispersion of data made only tentative conclusions possible. TXB_2 showed a significant ($p < 0.05$) relative increase after 10 minutes of both compressive and tensile force applications. Thus evidence was presented that thrombane A_2 may be involved in connective tissue remodelling in response to orthodontic-type forces.

(6). The conclusions of the prostaglandin studies were only tentative because of a wide data dispersion. This could be due to a variety of reasons, including prostaglandin analytical techniques, which could be improved by using the more specific radioimmunoassay methodology. But it may also be due to the sensitivity of the prostaglandin synthetic response, which could be triggered by a variety of stimuli, including culture prepara-

tion, and which is thus difficult to overcome.

(7). Analysis of cellular regulators and cell behaviour during orthodontic tooth movement has been difficult in the past because of masking and modulating influences in the in vivo situation, and because of the prohibitive cost of using expensive radiolabelled tracer molecules in live animals. The introduction of a periodontal organ culture system and a calvarial culture system, both of which are capable of receiving orthodontic-type forces, allows, for the first time, extensive study of a variety of hypothesized cellular regulators functioning during orthodontic tooth movement to be accomplished. To this end a proposed cascade of cellular events acting during orthodontic tooth movement has been advanced, and this hypothesis is discussed in relation to the results from this study, and in relation to proposed future studies.

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