THE EFFECTS OF COMPRESSIVE FORCES ON CELLS *IN VITRO*

A HISTOCHEMICAL AND AUTORADIOGRAPHIC STUDY

by

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SUMMARY

Many workers have shown that orthodontic tooth movement is a result of bone remodelling effected by mechanical stress, which is the basis of orthodontic practice. Mechanical stresses act on various forms of both terrestrial and aquatic life. For this reason much time has been spent in a literature review of the biological and medical fields discussing the compatibility between mechanical stress and life, and in the orthodontic field spotlighting various aspects of orthodontic tooth movement related to the effects of forces on alveola tissue components.

In order to better understand the mechanisms of orthodontic tooth movement, experiments were undertaken utilizing isolated cells in tissue cultures to which known forces were applied.

Compressive forces ranging from 10 gm/cm^2 to 80 gm/cm^2, which were provided by a specially fabricated apparatus, were directly applied to mouse fibroblast L-929 cells for periods ranging from 30 minutes to 4 hours.

The effects of the compressive forces on the cells were investigated with various cytochemical methods and with the use of tritiated nucleic acid and protein precursor autoradiography. As a result, cytoplasmic blister formation was observed following compression of cells with 10 gm/cm^2 for 30 minutes, increasing in

proportion with the magnitude of the forces and time. Succinic dehydrogenase and cytochrome oxidase activities were severely reduced by increasing compressive forces and time and acid phosphatase activity was reduced progressively with forces of 60 and 80 gm/cm^2 as the duration of compression increased. However, the Feulgen reaction, periodic acid-Schiff reaction, Sudan black B, glutamic, malic, α-glycerophosphate - and lactic dehydrogenases were not visibly affected under the experimental conditions.

Incorporation of H^3-thymidine, H^3-uridine and H^3-proline was not affected except for a slight decrease following the longest experimental period.

The results are discussed with respect to the biological relations of the techniques employed. It is considered that disturbance of even one cellular function could result in malfunction of other cell components leading to cell death. On these grounds it is concluded that any force applied for a long enough time could overstress cells in the strictest sense. However, if compressive forces must be used to accomplish a certain goal, possible tissue damages could be minimized by the use of optimal forces. Such considerations may have quite practical applications to orthodontics.

Finally, the present results are related to bone resorption and the mechanism of orthodontic tooth movement. Either direct or secondary effects of compressive forces may play an important role

in stimulating either the already existing cells capable of bone resorption, or precursor cells, to differentiate into mature osteoclastic cells. An attempt is made to explain the mechanism by a negative feedback system whose ultimate goal is to retain homeostasis. Before a completely satisfactory mechanism can be proposed, it is emphasized that more information on the milieu of bone resorption, particularly the micro-environment of osteoclasts, is required.

DECLARATION

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any University, and to the best of my knowledge contains no material previously published by another person, except where due reference is made in the text.

The results have been presented in part to a meeting of the Australian Society of Orthodontists, 1972. Some of the material is in preparation for submission to scientific journals for publication.

MASAAKI NAKAMURA

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1. THE PROBLEM

It is one of the great achievements of dentistry that malpositioned teeth in alveolar bone can be moved almost at will so as to bring them into correct occlusion.

In the fields of conservative dentistry, prosthodontia and oral surgery, occlusal rehabilitation often necessitates the use of artificial materials, the biological, chemical and physical properties of which have to be ascertained in order to achieve a final result compatible with physiological hard and soft tissue tolerance. On the other hand, orthodontic tooth movement, as one form of occlusal rehabilitation, utilizes 'self components' so that many of the considerations pertaining to the use of foreign materials are eliminated.

However, orthodontic tooth movement does present different problems. How are the teeth moved to a different location when force is exerted? What kind of responses occur in the tooth and/or its supporting tissues during this movement? A thorough understanding of these problems would seem mandatory in planning proper

treatment which otherwise could be disastrous to the patient.

There are many sites in our body which are subject to mechanical forces. Our legs bear the whole body weight and arteries carry blood which is pumped from the heart under pressure to every corner of our body. Teeth and their supporting tissues, which are the direct object of the present study, also receive various forms of mechanical force. In fact, in carrying out its main function, a tooth is necessarily subjected to considerable mechanical force which, fortunately, is distributed to the supporting tissues so as to permit normal function without damage to either. The integrity of the tooth and its supporting tissues is most effective against those forces which are more or less parallel to the tooth axis. Forces in a horizontal direction, i.e. which are more or less at right angles to the tooth axis, are not so well integrated with the supporting structures by virtue of the anatomical features of the periodontal membrane and the alveolar bone; excessive horizontal forces can easily cause irreparable damage to the periodontal hard and soft tissues. Orthodontic tooth movement frequently requires similar lateral forces.

Based on a knowledge that we can move a tooth in its alveolar socket with mechanical force, the currently available orthodontic appliances aim to achieve the goal of how to effectively apply these forces to obtain optimal tooth movement. In other words, application of a mechanical force is an essential component of today's ortho-

dontic treatment system. As it is unlikely that better alternatives will be forthcoming in the near future, it seems that the best approach at present to attaining optimal orthodontic tooth movement, is to refine the present system. At the outset, then, it is necessary to clearly define the forces applied which terminate with the movement of the tooth into the desired site.

Orthodontic textbooks (for example, GRABER, 1966) accept the principle that tooth movement, as a result of applied force, is due to resorption of alveolar bone by the osteoclasts on the pressure side and to the building up of new bone on the tension side by osteoblastic activity. But the biological mechanisms responsible for the above phenomena remain unresolved, despite many investigations on the subject.

A workshop on malocclusion conducted by the United States National Institute of Dental Research considered the ramifications of research related to malocclusion (MOORREES *et al.*, 1971). Existing knowledge was reviewed and attention directed to many unresolved problems. In its conclusions, the workshop particularly emphasised the importance and need for further investigations on tissue and cellular responses to force derived from orthodontic appliances.

In this respect two experimental approaches are feasible:

1. *in vivo* analysis of overall tissue responses following orthodontic tooth movement, or

2. *in vitro* analysis of isolated cell systems during or after the application of known forces.

Many publications are available which describe the *in vivo* approach. It is, however, extremely difficult to objectively analyse cell or tissue responses to compressive forces using this approach and it is not surprising that the results of *in vivo* experiments show wide variations due to the large numbers of uncontrollable parameters involved.

If one accepts that the cellular components of the tissue play an important role in alveolar bone remodelling in orthodontic tooth movement, then the question must arise - How do the involved cells react to the mechanical stress? An approach utilizing isolated cell systems seems appropriate for providing some answers. Obviously, elucidation of the mechanisms governing bone resorption would prevent possible damage to the tooth or its supporting tissues and make the development of more efficient appliances possible.

It is the purpose of the present study to investigate the direct effects of mechanical stresses on cells; known compressive forces have been applied to cells in a controlled *in vitro* environment.

2. REVIEW OF THE LITERATURE ON THE EFFECTS OF STRESS ON TISSUES

A. In Biological Systems

In the general field of biology the effect of mechanical stress has been investigated in a number of ways. The beginning of investigations on this problem appears to have stemmed from the realization that many organisms live in deep sea where the hydrostatic pressure must be very high.

REGNARD (1885a, b, c) observed the influence of various pressures on the activity of small aquatic organisms such as Cyclops, Daphnia and Gammarus pulex. With an initial application of pressures not exceeding 100 atmospheres (see Appendix for force units, which have been converted to standard terminology from the arbitary units employed by each investigator), the animals became agitated. Above this pressure they became tetanized and fell slowly to the bottom of the vessel, apparently incapable of movement. If the period of compression was short, the animals instantly recovered their activity upon the sudden release of the pressure. Longer periods of compression produced a coma, recovery from which required several hours at normal pressure. Still longer periods of compression caused death.

Since the time of REGNARD the trend of research on the biological effects of hydrostatic pressure has been influenced to a considerable

extent by progress in solving the engineering problems of achieving high pressures of up to 100,000 atmospheres in the laboratory (JOHNSON *et al.*, 1954).

LEPESCHKIN (1927) applied weights of 1.5 kg directly to Spirogyra neglecta, which resulted in coagulation of protoplasm. He also observed that granules appeared slowly in the homogeneous substance of the protoplasm and nucleus and they increased rapidly until the death of the Spirogyra. The mechanical stress led to a loss of selective permeability, observable in the first instance by increased permeability for water and water-soluble substances, and diffuse vital staining of protoplasm.

LINSBAUER (1929) applied mechanical pressure to Chara foetida with a specially designed "lateral compressor" clamp and observed protoplasmic flow caused by the stress.

According to CAMERON (1952), protoplasmic coagulation resulting from mechanical stress is reversible if not too severe, disappearing in 10 to 15 hours following removal of the stress, and is influenced by temperature and light.

High hydrostatic pressures have been applied to various biological entities. BROWN (1934) applied pressures of 403 atmospheres to the eggs of Arbacia punctulata in a pressure-centrifuge. He observed a decrease in the viscosity of the egg as the pressure increased.

MARSLAND and BROWN (1936) devised a special chamber to allow rapid compression of Amoeba dubia and Amoeba proteus. The compression forces ranged up to 450 atmospheres. The first effect noted was a cessation of amoeboid flow by sudden compression to about 250 atmospheres. This was followed by a sudden shrinkage of the long pseudopodia and reorganization of the pseudopodia into spherical shapes within about 5 minutes. They noted that these effects were largely dependent on the speed of compression.

In further experiments, MARSLAND (1938, 1939,a,b, 1944) observed the effects of pressure on the cleavage division of Arbacia eggs from which they suggested that the plasmagel band in the furrow region of the egg played an important role in egg division.

PEASE and KITCHING (1939) used a pressure bomb in which it was possible to apply pressures of up to 10,000 pounds/inch^2(psi) to the biological materials. When 1,000 psi or more was applied to Mytilus gills they observed an immediate increase in the frequency of the beat of the lateral cilia. At pressures of 5,000 or 6,000 psi, the frequency of ciliary beat fell rapidly and permanent injury was observed.

PEASE (1940a) observed the effects of pressure on protoplasmic streaming in Plasmodia. With pressures of 1,000 to 3,000 psi, smoothening of the surface and acceleration of flow of the plasmagel were observed, while a dramatic decrease of flow was reached at a

critical pressure level of 4,000 psi.

PEASE (1940b) also applied stress, equivalent to between 11,000g and 123,000g, to Cumingia and Chaetopterus eggs by centrifuging. He found a shift of the cleaving polar axis of the eggs at high pressures.

MARSLAND and BROWN (1942) investigated the effects of pressure on the sol-gel equilibria of various protoplasmic gels. They found that gelation and solation reactions occurred with much greater velocity in myosin and other protoplasmic systems compared to that found in methylcellulose and gelatin systems.

PEASE (1946) later observed that with pressures of 5,000 to 7,000 psi the spindle of Tradescantia pollen mother cells reversibly liquefied, while condensed chromosomes were softened at these pressures and liquefied by pressures of 15,000 psi.

MARSLAND (1950) later utilized both the pressure chamber and the pressure-centrifuge to exert pressures of up to 16,000 psi in temperatures ranging between 0^{o} and $35^{o}C$. He found that egg division in Arbacia punctulata was susceptible not only to increasing pressure, but also to decreasing temperature.

LANDAU and MARSLAND (1952) studied the effects of pressure on the muscle contraction rate of tissue culture explants of the heart of Rana pipiens. Again, they found the effects were largely dependent on temperature. With pressures of 4,000 psi the contraction

rate was decreased at temperatures of 14°C to 16°C or lower and increased above this temperature range up to 35°C.

In subsequent publications this group of investigators confirmed the antagonistic relationship between pressure and temperature as they affected biological systems (LANDAU *et al.*, 1954; MARSLAND, 1956).

In order to study sol-gel changes in mammalian cells, PADAWER *et al.* (1958) applied high hydrostatic pressures of 7,000 to 13,000 psi to rat peritoneal mast cells. They observed deformation consisting of cell elongation, sedimentation of the cytoplasmic granules and cell fragmentation, which were reversible. The behaviour of mammalian cells was similar to that of the non-mammalian cells previously studied.

ZIMMERMAN *et al.* (1958) investigated the effects of certain chemicals on the responses of cells subjected to high pressures. They found a consistently higher stability of pseudopodia of Amoeba proteus treated with adenosine triphosphate and adenosine monophosphate at pressures of 5,000 psi, while adenosine, dinitrocresol, or a combination of adenosine mono- or triphosphate and dinitrocresol had no influence.

LANDAU (1960) further investigated the effect of high hydrostatic pressures on mammalian cells. He applied pressures of up to 10,000

psi to embryonic chick heart fibroblasts grown under *in vitro* conditions at 35°C. He observed that below 7,000 psi there occurred little or no change in cell shape, but an increasing number of cells became round or ovoid as the pressure was raised; between 75 per cent and 80 per cent of the cells being changed in this manner at 9,000 and 9,500 psi; marked irreversible effects were seen beyond this pressure.

LANDAU (1961) applied the same order of pressures at 35°C to human cells in primary and continuous cultures, such as primary cultures of amnion cells, continuous cultures of Chang conjunctiva cells, foreskin cells, sternal marrow cells, FL-amnion cells, HeLa cells, S-3 HeLa cells and sternal marrow (Detroit-6) cells. He found almost identical changes to those previously reported, namely, rounding of cells, the extent of which was proportional to the amount of pressure; these effects were completely reversible at the pressures used.

In addition to the largely morphological studies quoted above using mammalian whole systems, a number of biological materials such as proteins, enzymes, viruses, antigens, antibodies and micro-organisms, were also investigated with respect to the effects of hydrostatic pressures in excess of 1,000 atmospheres on their activity.

BRIDGMAN (1914) recorded a slight stiffening of albumin at 5,000 atmospheres and complete coagulation at 7,000 atmospheres, while

BRIDGMAN and CONANT (1929) found that carboxyhemoglobin coagulated at 9,000 atmospheres.

DOW *et al.* (1940) found coagulation of insulin without loss of physiological activity at 10,000 atmospheres.

LANDAU (1966) demonstrated that incorporation of C^{14}-leucine and C^{14}-glycine was markedly reduced in E.coli at pressures of 10,000 psi but were similar to the controls at 6,000 psi. Increased amino acid incorporation compared to controls was found at 4,000 psi. Incorporation of C^{14}-adenine was similar to the two amino-acids. However, two important differences were noted; at 10,000 psi, protein synthesis was completely inhibited after 20 minutes, while nucleic acid synthesis was maintained, although at a greatly reduced rate.

POLLARD and WELLER (1966) studied the effects of hydrostatic pressure on protein and nucleic acid synthesis, as well as on induced enzyme synthesis in E.coli. They found that there was a cessation of the formation of β-galactosidase at pressures of 450 atmospheres and greater. At these pressures, general protein synthesis was affected more than the incorporation of thymine and uracil into nucleic acids.

LANDAU (1967) also investigated the induced synthesis of β-galactosidase in E.coli under pressure. He reported that transcription seemed least affected, being normal at 265 atmospheres and continued at a reduced rate at 670 atmospheres; induction was totally inhibited above 265 atmospheres.

YAYANOS and POLLARD (1969) reported that in E.coli the rate of C^{14}-leucine incorporation diminished to zero at about 580 atmospheres and that of C^{14}-uracil incorporation to zero at about 770 atmospheres. C^{14}-thymine incorporation was observed at pressures below 500 atmospheres, but no further incorporation was observed with higher pressures.

Utilizing autoradiographic procedures, ZIMMERMAN (1963) and ZIMMERMAN and SILBERMAN (1964) found DNA synthesis in fertilized Arbacia eggs was unimpeded at pressures as high as 5,000 psi even though morphological mitotic activity had ceased. At pressures of 7,500 psi and greater incorporation of H^3-thymidine was inhibited.

Utilizing pulse labelling with H^3-uridine, YUYAMA and ZIMMERMAN (1969) reported that hydrostatic pressure equally suppressed the synthesis of all major classes of ribonucleic acid (RNA) obtained from the fractionation of Tetrahymena pyriformis GL by either methylated albumin Kieselguhr chromatography or by sucrose gradient centrifugation.

Recently, LANDAU (1970) studied the effects of hydrostatic pressure on RNA and protein synthesis in cultures of S-3 HeLa cells. He found that the incorporation of labelled amino acids at pressures of 4,000 psi was comparable to that at atmospheric pressure, while at pressures of 5,000 psi and greater, the incorporation was markedly decreased. Incorporation of C^{14}-uridine into RNA decreased pro-

gressively with pressures ranging from 2,000 to 10,000 psi. Immediately upon decompression, the rate of incorporation of amino acids and uridine returned to normal.

MACHEBOEUF and BASSET (1934) investigated the effects of high pressure on the activity of enzymes. They found that the activity of enzymes such as yeast saccharase, various lactases, trypsin and amylase remained unchanged at pressures below 5,000 - 6,000 atmospheres, some were destroyed at 8,000 - 9,000 atmospheres while others required still higher pressures for inactivation.

CURL and JANSEN (1950a,b) applied a pressure of 7,600 atmospheres to trypsin, chymotrypsin, pepsin and chymotrypsinogen. As might be expected the effects were pH dependent when pressure was applied. They found that inactivation of pepsin commenced at pH's below 5.2 and that it became less stable with increasing acidity. Trypsin and chymotrypsin were scarcely affected at pH 3.0 but partially inactivated at pH 7.6, and chymotrypsinogen was partially inactivated at pH's ranging between 3.1 to 7.6 at this pressure.

MORITA and ZOBELL (1956) reported that some inactivation of E.coli succinic dehydrogenase occurred at 200 atmospheres and the amount of inactivation increased progressively with the time of compression. Approximately half of the enzyme was inactivated after 4 hours at 600 atmospheres and virtually all was irreversibly inactivated after 4 hours at 1,000 atmospheres.

MORITA (1957) obtained similar results with E.coli formic and malic dehydrogenases under pressure.

MORITA and HOWE (1957) studied the phosphatase activity of marine bacteria under hydrostatic pressure. They reported considerable variations in the sensitivity of phosphatase to pressure, i.e. the enzyme activity of some marine bacteria increased as the pressure increased, the activity of others decreased, while in some it remained relatively constant.

SUZUKI and KITAMURA (1963) reported that the rate of inactivation of the α-amylase of Bacillus subtilis became progressively greater as pressures increased up to 5,000 kg/cm^2.

HILL and MORITA (1964) tested the activity under pressure of isolated mitochondria dehydrogenases obtained from Allomyces macrogynus cells. They found that increased hydrostatic pressures brought about a decrease in mitochondrial dehydrogenase activity in all cases. From this, they postulated that at pressures of 600 atmospheres or more, operation of the tricarboxylic acid (TCA) cycle in Allomyces macrogynus would cease.

WEIMER and MORITA (1968) reported decreased gelatinase activity in marine Vibrio between 1 and 200 atmospheres, and a great fall in activity between 200 and 300 atmospheres. However, increasing the pressure up to 600 atmospheres had no further effects on the enzyme.

BASSET *et al.* (1935) found inactivation of viruses, such as Herpes virus (rabbit) at 3,000 atmospheres, Fièvre aphteuse virus (guinea pig) at 4,000 atmospheres, Tobacco mosaic virus at 8,000 atmospheres and attenuation of Yellow fever virus (monkey) at 3,000 atmospheres and Rabies virus (rabbit) at 4,000 atmospheres.

BASSET and MACHEBOEUF (1932, 1933) investigated the effects of high pressure on the activity of certain antigens and antibodies. They found that with pressures of 13,500 atmospheres, cobra venom and tuberculin remained potent antigens, while diphtheria toxin and equine antitoxin were partially inactivated and tetanus toxin was inactivated. With pressures of 17,600 atmospheres they found diphtheria toxin to be completely inactivated.

ROGER (1895) applied pressures of 3,000 atmospheres to micro-organisms. He found that Colon bacteria and Staphylococci were unaffected, Streptococci were killed or retarded in growth and the Anthrax bacillus lost some virulence.

LARSON *et al.* (1918) found that the vegetative cells of various bacillus species of bacteria were killed with pressures of 6,000 atmospheres, and that the spores were killed with pressures of 12,000 atmospheres.

JOHNSON *et al.* (1942) applied pressures of 476 atmospheres to the luminescent bacteria, Photobacterium phosphoreum and Vibrio phosphorescens. They found that luminescence intensity was closely

dependent upon the temperature-pressure relationship. According to them, there are optimum temperature levels for the expression of luminescence in these bacteria, namely, approximately 20°C for the Photobacterium and 30°C for the Vibrio. At temperatures above the optimum, the effect of pressure was to increase luminescence. At temperatures below the optimum, on the other hand, the effect of pressure was to decrease the intensity; at the optimum temperature the effect of pressure was negligible.

At the time, these results suggested the practical possibility of using high pressures for the sterilization of solutions or for separating and/or distinguishing between pressure-sensitive and pressure-resistant biological components.

The ultrastructure of cells under high hydrostatic pressure has also been investigated. LANDAU and THIBODEAU (1962) applied a pressure of 8,000 psi to Amoeba proteus for 20 minutes at 15°C. They observed loss of the Golgi complex and pinocytosis channels, but otherwise there was little change in the appearance of the mitochondria and other cytoplasmic structures.

TILNEY *et al.* (1966) examined the microtubules of Actinosphaerium nucleofilum subjected to pressures. They found disintegration of microtubular elements of the axopodia and cytosome under pressures ranging from 4,000 to 8,000 psi, but little changes in other organelles.

KENNEDY and ZIMMERMAN (1970) also observed a breakdown of the central ciliary microtubules of Tetrahymena pyriformis at pressures of 7,500 or 10,000 psi.

BOATMAN (1967) reported on the ultra structural changes of bacteria under high pressure. He observed numerous invaginations of the cell wall and a reduction in the number of ribosomes of E.coli at pressures of 400 atmospheres. On the other hand, he found an increased amount of nuclear material and decreased numbers of ribosomes in a species of Corynebacterium at the same pressures. At 270 atmospheres, there was loss of nuclear material in a species of Vibrio and scarcely discernible mesosomes and thicker cell walls in Bacillus mycoides.

Marine and other cells were also investigated for ultra-structural changes under high pressures. ZIMMERMAN and PHILPOTT (1968) reported the complete disorganization of the cytoplasmic microtubules of Arbacia eggs in metaphase under pressure of 10,000 psi for 1 minute.

TILNEY and GIBBINS (1969) found that the ciliary microtubules from Arbacia gastrulae under pressures of 6,500 psi for 1 hour were unaffected, although the cytoplasmic microtubules were no longer visible.

TILNEY and MARSLAND (1969) reported that, with a pressure of

6,000 psi, the nuclei of Arbacia eggs remained intact and no sign of furrow induction was seen, while with 10,000 psi vigorous furrow induction was observed and the nuclei were absent from almost all the eggs.

TILNEY and CARDELL (1969) applied a hydrostatic pressure of 6,500 psi to segments of the small intestine of the salamander and found a reduction in the number of microvilli and a loss of the terminal web in the epithelial cells. On the termination of compression, they observed the reformation of the microvilli and the terminal web.

B. In Medicine

(i) Mechanical Stresses

In the medical field, the effects of mechanical stress on tissues is a major issue related to growth and development of bone and other tissues of particular concern to orthopaedic surgeons. Bone is the tissue best described in the body from the viewpoint of the effects of mechanical stress, because, as the main supporting system, it receives various forms and magnitudes of mechanical stress. Bone structure has long been explained by the trajectorial theory first advanced by MEYER in 1867 (quoted by MURRAY, 1936), the fundamental principle of which can be paraphrased as follows: if a block of homogeneous elastic material is subjected to force, then at any point

in the material, there are two directions of principal stress, at right angles to each other, which generally correspond to the directions of maximum tension and pressure. The closer these imaginary lines, known as "trajectories" in anatomical theory, are to each other, the greater will be the principle stresses in a given region.

For a structure to best resist the deforming action of an external force the composite material should be so arranged that its greatest mass is concentrated where the stress is greatest and orientated along the lines of principle stress. The fundamental idea in the trajectorial theory of bone structure is that the trabeculae of cancellous bone follow the lines of trajectories in a homogeneous body of the same form as the bone and stressed in the same way (MURRAY, 1936).

WOLFF (1892) extended the trajectorial theory to cover the alterations of bone in various environments. Based on the study of a large number of specimens, he concluded that bone structure which was originally modelled by the trajectories produced by normal stresses, could be modified drastically by subsequent mechanical stresses producing a new trajectorial system.

JANSEN (1920) also studied a number of bones whose structures had been modified in various ways, and found that increased pressure always led to strengthening of the bony structure, while increased tension led to atrophy. He concluded that, although the shape of a particular bone is genetically determined, the elements of each

bone will align themselves according to the direction of functional pressure on the bone. This accords with WOLFF's view.

Since then, many experimental approaches to the problem have been made. JORES (1920) applied fixed weighted bags for more than 100 days over the spines of the thoracic vertebrae behind the shoulder blades of young guinea pigs and rabbits, thus exerting pressure on the spinous processes through the skin. He found that bone, when constant pressure was applied, underwent atrophy, but that removal of the pressure was followed by active growth of the bone; intermittent pressure clearly increased bone growth.

LOESCHKE and WEINNOLD (1922) studied a number of skulls which showed that resorption of bone from the inner side of the skull wall was associated with the weight of the skull contents exerting pressure on the bone, while deposition of bone appeared to be dependent on the intra-cranial fluid pressure. They observed that, in a young growing skull, both inner and outer walls were covered with secondary bone, but the impressiones digitatae regularly showed resorption. In older skulls, on the other hand, there was more active apposition of new bone on the inner surface of the skull roof and the impressiones digitatae disappeared; on the floor of the skull resorption continued. The authors believed that the vascular system was directly responsible for skull bone remodelling when pressure was exerted either by brain growth or by gravity effects.

GRÜNBERG (1937) studied tooth eruption in the grey-lethal mouse strain and discussed the relation of external and internal factors in bone development. He considered that bone reacted by absorption to pressure if the hereditary basis for doing so was undisturbed, and concluded that the relation between stimulus and response was not direct, but subject to conditions inherent to the constitution of an animal.

GLÜCKSMANN (1938, 1939, 1942) cultured chick endosteal tissue, derived from the tibiae, between two ribs *in vitro*. During the subsequent culture period the ribs approached each other thus exerting pressure on the endosteal tissues, which produced a reorientation of the embryonic tissue. This was more readily produced in less ossified than in highly ossified cultures. He noted that mechanical stresses caused cartilage formation in the perichondrium and periosteum. He thought that pressure was not an essential factor in chondrogenesis but that it could act as a mechanical initiator of cartilage formation and set up conditions favouring the formation of hyaline ground substance. In 1942 he repeated his initial protocol for exerting pressure on cartilaginous rudiments and demonstrated that pressure and tension stresses exerted on cartilage *in vitro* caused the reorientation of the cartilage cells, promoted bone formation in osteogenic tissue and determined the pattern of osseous architecture.

AMPRINO (1958) investigated the physical properties of pieces of

compacta obtained from the diaphysis of the long bones of birds, calves and horses. Applying forces of 15 to 300 gm he recorded microhardness values that were 20 to 25 per cent higher when the measurements were made on a parallel plane to the long axis than when the forces were applied at right angles to the long axis of the collagen fibres. Microhardness values were generally higher as the degree of calcification of the bony tissue increased. Drying of the bones resulted in increased microhardness, as compared with a decrease after carbonization.

BASSETT and HERRMANN (1961) studied the effects *in vitro* of mechanical stresses and oxygen tension on chick embryonal tibia cortex. They found that tibia cultures under pressure and high oxygen tension resulted in bone tissue in 100 per cent of specimens. On the other hand, under mechanical pressure and low oxygen tension they observed no bone tissue, but hyaline and fibrocartilage.

In a review of the subject, BASSETT (1962) emphasized the influence of environment on cell function during osteogenesis.

LISKOVA (1965) applied mechanical stresses such as pressure, traction and bending to the diaphysis of rabbit tibia. Forces applied ranged between 0.15 to 3.65 kg/mm^2. The author found no significant changes in the form and size of the bone brought about by any of the stresses applied.

Recently, bone has been investigated more from the biomechanical

point of view. ZAREK (1966) pointed out that bone was not homogeneous as an engineering material and that it showed a marked degree of anisotropy, which indicated that the elastic properties of bone were uniformly identical throughout any one specimen. Studies of bones fractured under dynamic conditions showed that bones such as the femur, the skull or the pelvis represented excellent energy absorbing structures.

SEDLIN and SONNERUP (1966) studied the effects of stresses on samples of wet femoral cortical bone from a rheological viewpoint. Their results showed that bone was a more complex material than could be characterized by such idealized models as represented by the perfectly elastic body, the perfectly viscous body, the rigid plastic body and the perfectly plastic body. They formulated a model for the behaviour of bone under moderate load which enabled them to predict bone behaviour under complex circumstances that cannot be tested in the laboratory.

BAUER and KOSHINO (1968), KOSHINO and RANAWAT (1970) and KOSHINO (1971) employed radioisotopes to investigate the effect of weightbearing forces on the healing process following tibial osteotomy in man. Strontium^{85} levels were counted in 27 knees before and 1 to 3 years after tibial osteotomy for the correction of various angular deformities associated with arthritis. They found higher counts in osteotomy regions. Within the plane of the osteotomy incision they observed a significantly higher uptake of strontium^{85}

where normally the greater weightbearing force was applied. They interpreted the results to mean that pressure on bone accelerated the rate of bone formation.

HERT (1969) studied the influence of continuous bending forces on the tibia of rabbits. He found a slight curving deformation of growing bones, but no deformation or other reactions in mature bone even after application of bending forces for 9 months.

EVANS and VINCENTELLI (1969) investigated the relationship between collagen fibre orientation and the mechanical properties of human cortical bone. They observed significant correlations between the different osteon systems in which the predominant direction of the collagen fibres was visible, and the mechanical properties.

PERREN *et al.* (1969) studied the reaction of cortical bone to compression, in conjunction with bone healing following fracture. They found no pressure necrosis at the fixed fractured joints which received compressive forces of up to 400 kg/cm^2, but rather, direct Haversian remodelling at the osteotomy region.

RODBARD (1970) reviewed the effects of mechanical stresses on the connective and cardiovascular tissues. Based on investigations of cardiovascular systems subject to various mechanical stresses, he applied the concept of negative feedback to explain the induction of the tissue changes to accommodate stresses, as follows:

Certain cells of the cardiovascular system are sensors for specific mechanical stresses such as tension, compression, etc. and respond by elaborating and orientating specific structural materials to overcome the effects of these stresses.

CHAMAY (1970) compressed dog ulnae in their long axis to the point of fracture and recorded the force deformation curves of the elastic and plastic deformation phases. He observed histologic and electron microscopic slip lines in the compressed bone cortex, which he proposed was a manifestation of plastic bone deformation.

CURREY (1970) reviewed the mechanical properties of bone, notably such features as the modulus of elasticity, compressive strength, bending strength, hardness and fatigue. He pointed out the difficulty of developing approaches for understanding the properties of a complex structure like bone which was composed of two such dissimilar materials as collagen and hydroxyapatite.

RADIN *et al.* (1970) applied compressive forces to bovine subchondral bone and articular cartilage and compared their ability to transmit forces. They found that both were capable of deforming under pressure and considered that this contributed to the attenuation of applied forces on a joint so as to efficiently support the required loads.

CHAMAY and TSCHANTZ (1972) applied intermittent, static and overload compressive forces to dog ulnae. They observed adaptive

hypertrophy of the long bones on the concave side only under conditions of intermittent compression.

Investigations concerning the effects of mechanical stress on soft tissues have been made with reference to the bed-sore problem.

HUSAIN (1953) applied pressures of between 100 and 800 mm Hg by means of a plethysmograph and a pressure cuff to guinea pig legs and rat tails. He found that the ischaemia caused by pressure led to local lesions. Low pressures, maintained for long periods of time, produced more tissue damage than high pressures for short periods. He also found that the same pressure evenly distributed over the body was less damaging to the tissues than pressure distributed only over a localized area.

KOSIAK (1959) produced ischaemic ulcers in dogs with pressures of up to 550 mm Hg for periods of 1 to 12 hours. He found microscopic pathologic changes with pressures as low as 60 mm Hg for only one hour.

LINDAN (1961) applied pressures of 20 to 100 mm Hg to rabbit ears. He observed no appreciable damage with pressures of 20 and 40 mm Hg, but severe damage with pressures of 60 and 100 mm Hg.

In the dental field, the effects of mechanical stress have also been investigated. NIKOLOW and JORDANOW (1969) applied compressive forces to dog hard palates and observed narrowing of the

lumina in the large veins and dilatation and deformation of the small veins and venules in the connective tissue of the mucosa.

KYDD *et al.* (1969) applied pressures of 5 and 20 gm/mm^2 to the palatal mucosa of dogs for periods of between 15 and 360 minutes. They found the original thickness of the oral mucoperiosteum reduced by 45 per cent with forces of 5 and 20 gm/mm^2 applied for 6 hours with vacuolation and decreased staining of the cytoplasm, cellular swelling, increased nuclear size and intercellular oedema of the middle and upper layers of the stratum spinosum. The changes in the lamina propria and sub-mucosa were more severe with the application of 20 gm/mm^2 than with pressures of 5 gm/mm^2. There was no osteoclastic activity at the surface of the bone directly below the pressure point or in the surrounding area.

Investigating alveolar bone resorption under full dentures, which distributed the biting force over a wide area, KELSEY (1971) examined the effects of factors such as occlusal forms, alignment of the teeth, deformation of the denture bases, loss of occlusal vertical dimension, etc. Apart from the loss of volume and shape of the residual alveolar ridges which is common and considered to be a normal physiological process, he was unable to explain the cause of the bone resorption in patients wearing complete artificial dentures.

(ii) Effects of Electric Currents and the Piezoelectric Phenomenon

Consideration of the electric effects produced by stresses has assumed greater importance in the field of calcified tissue research in more recent years. It is well known that small electric currents of the order of 10^{-9} c.g.s.e.s.u. (centimeter-gram-second electrostatic units) are produced when bone is stressed. This piezoelectric phenomenon has been investigated as a possible trigger mechanism for remodelling bone under stress. This section, therefore, considers the literature on this subject.

YASUDA *et al.* (1955) first demonstrated that electrical potentials were generated when bone was compressed. Stimulated by KÜNTSCHER's work (1935), which had postulated that calluses were formed by mechanical, thermal, and chemical stimuli without necessarily leading to fractures, they fitted a device to rabbit femurs to exert compressive forces. They found negative potentials in the compressed portions of the long bones and positive potentials in those parts under tension. As they observed similar electric potentials in boiled and in decalcified bones under compression, they thought the electrical phenomena were caused by the strain of the collagen fibres. They considered the possibility of forming callus merely by electrical energy. Using a 1.5 volt battery attached to rabbits they maintained a current of 1 μA through the femurs for 3 weeks *in vivo*. The resulting callus was orientated in the periosteum between the anode and

cathode, but more callus was formed nearer the cathode. From these experiments the authors concluded that dynamic energy exerted upon bones may generate electrical effects which could play an important role in bone remodelling, even to the extent of causing callus formation. This work led to much research on the effects of electrical current on bone remodelling.

IIDA *et al.* (1956) reported increased bone formation in rabbit femurs stimulated with alternating currents.

FUKADA and YASUDA (1957) published their now famous article "On the Piezoelectric Effect of Bone". They undertook measurements of the magnitude of the piezoelectric currents in bone and found that the piezoelectric effect was manifest only when the shearing forces were such as to cause the collagen fibrils to glide over each other. The magnitude of the piezoelectric currents depended on the angle between the applied pressure and the axis of the bone. The authors concluded that the origin of piezoelectricity in bone might be ascribed to the crystalline micelle of the collagen molecules. Similar findings were reported by BASSETT and BECKER (1962), BASSETT *et al.* (1964) and PAWLUK and BASSETT (1970).

SHAMOS *et al.* (1963) confirmed the presence of electrical effects in a number of whole bones from different anatomical sites and species when the bones were subjected to bending and compression stresses.

In 1964 FUKADA and YASUDA studied piezoelectric effects on collagen, which they considered as the origin of the electric responses in bone under compression. Using ox and horse Achilles tendons, in which the collagen molecules were highly oriented and crystallized, they observed direct and converse piezoelectric effects. The authors suggested that the piezoelectric effect was due to the polarization or displacement of the interchain hydrogen bonds in the collagen crystals.

According to BASSETT (1965, 1966) using WOLFF's law (WOLFF, 1892) as a basis, the orientation and mass of bone structures are controlled by a negative feedback system in which the essential factors are:

(i) the initiating environmental signal,

(ii) a sensor to detect and convert the signal to a meaningful biologic response (a transducer),

(iii) a sensor to translate this response to a reaction which will either stop or correct the original signal,

(iv) cellular activity proportional to the translated signal, and

(v) structural changes appropriate to stabilize the reduction of the original signal. The system recycles until the magnitude of the original signal reaches a point below the threshold level for the system.

BASSETT (1965, 1966) and BECKER (1966) postulated that the

collagen-hydroxyapatite junction of the bone acted as the transducers for the system, and in effect could be regarded as having semi-conductor properties. According to them, collagen behaves as a negatively charged carrier and hydroxyapatite as a positive carrier. As a result, these oppositely charged semiconductors formed a highly sensitive PN (positive-negative) junction diode which could transduce and translate incoming signals to bone cells. Their hypothesis differs from that of FUKADA and YASUDA (1964) who considered that the origin of piezoelectric effects in bone was the polarization or displacement of hydrogen bonding in the polypeptide chains of the collagen crystals.

CERQUIGLINI *et al.* (1967) subjected whole living plants and bones of various animals *in vivo* and *in vitro* to bending forces. They considered that the electrical effects so produced were analogous to the setting up of potential differences in veins due to the flow of a liquid under pressure in contact with the "solid" vessel wall(streaming potential). The potential difference so produced was linearly dependent on the electro-kinetic potential that existed at the solid-liquid interface.

BODEMER (1964) demonstrated regeneration by electrical stimulation of the nerve supply in amputated forelimbs of adult Anuran.

SMITH (1967) amputated the forelimbs of adult frogs, implanted an electrode and applied a low electric current for 3 months. He observed significant regenerating effects in adult Anuran limbs with

small local electrical potentials. He considered either one or a combination of three factors as being responsible for the induction of the regeneration:

(i) direct stimulation of the cells,

(ii) electrophoretic transfer of information-bearing molecules, or

(iii) stimulation of nerves.

BECKER and MURRAY (1967, 1970) investigated the effects of small electrical currents on cellular dedifferentiation *in vitro* using amphibian erythrocytes and healing fractures. They observed hard tissue regeneration and various sequences of morphological changes in the erythrocytes, which they assumed were directly due to the applied electrical currents.

McELHANEY (1967) mapped the electrical charge distribution over human femurs stressed *in vitro*, and noted a wide variation over the bone surface, even in different parts of concave or convex sides. He considered that the charge variations between areas arose from the complicated three-dimensional orientation of the collagen matrix.

ANDERSON and ERIKSSON (1968) studied the electrical properties of wet collagen derived from human Achilles tendon in an environment mimmicking the *in vivo* situation and were unable to demonstrate a piezoelectric effect in completely wet collagen. They repeated the experiments of FUKADA and YASUDA (1957, 1964) who had used dried materials, and confirmed their results. ANDERSON and ERIKSSON con-

sidered that water increased the symmetry of the collagen molecule, therefore preventing piezoelectric effects. They agreed with CERQUIGLINI *et al.* (1967) that streaming potential was the origin of electric currents when bone was stressed *in vivo* and not the piezoelectricity of collagen, nor a semiconductor system. Their results are interesting, because bone collagen *in vivo* is in a fully hydrated condition bathed in tissue fluid.

FRIEDENBERG and KOHANIM (1968) applied direct current to rabbit tibias *in vivo*. They observed destructive changes occurring at the positive electrode and minimal changes in the connective tissue, cartilage and bone at the negative electrode. They concluded that the application of physiological direct currents does not stimulate the growth of rabbit tibias when the electrodes are place on either side of the epiphyseal line.

FUKADA (1968) found piezoelectric effects in dehydrated blood vessels, the intestine and trachea.

STEINBERG *et al.* (1968) found that bone either *in vivo* or following excision,consistently generated electrical potentials when stressed. Prolonged storage or freezing of bone resulted in the generation of nearly identical electric potentials, but none could be detected in dry, decalcified or deproteinized bone. Their results differ from those already reported by other workers.

O'CONNOR *et al.* (1969) applied a low level of direct current to bone *in vivo* and reported abundant bone formation at the cathode, thus confirming the work of BASSETT *et al.* (1964). However, they noted a considerable biological variation in the response of bone to electric currents even within a single experimental group.

LAVINE *et al.* (1969) discussed the effects of the experimental methods used on the response of bone *in vivo* to electric currents. They pointed out that some of the discrepancies between the results of BASSETT *et al.* (1964) and those of O'CONNOR *et al.* (1969) could be explained by foreign body tissue reactions to the implanted experimental materials, or to the overheating effect of the current, or to variations in the current applied during the experiments.

When tibias were fractured FRIEDENBERG and SMITH (1969) recorded an increase of electronegativity over the entire bone with the greatest increase at the fracture site.

MARINO and BECKER (1970a) discussed some possible direct effects of piezoelectric current on cellular activity. They proposed that current produced by physical processes, such as stress or fracture, might influence the bone cells to change from one specialized function to another. They (1970b) applied low levels of electric current through rat tail collagen solutions and observed the formation of an opaque band (which they maintained was due to electrolysis which begins above 1.7 volts) in the vicinity of the cathode at times

inversely dependent on the applied voltage. The bone stressing potentials were too small to produce an orientation or aggregation of the tropo-collagen molecules. They suggested that if the electric potentials were related to the growth of bone they acted through some other mechanism.

DWYER and MATTHEWS (1970) observed electrical responses of dried, recently excised, and living bones when subjected to stress; drying reduced the voltage response.

ANDERSON and ERIKSSON (1970) studied both dry and wet bones and confirmed their previous results (ANDERSON and ERIKSSON, 1968) which showed that the main source of electrical effects in wet bone subjected to mechanical stress was the streaming potentials. However, they considered that some piezoelectricity would continue to be generated when fully hydrated bone was stressed, due to interference with water access to the collagen by the hydroxyapatite.

WILLIAMS and PERLETZ (1971) tested the validity of the PN junction semiconductor theory proposed by BASSETT (1965, 1966) and BECKER (1966). They did not obtain a measurable voltage when the PN junction diode was compressed. They considered that, theoretically, one should not expect electrical effects from semiconductors and challenged the concept that the origin of the piezoelectric effect in bone was the stress-sensitive PN junction.

More recently, MARINO *et al.* (1971) concluded that the collagen component of bone contributed most, if not all, the piezoelectric effects, as had been previously proposed by FUKADA and YASUDA (1957, 1964).

HAMBURY *et al.* (1971) applied low electric currents to bone *in vivo*. Using strontium^{85} absorption rates they were unable to detect differences between radioisotope uptake at the anode or cathode at the electrode implant sites. They found no consistent evidence of increased bone growth at either electrode as assessed by macroscopic, histologic or radiological examination. This data challenges the hypothesis that piezoelectric currents act as a triggering stimulus for bone remodelling.

On the other hand LAVINE *et al.* (1972) applied a direct electric current across a human pseudoarthrosis defect in the tibia. By radiograms, histological techniques and electron microscopy they observed newly formed bone in the defect region, which they ascribed to the effects of the electric current.

C. In the Orthodontic Field

(i) Histological Findings

In the orthodontic field, tissue changes incident to orthodontic tooth movement, i.e. the effect of mechanical stress on dental tissues,

have been investigated histologically since the beginning of this century. SANDSTEDT published his experimental works on the subject in 1904 and 1905. He used dogs in his experiments and placed labial arch wires on the maxillary teeth. Orthodontic tooth movement of the anterior teeth was carried out by tightening screws at both ends of the arch wire. After 3 weeks the dogs were sacrificed and histological sections of the teeth and supporting structures were made. Bone deposition was observed on the tension side irrespective of the amount of force applied. Bone was formed along the stretched periodontal fibres and a distinct line could be found at the border between the newly deposited and old bone. On the pressure side, on the other hand, with light force, resorption was observed on the alveolar bone surface; the tooth root appeared to be intact. With heavier force, however, the periodontal membrane was compressed onto the bone surface and bone resorption did not occur at the compressed front. Instead, active bone resorption appeared in the bone marrow of the compressed alveolar bone. The resorption was directed towards the compressed periodontal membrane and resulted in disappearance of the underlying bone; at this stage the teeth assumed their new position. SANDSTEDT defined this bone resorption as undermining bone resorption.

SANDSTEDT's findings formed the basic concepts on the subject, and were repeated and confirmed by many histologists and orthodontists.

In 1911 OPPENHEIM published his results using monkeys as experimental animals. They differed somewhat from those of SANDSTEDT in that, with intermittent light force, the fulcrum was closer to the root apex compared with STANDSTEDT, who estimated the fulcrum to be slightly apical from the midpoint of the root. Basically, however, OPPENHEIM's results were identical to those of SANDSTEDT, i.e. bone resorption occurred on the compressed side and bone formation on the stressed side.

In 1926, JOHNSON *et al.* observed an increased number of osteoclasts in the alveolar bone on the compressed side and bending, as well as resorption of the root apex of the tooth that had been moved.

GOTTLIEB and ORBAN (1931) reported bone and teeth resorption with traumatic pressure in dogs.

SCHWARZ (1932) produced orthodontic tooth movement in dogs by placing lingual arches with finger and loop springs on the maxillary and mandibular jaws and confirmed SANDSTEDT's findings. He classified the tissue responses into four stages, and maintained that the most biologically favourable response occurred when the force did not exceed the capillary pressure. According to him, the optimal force to apply was between 15 and 20 mm Hg in most mammals, including human beings, this being equivalent to 20 to 26 gm per square centimeter. Under these conditions, continuous resorption took place in the alveolar bone at the regions of pressure.

After the forces were discontinued the periodontal membrane and alveolar bone was anatomically and functionally reconstituted, and there was no evidence of resorption on the tooth root. In fact, these values have long been regarded as fundamental for optimal orthodontic forces, although SCHWARZ did not provide any experimental evidence as to how he arrived at his figures. Unlike OPPENHEIM, he advocated strongly the application of light, continuous forces for orthodontic tooth movement.

HERZBERG (1932) was the first to utilize human materials in his experiments. As his results paralleled those found in animals by earlier workers, he suggested that further animal experiments would be justified.

MARSHALL (1933) found that monkeys on deficient diets had greater resorbed areas and much slower repair compared to animals on a full diet using the same appliances.

In 1936 ORBAN reviewed the then available literature on this subject and warned that any orthodontic movement of teeth by means of appliances overstressed the tissues in the biological sense. However, he concurred with the use of light forces equivalent to those which SCHWARZ had recommended, if teeth were to be moved orthodontically.

OPPENHEIM (1944), applying intermittent forces to monkey teeth, found three kinds of osteoclasts, namely primary, secondary and

tertiary osteoclasts which appeared respectively at the compressed bone front, at the undermining bone resorbing front, and at the traction side. According to him, the primary osteoclasts persisted for at least four days at the compressed front. As long as these osteoclasts persisted the use of further force (stimuli) was unnecessary and, indeed, may cause damage to the periodontal membrane, bone, cementum and pulp. This work formed the basis for his advocacy of the use of light, intermittent forces.

In 1951, REITAN published the results of his exhaustive experiments using dog and human materials with fixed and removable appliances. In general, he confirmed STANDSTEDT's findings.

MACAPANPAN *et al.* (1954) reported on early tissue changes and mitotic activity following enforced tooth movement in rats. They simulated orthodontic forces by placing strips of rubber dam between the teeth. According to them, in the first 12 hours, the changes in the periodontal membrane on the pressure side consisted of pyknosis of the fibroblast nuclei, disorganization, and hyalinization of the periodontal fibres, and disappearance of osteoblasts. After 60 to 72 hours, resorption was observed. On the tension side, on the other hand, mitotic activity among the fibroblasts was markedly increased at between 24 to 36 hours after the initiation of the tooth movement, and fell to zero after 48 hours. They considered that fibroblast proliferation was part of the repair process which allowed the damaged fibre bundles to readapt to

the changed relations between tooth and bone.

YEN and ROTHBLATT (1955), using similar techniques, reported on early tissue changes following tooth movement in the rat. Histological observations were made at varying periods of time after the application of stress, i.e. from 4 to 24 hours later. They observed cellular changes on both pressure and tension sides as early as 6 hours, which increased in extent up to 24 hours. They drew attention to the rapidity with which early tissue changes accompanied tooth movement.

HUMERFELT and REITAN (1966) reported that hypercementosed teeth moved with greater difficulty when orthodontic forces were applied.

Orthodontic tooth movement of deciduous teeth did not affect the tooth germs of the permanent teeth (KALAMKAROV, 1968).

GAUDET (1970) studied the tissue changes following root torque with a light wire appliance. He found a fairly high incidence and varying degrees of root resorption in these cases.

AZUMA (1970) investigated changes in the periodontal membrane during experimental tooth movement in rats. He observed a remodelling process on the pressure side which was characterised by hyalinization of the periodontal membrane after 6 to 12 hours and its replacement by granulation tissue after 14 days, followed by regeneration of the periodontal fibres after 21 days. Moreover, he found that tooth displacement occurred in two phases of accelerated

activity over a given period of time. The first, hyperactive phase took place up to six hours from the initial application of force, which he considered to be related to the viscoelastic properties of the periodontal membrane. The second phase of increased tooth displacement occurred after 17 hours.

Following the earlier descriptions of the histological responses in the tissues consequent to orthodontic tooth movement, more effort was directed from the mid 1950's onwards to the elucidation of various practical problems related to orthodontic tooth movement, despite the fact that the underlying mechanisms thereto remained unresolved. Some of the practical problems related to orthodontic tooth movement will be reviewed hereunder.

(a) Orthodontic Techniques and Methods of Applying Forces to Teeth

Efficient ways of applying forces to teeth have long been explored Since orthodontic treatment utilizes both biological and physical principles, a thorough understanding of these two fields is essential. One approach has been directed towards refining orthodontic appliances so as to optimally utilize appropriate physical principles. So long as the application of force is an inevitable means of obtaining tooth movement, an essential goal is to arrive at an understanding of the optimal forces that can be applied to tissues. Another approach has been directed towards ascertaining optimal forces by studying tissue reactions to various magnitudes of force.

To date, literally hundreds of orthodontic appliances have been developed - appliances such as the Pin and tube (ANGLE, 1912); Ribbon arch (ANGLE, 1916); Labio-lingual (MERSHON, 1918); Edgewise (ANGLE, 1929; TWEED, 1966); Twin-wire (JOHNSON, 1932); Universal (ATKINSON, 1937; CUCALON, 1965); Light differential wire (BEGG, 1954, 1961, 1965; SIMS, 1964; JARABAK and FIZZELL, 1963), and Removable appliances (ANDRESEN and HÄUPL, 1942; BJÖRK, 1951; ADAMS, 1964; SCHWARZ, 1966; BIMLER, 1961; FRÄNKEL, 1967) just to mention a few. Despite, or because of, these technological advances there is a tendency, in present day clinical practice, to become obsessed with the mechanical advantages of each technique and to overlook the other important principle, namely, the biological aspect.

MOYERS (1950), MOYERS and BAUER (1950), HALDERSON *et al.* (1953) discussed the practical mechanics of the then available appliances, such as round wire, edgewise rectangular wire, twin-wire and universal appliances from the viewpoint of known histological responses. They demonstrated that with the edgewise rectangular wire appliance, the forces applied on the periodontal membrane were completely beyond the limits of physiological tolerance, although they acknowledged that forces could be applied to any tooth in any direction with this appliance. From their histological findings, they recommended the use of a light round arch wire prior to the application of the rigid, rectangular arch wire in order to prevent tissue damage. In fact, this technique has been the most popular of the multibanded techniques.

Their studies provided a great stimulus to orthodontics and resulted in the routine application of light arch wires before the rectangular arch wire and in the rapid development of the light differential wire techniques.

The concept of an optimal force value has been discussed from time to time. As already described, SCHWARZ (1932) considered the optimal force should not exceed the capillary pressure, equivalent to between 20 and 26 gm per square centimetre. However, the means by which he arrived at this value are obscure and his recommendations must be regarded as rather hypothetical.

STOREY and SMITH (1952) and SMITH and STOREY (1952) were the first to present objective data on the relationship between force value and tooth movement. Using retraction appliances on human cuspids, they applied two kinds of force, light forces of 175 to 300 gm and heavy forces of 400 to 600 gm. With light forces the cuspids moved rapidly until the force was reduced to 135 - 180 gm. On the other hand with heavy forces, the molars which could function as anchor teeth for the appliance, moved until the force was reduced to 200 - 300 gm. after which the cuspid started to move in the same manner as was observed in the case of the application of light forces. From these findings, they concluded that each tooth had its optimal force range for the most effective movement; that is, forces optimal for the movement of one tooth may not be applicable to other teeth.

This pioneer work provided the theoretical basis for the develop-

ment of the light differential wire techniques (BEGG, 1965). BEGG described three kinds of force values for a given tooth used in the light differential wire technique, namely, low, optimal and excessive forces. Due to the differences in tissue reactions between teeth subjected to a given force, BEGG suggested that excessive forces on particular teeth could be used to form an anchorage for the movement of another tooth when necessary. However, generally he recommended the use of light forces throughout the whole treatment period unless the need for the other kinds of force arose.

STOREY (1953, 1954, 1955a,b) published his experimental findings on tooth movement and bone changes and confirmed that there was an optimal range of force required to produce a maximum rate of mature bone growth. He concluded that with heavy forces, highly cellular, poorly calcified bone was laid down, while light forces resulted in well formed, mature bone.

REITAN (1957) enumerated the main factors determining the evaluation of forces as (i) the individual variation in tissue reaction, (ii) the type of force applied, and (iii) the mechanical principles involved.

BURSTONE *et al.* (1961), BURSTONE (1962, 1969) and CHRISTIANSEN and BURSTONE (1969) also emphasized the necessity of a thorough understanding of the biomechanics of tooth movement. They investigated the characteristics of orthodontic wires necessary to produce

light continuous forces, and concluded that by using wires of various diameters in one arch rather than a uniform diameter arch wire, optimal force could be applied to individual teeth, because wires of different diameters have different elasticity and load deflection rates. From these findings BURSTONE developed the segmented arch wire technique.

JARABAK and FIZZELL (1963, p.192-280) maintained that the most effective pressures for tooth movement were likely to be between 2.0 and 2.5 gm/mm^2 of the tooth root area.

LEE (1965a,b, 1968) wrote that the critical factor in determining the rate of tooth movement was not the force applied to the crown, but the force exerted by the tooth root on the surrounding bone-periodontal membrane complex. He thought that the optimal forces which should be applied to the crowns should be between 150 and 260 gm.

As each investigator recommends different values for the optimal force it is obviously necessary to determine the minimum force which can initiate tooth movement, i.e. the threshold force level. BURSTONE and GROVES (1960) tried to obtain a threshold force value for maxillary anterior teeth. They were unable to determine a threshold when forces between 25 to 150 gm were used. They considered that a force value considerably lower than those employed would be the threshold if it existed. They reported that the optimum value was 50 to 75 gm.

WEINSTEIN (1967) concluded that muscle forces of such low values as 1.68 gm above the resting muscle force, if acting for a sufficient time, were capable of moving teeth.

LEAR and MACKAY (1972) and LEAR *et al.* (1972) studied threshold levels for tooth displacement and found that quite small forces, ranging from 1.6 to 3.5 gm were sufficient to cause displacement.

HIXON *et al.* (1969, 1970) and HIXON and KLEIN (1972) cast serious doubts on the concepts of optimal and differential forces. They considered that the concepts of physiologic force, optimal force, and differential force were clinically meaningless; osteoclasts responded only as a function of pressure with time and not as a function of the technology of the appliances.

(b) Observations on Bodily Tooth Movement and Tipping Tooth Movement.

There are two means by which teeth can be moved horizontally; by bodily tooth movement and tipping tooth movement. In 1947 REITAN repeated his findings on continuous bodily tooth movement by using appliances which consisted of bands with horizontal tubes in which were placed short arches provided with coil springs. Forces used ranged from 45 to 85 gm. With forces of from 68 to 85 gm he found a small amount of root resorption, but not with forces of 45 to 55 gm. On the other hand, he found necrotic pressure areas and resorption of the roots in cases of teeth moved mesially

with a tipping force of 45 gm. After further investigations (REITAN, 1957, 1964 and 1966) he maintained that bodily tooth movement was preferable to tipping tooth movement because in a tipping movement the tooth would act as a two armed lever in which the forces applied are concentrated in a small area near the alveolar crest. In a bodily movement the force applied is not augmented by the mechanics involved and furthermore is distributed over the whole root. He concluded that tipping movements resulted in more frequently over-compressed cell free areas in the periodontal membrane.

BEGG (1965, p.105-120) recommended the use of light continuous tipping movement; in fact, tipping movement is the very essence of the light wire technique. In most cases with this technique the tooth crown is tipped lingually or distally first, followed by tooth uprighting for subsequent root paralleling. BEGG maintained that a relatively light and continuous arch wire with rubber ligature forces and brackets that allow tipping, produced the most rapid movement of the anterior teeth with the least disturbance of the tooth-investing tissues and least discomfort to patients. He provided no histological evidence to support his claims other than to refer to the work of STOREY (STOREY and SMITH, 1952; SMITH and STOREY, 1952).

(c) The Influence of Function on Orthodontic Tooth Movement

Among the main functions of a tooth is that of occlusion. The

periodontal tissues support this function. In this respect, one must question the influence of function on orthodontic tooth movement. Since the tooth continues to function even while it is moved orthodontically, the effect of a functional factor cannot be overlooked.

From this viewpoint, ESCHLER and HÄUPL (1940) performed interesting experiments in conjunction with their theory of jaw orthopaedic therapy. They applied different orthodontic forces to dogs following the removal of all muscular and functional effects by resecting the mandibular third trigeminal, hypoglossal and facial nerves. Two days later, the animals were sacrificed and the teeth and supplementary tissues histologically examined. They could not find any changes in the bone surrounding the teeth, despite the fact that the teeth had been exposed to both pressure and tension by orthodontic appliances. They repeated the experiments, but instituted simulated normal muscular movements in the functionally inert areas. Normal bone deposition and bone resorption which would be expected to follow the application of the orthodontic appliance was observed. Their results indicated that lack of function prevents bone remodelling.

REITAN (1951a,b) applied orthodontic forces to two groups of dogs, one of which was allowed free jaw movement, and the other was kept in a non-functioning state. His results may be summarized as follows: Bone resorption and apposition might take place incidentally

to orthodontic tooth movement, even if the structures involved are non-functional, because extensive bone resorption and deposition occurred in both the non-functioning and in the normal functioning groups of animals. He obtained identical results in humans. In other experiments, he used attached plates and activators which are believed to utilize muscular forces to determine the influence of the functional factor. He was unable to demonstrate differences in tissue reactions caused by the attached plates and activators, as far as movement of individual teeth was concerned. He suggested that activators might cause considerable changes in the jaw structures as a whole, including the muscles and the temporo-mandibular joint, which he regarded as a separate problem.

As the results of ESCHLER and HÄUPL are contrary to those of REITAN, further information is required on this subject.

(d) Age and Sex Factors Relating to Tooth Movement

The effects of age and sex on the tissue changes incident to orthodontic tooth movement have been investigated. REITAN (1951a, 1954) utilized young individuals, aged 11 to 13 years and an adult, aged 39 years, for his investigations. He found that young individuals frequently exhibited a maximal tendency to cell proliferation in the periodontal membrane within 45 hours of the application of force. In the case of the adult, bone resorption was not observed before four days, and bone deposition not before two to three weeks.

REITAN considered that the necessary potential for rapid cell proliferation was present in young individuals, but in adults the periodontal structures were usually in a state of relative rest and a lapse of time would be needed to attain a proliferative stage.

STOREY (1955c) found that, when the same force was used, there was a decrease in the rate of mechanically produced tooth movement with increasing age in the rabbit and guinea pig. He assumed that this was associated with a decrease in the rate of cellular activity.

He further discussed the influences of the menstrual cycle (1954) and sex (1955c) on tooth movement and concluded that there was a variation in the rate of tooth movement related to the menstrual cycle. During the first part of the cycle, there was a significant decrease in the rate of tooth movement, while during the second half of the cycle the rate increased, followed by a significant fall before or at menstruation.

As for sex influences, differences in bone responses following tooth movement in male and female guinea pigs of the same age were slight but significant and appeared to be related to the different degrees of bone maturation, the male being relatively immature compared to the female. STOREY explained the differences in bone responses during the menstrual cycles or between sexes as being under the influences of the oestrogenic hormones, and that the rhythmical variations in cellular activity of the anterior lobe of the pituitary

gland exercised an overall control on bone responses.

(e) Special Effects of Rotational Forces

It is interesting to note the tissue changes resulting from rotational tooth movement. SKILLEN and REITAN (1940) and REITAN (1959) have reported on this subject. Among the various factors involved, in rotational movements, REITAN emphasised the necessity to consider the anatomical and mechanical aspects of the teeth. He pointed out that, except for the upper central incisor and to some extent the first and second lower premolars in man, most roots, seen in cross section, are oval. Due to this anatomical feature, most teeth to be rotated would form two pressure areas and two tension areas. Another anatomical detail of importance is the arrangement of the periodontal fibres on the marginal, middle and apical thirds of the root. REITAN stressed that differences in the attachment of the fibre bundles was of great importance, particularly during the retention period. At the pressure and tension sides all the changes related to tooth movements, such as direct bone resorption, undermining bone resorption and bone deposition take place. The periodontal fibre bundles and new bone layers at the middle and apical thirds of the moved teeth rearrange themselves after a fairly short retention period, but the free gingival fibres remain stretched and displaced for as long as 232 days, and possibly longer. He considered that this was mainly responsible for the almost inevitable relapses which follow rotational tooth movement.

SCHULTZ (1967) investigated the tendency to relapse of orthodontically rotated teeth in dogs. From clinical and roentgenographic observations, he concluded that the absence of occlusal and muscular influences would cause orthodontically rotated teeth to relapse. Failure to obtain attachment of the gingival and transseptal fibres of the periodontal membrane caused relapse in at least fifty per cent of cases.

EDWARDS (1968) considered that oxytalan fibres of the periodontal membrane were responsible for the relapse of orthodontically rotated teeth.

To reduce the incidence of relapse in orthodontically rotated teeth, WISER (1966), BOESE (1969), BRAIN (1969) and ITO *et al.* (1971) favour surgical resection of the gingival fibres, while REITAN (1969) proposes early case treatment, over-correction, and immediate insertion of a retainer following the removal of the bands. Retention should be extended for some time.

(f) The effect of Extrusion and Intrusion Forces

Extrusion and intrusion are necessary steps in the orthodontic correction of malocclusions involving vertical dimension changes, but investigations of the tissue changes incident to vertical tooth movements are few.

OPPENHEIM (1940) orthodontically extruded teeth and observed bone formation at the alveolar crest region and fundus of the alveolus with concurrent pulp damage. He recommended the use of light, intermittent forces for this type of tooth movement as well.

REITAN (1957, 1967, 1969) also demonstrated bone deposition at the alveolar crestal region and fundus following the extrusion of teeth. According to him, stretching and displacement of the fibres of the periodontal membrane could be observed along the entire root surface, but the supra alveolar fibre components were involved for a longer period than the principal and apical third fibres which were completely rearranged after two to three months retention. As in the case of orthodontically rotated teeth, extrusion cases are also prone to relapse, the cause of which is the free gingival fibre component.

STENVIK (1971) reported circulatory disturbances and the appearance of small epithelial islands in the pulps of some extruded teeth. DELLINGER (1967) intruded monkey premolars for 60 days with forces of 300, 100, 50 and 10 gm. He found slight to moderate root resorption with forces of 10 and 50 gm, an increased amount of root resorption with 100 gm and unacceptable, severe root resorption with 300 gm. He concluded that, with properly directed and managed forces, teeth could be intruded in their alveolar sockets with minimal root resorption and tissue damage. Under these conditions, the tissue reaction allowing intrusion did not appear to be root resorption,

but rather, that of bone resorption at the site corresponding to the root apex and bone deposition at the alveolar crest. In the monkey the optimum intrusive force was 50 gm.

In experiments on dogs, REITAN (1969) found that forces ranging from 100 to 200 gm resulted in apical root resorption of dog maxillary anterior teeth, but with light forces ranging from 15 to 50 gm, there was insignificant apical resorption with no shortening of the roots. His results paralleled those of DELLINGER. REITAN concluded that teeth in young patients may be intruded more readily and with less tendency to shortening of the apical portion of the root than in older patients.

MJÖR and STENVIK (1969), STENVIK (1969) and STENVIK and MJÖR (1970) intruded teeth in children aged 10 to 13 years old with fixed appliances for 5 to 28 days. They observed vacuolization, circulatory disturbances and abnormal predentine formation in the pulp. Teeth with closed apices exhibited more severe changes in the dentine than teeth with open apices; if the root was not fully formed there was abnormal root development with a high frequency of pulp stone formation which STENVIK considered to be the result of disturbances in the epithelial root sheath of HERTWIG during intrusion.

(g) Changes following Reversed Tooth Movement

It is essential to preserve good anchorage in orthodontic tooth movement. Without anchorage orthodontists can not move the teeth as desired. Among the various means available for obtaining anchorage reversed tooth movement is regarded as an effective technique by some orthodontists, the rationale behind which is as follows: It is well known that bone deposition occurs at the tension side when teeth are moved. At the commencement of treatment, highly calcified bone is not laid down, but rather a highly organic material, low in inorganic salts, i.e. osteoid tissue, is laid down initially, which will eventually become highly calcified. As this osteoid tissue is apparently not resorbed by osteoclasts, there seems merit in utilizing osteoid tissue for increasing the resistance of anchor teeth. In practice, the anchor teeth move in the opposite direction to that of the main tooth movement, as osteoid tissue will have been deposited on the tension side. Then the newly moved teeth will act as anchor teeth opposing the reverse force direction.

REITAN (1953, 1962) investigated this hypothesis by moving teeth labially for 8 days and then lingually for periods ranging from 1 to 21 days in 12 year old patients. The force used was 70 gm. According to his results osteoid deposited along the internal alveolar bone surface during the first 8 day period caused a delay in the onset of direct bone resorption, up to an experimental period of 3 days, but not later than this time. He concluded that osteoid, formed

during the distal tipping of anchor teeth, could not be considered a factor of any significance in stabilizing the anchor teeth against mesial movement.

(h) Tooth Movement into Alveolar Sockets following Extraction

Surprisingly enough, investigations on the tissue changes incident to tooth movement into extraction sockets are very few, although a large number of cases are orthodontically treated now following planned tooth extraction.

It is a major clinical problem to determine when orthodontic tooth movement should be started following extraction. According to BEGG (1965, p.130), generally tooth extraction should not be performed more than three weeks before commencing treatment with orthodontic appliances, because quite often posterior teeth will migrate mesially rapidly after contact of the proximal tooth is lost following the extraction. There is no available histological evidence to support this view.

NIWA (1969) applied orthodontic forces to monkey teeth with a pin and tube appliance 2, 7, 14, 21 and 28 days following extraction. He found that in those groups where orthodontic force commenced within 14 days of the extractions, the rates of tooth movement remained uniformly high, compared with the groups where the forces were applied at 21 or 28 days post extraction. In a prolonged

experiment, root resorption was observed occasionally on the marginal and mid-root regions of the pressure side, and on the apical thirds of the tension side. He also found there was no difference between experimental and control sides in the extraction wound healing.

(i) Hormonal Influences on Tooth Movement

STOREY (1958) first investigated the influence of hormones on tooth movement. He injected rabbits and guinea pigs daily with cortisone acetate and adrenocroticotropic hormone (ACTH) while moving the incisors with a force of 50 gm. He found increased resorption of bone and connective tissues on the pressure side in rabbits treated with cortisone and resorption and inhibition of bone formation on the tension side. ACTH had little effect. In guinea pigs, treatment with either hormone had little influence on bone formation or resorption.

GIANELLY and SCHNUR (1969) used parathyroid hormone to promote bone resorption. Prior to tooth movement, they administered the hormone locally to the anterior portion of the maxilla of rats where the tooth movement was taking place. They observed resorption of the alveolar bone in the treated group and significantly enhanced tooth movement. In particular, there were differences between treated and control groups where tensive forces were applied. They suggested the hormone could be used locally to

assist tooth movement by overcoming the lag phase between the application of force and the onset of tooth movement.

DECKER (1971) injected rats daily with the growth hormone somatotropin during the period of tooth movement. He found a definite increase in osteoblastic activity in the treated groups, but no change in the osteoclastic activity.

DAVIDOVITCH *et al.* (1972) injected one group of cats daily with parathyroid hormone (PTH) and another group daily with cortisone acetate, while moving the maxillary canines with forces of 100 and 200 gm. The greatest tooth movement occurred in PTH treated cats, followed by the control group and the least in cortisone treated cats. Application of the forces for longer periods resulted in an increased amount of tooth movement, but the magnitude of the force had little effect.

(ii) Application of some Recent Techniques to the Problems of Orthodontic Tooth Movement

(a) Autoradiography

Autoradiography is a technique which utilizes the incorporation of radioactive labelled compounds (generally essential precursors or metabolites) into various cellular constituents in such a way that the radioactivity can be localized on a film emulsion to specific cell or tissue sites. Isotopes of hydrogen or carbon are the most

popular radioactive carriers used in biological investigations. This is because they are ubiquitous atoms in essential metabolic pathways and their low energy emission of β-rays allows us to investigate the incorporation at cellular as well as sub-cellular levels. Tritium, the hydrogen atom isotope is widely used today.

Tritiated thymidine (H^3-thymidine), one of the components of deoxyribonucleic acid (DNA), was first applied by DALE to orthodontic problems (DALE *et al.*, 1964) studying the effects of hypothyroid conditions in rats during tooth movement. He observed cell proliferation on the tension side within four hours of the application of force in both the normal and hypothyroid rats. In areas of maximum pressure, labelled cells were not observed for 5 days, but peripherally to this where the pressure forces were disseminated, labelled cells were seen within 12 hours. Zones of excessive pressure were characterized by contact between tooth and alveolar bone, root resorption, and absence of cell proliferation.

In orthodontically treated rats, STALLARD (1967) observed an initial drop in the number of H^3-thymidine labelled cells as early as 24 hours at the pressure zones within the periodontal membrane following the application of forces and at 48 to 72 hours in the case of H^3-proline. After the compressive force had been equalized through bone resorption and movement of the teeth, an appreciable

increase in the number of H^3-thymidine and H^3-proline labelled cells accompanied by rapid ingrowth of new capillaries was noted. STALLARD considered these changes were due to alterations in the vascular supply. However, no information with respect to the magnitude of the compressive forces or their duration was provided.

BAUMRIND (1969) and BAUMRIND and BUCK (1970) applied orthodontic forces to rat molars and studied the uptake of H^3-thymidine, H^3-proline and H^3-uridine in the affected tissues 2 hours prior to sacrifice. They counted the number of labelled cells per unit area of the periodontal space at given times and found that the metabolic rates of H^3-thymidine and H^3-uridine increased on the pressure and tension sides whereas the rates of H^3-proline incorporation decreased in both areas. In no case did they observe a difference in the metabolic rates of the isotopes between the pressure and the tension sides. Their findings are contrary to predictions expected of the pressure-tension hypothesis which has long dominated the field of tissue changes incident to orthodontic tooth movement. As the pressure forces were applied by the insertion of stretched rubber dam between the teeth (which MACAPANPAN *et al.* (1954) and WALDO and ROTHBLATT (1954) first used), it was not possible to correlate the amount of force applied and the consequent tooth movement. In the field of research under discussion, it is essential to know the relationship between the force applied and its effect on the tissues.

KVAM (1969) used H^3-thymidine to study the "cell-free zone" which often appears in areas of maximum pressure within the periodontal membrane following experimental tooth movement in rats. He observed labelled cells surrounding the cell-free zone adjacent to root surfaces, but not in the cell-free zone. Concurrent with bone resorption, labelled cells begin to infiltrate the cell-free zone.

CRUMLEY (1964) used H^3-proline to investigate the rate of collagen formation in different parts of the periodontal membrane, cementum and alveolar bone of rats following the application of continuous pressures produced by inserting stretched rubber dam between teeth. Observations were made from 30 minutes to 72 hours after the injection of the radiochemical. The alveolar bone and periodontal membrane were heavily labelled. However, there were few if any differences between the treated and control sides.

KOUMAS and MATTHEWS (1969) utilized H^3-proline to investigate the effects of pressure on collagen formation in the periodontal membrane of guinea pigs. Pressure was produced with a helical loop on the lower incisors, but the actual magnitude of the forces applied was not recorded. They found high isotope incorporation rates, presumably due to the stimulus provided by the application of external force, in the periodontal membrane near the alveolar bone on both the pressure and tension sides, but not

in control animals.

DeANGELIS (1970) used H^3-proline to investigate the response of alveolar bone to orthodontic forces produced by inserting a piece of rubber dam between the maxillary incisors of rats and, somewhat surprisingly, found heavy labelling on the tension side but scant labelling on the pressure side.

Calcium-45 has also been used in bone resorption studies. JUDA *et al.* (1963) used Ca^{45} to evaluate calcified tissue changes during orthodontic tooth movement in the dog. They applied 18 ounces of force with a spring between the maxillary cuspid and third incisor and found an increase of calcium metabolism in both tension and pressure sides. They also observed bone formation on the tension side and tooth and bone resorption on the pressure side. Their concomitant tetracycline fluorescence study correlated with the calcium autoradiography data.

Utilizing Ca^{45} autoradiography SKLAROFF and RABINOWITZ (1968) could find no differences in Ca^{45} uptake in animals subjected to light continuous force compared to those in which heavy intermittent forces had been applied. They observed higher Ca^{45} uptake on the tension side compared with the pressure side; the Ca^{45} metabolism of the latter was approximately the same as in the control.

(b) Histochemistry

TAKIMOTO *et al.* (1966) observed succinic dehydrogenase activity in osteoclasts following experimental tooth movement. They found strong enzyme activity in the osteoclasts under both normal and experimental conditions.

DEGUCHI and MORI (1968) inserted stretched rubber dam between the teeth of rats and investigated the levels of oxidative enzymes such as succinic, malic, lactic, glutamic, isocitric, α-glycerophosphate, β-hydroxybutyric acid and glucose-6-phosphate dehydrogenases. They observed intense activity of lactic, malic, isocitric and glucose-6-phosphate dehydrogenases and moderate activity of succinic dehydrogenase in the osteoblasts; intense activity of succinic, lactic and malic dehydrogenases and a moderate activity of isocitric and glucose-6-phosphate dehydrogenases in the osteoclasts, but the enzyme activities in periodontal membrane fibroblasts of the experimental animals were of the same order as seen in the controls.

TAKIMOTO *et al.* (1968) used similar methods to detect acid and alkaline phosphatases. They found decreasing alkaline phosphatase activity on the pressure side of the periodontal membranes and strong acid and alkaline phosphatase reactions on the tension side.

MOSKOWITZ and KRONMAN (1969) and KRONMAN (1971) investigated changes in the ground substance, acid and alkaline phosphatase, and

lactic and succinic dehydrogenase activities in hamsters following tooth movement effected by rubber dam strips. However, their results were equivocal.

DEGUCHI (1969a) studied acid and alkaline phosphatases and succinic, lactic and glucose-6-phosphate dehydrogenase responses in parathyroid hormone and cortisone treated rats following orthodontic tooth movement. He concluded that in both parathyroid hormone and cortisone treated rats there was increased alkaline phosphatase activity on both the pressure and tension sides compared to non-treated rats. He made no comment on the changes in dehydrogenase activities.

In another study DEGUCHI (1969b) induced hyalinized tissue changes with heavy orthodontic forces and observed somewhat increased acid phosphatase and β-glucuronidase activity in the hyalinized areas, but no change in the succinic, lactic and glucose-6-phosphate dehydrogenase activities.

KAMEDA (1970) studied changes in the fibrous components of the periodontal membrane of rats due to experimental tooth movement. There was a rapid change in the direction of the SHARPEY's fibres when force was applied with less marked changes in the orientation of the oxytalan fibres.

FURSTMAN *et al.* (1971) noticed differential responses to force between teeth in the maxilla and those in the mandible. They found

faster tooth movement in the maxilla which they considered to be due to differences in the architectural structure of the two alveolar bones.

(c) Tetracycline Tracing

Tetracycline is incorporated into growing bones and teeth, although the exact mechanism is as yet unknown. One can observe the sequence of bone formation under ultraviolet light following labelling with the drug. This technique has been used to investigate calcium metabolism for some time and was employed by BUCK and WEAVER (1965) to demonstrate new bone formation during the period of tooth movement.

UTLEY (1968) employed tetracycline labelling to investigate changes in alveolar bone incident to orthodontic tooth movement in cats. He used light forces of 40 to 60 gm, medium forces of 135 to 164 gm and heavy forces of 400 to 560 gm. With respect to the direction of forces acting on the alveolar processes, he observed bone deposition at the trailing walls of the bone marrow cavities and resorption on the leading walls; resorption of the alveolar lamina dura occurred in the area of compressive forces and bone deposition in those areas on which tensile forces acted.

MURPHEY (1970) compared the bone responses in extraction sites immediately after the space was created and into which teeth were

being orthodontically moved with those found 7 weeks following healing of the extraction wound. There was greater tooth movement in healed bone. Despite the fact that a force of 250 gm was used for tooth movement, it was not possible to relocate the interseptal bone into the fresh extraction site. He suggested that bone resorption and formation was necessary in order to effect tooth movement into extraction sites and that there was no advantage in commencing tooth movement immediately after extractions.

(d) Electric Phenomena

Applying forces of up to 10 kg to the teeth in transverse sections of bovine mandibles, COCHRAN *et al.* (1967) and GILLOLY *et al.* (1968) recorded piezoelectric responses of 0.5 to 5 mV which they maintained were of possible biological significance; the negative charge was on the concave compression side. From these observations they maintained that similar currents were generated during mastication, deglutition and orthodontic movements which played a significant role in the physiology of the tooth and surrounding structures.

NORTON and MOORE (1972) and NORTON and KRAMER (1972) applied an unidirectional pulsating electric current to the culture vessel in which rat calvaria were grown. Uptake of H^3-glycine, H^3-thymidine, C^{14}-proline and H^3-tetracycline was used as an indicator of bone growth. Alternating patterns of bone growth as indicated by

differential uptake of H^3-glycine and C^{14}-proline were observed which they considered was positive evidence for a role of electrical effects in stimulating osteogenesis. They extended their results to explain orthodontic tooth movement.

(e) Electron Microscopy

With the rapid advancement of electron microscopic techniques, structures and phenomena at the subcellular level can now be studied. KOUMAS and MATTHEWS (1969) made electron microscopic observations of the periodontal membrane collagen bundles under pressure. They observed the characteristic 640 Å axial periodicity of the periodontal fibres but no signs of resorption or disruption of the fibres. Their studies did not provide information on the effects of pressure in the periodontal membrane.

RYGH (1972) investigated the ultrastructural vascular changes of the periodontal membrane in rats with pressures ranging from 5 to 25 gm provided by a fixed appliance for from 30 minutes to 28 days. He found retardation and stasis as early as 30 minutes after application of the force, fragmentation of erythrocytes at 2 hours to 3 days and disintegration of the walls of the blood vessels and release of their contents into the surrounding tissue after 1 to 7 days. Regenerative processes commenced after 7 days and continued to 28 days. Interestingly enough, he found severe changes as a result of applying a 10 gm force for 2 hours; a force believed

to be well within the optimal level for orthodontic tooth movement.

(f) Biochemical Investigations

Several attempts have been made to apply biochemical analyses to the problems of orthodontic tooth movement. KAWATA *et al.* (1965) investigated the effects of cortisone on adrenal ascorbic acid levels during experimental tooth movement in the rat. They found that administration of cortisone prior to applying a non-specific stress, in this case force, inhibited the anticipated fall in the adrenal ascorbic acid level.

TAKIMOTO *et al.* (1966) investigated changes in the levels of the 17-ketosteroids in blood during experimental tooth movement in rats. They found a significant increase in steroid levels within 4 to 24 hours following the application of force.

YATABE (1971) applied orthodontic forces of 25 gm, 50 gm and 100 gm to rats to investigate the adrenal responses induced by experimental tooth movement. He found a typical adrenal response pattern in which the adrenal cortico-steroid levels were decreased initially followed by increased levels, finally recovering to normal. With increased forces, the changes in the cortico-steroid levels became more prominent and recovery to normal levels took longer, i.e. 4 to 10 hours with forces of 50 and 100 gm compared to 1 to 2 hours when a force of 25 gm was applied.

OZAKI *et al.* (1971) studied collagenolytic activity during tooth movement in rabbits. They found increased collagenolysis on the pressure side, which they thought resulted from the resorption of bone collagen or from degenerated periodontal membrane.

(g) Physiological Investigations

Tooth mobility, particularly following the short term application of forces has been measured with a variety of devices, such as miniature dial gauges (MÜHLEMANN, 1951), linear variable inductance transformers (PARFITT, 1960), strain gauges (SCHÖHL, 1960; PICTON, 1963) and capacitance devices (KÖRBER, 1962).

MÜHLEMANN (1951) classified tooth mobility in the horizontal direction into two phases: one was a phase of relatively free movement obtained with forces below 100 gm and the other a phase of restricted displacement which followed forces up to 1.5 kg. He concluded that the upper limit of physiological tooth mobility for incisors was around 0.2 mm.

To investigate the physiological mobility of individual teeth in an axial, i.e. vertical direction, PARFITT (1960) applied a force of 500 gm in a series of pulses applied within a few seconds of each other. He demonstrated about 0.02 mm of tooth mobility following the initial application of force and proportionally reduced ability to return to the original location after each successive pulse.

KÖRBER (1962) and KÖRBER and KÖRBER (1967) used capacitors to record the complex course which a tooth would necessarily take on returning to its primary location after the application of force.

PICTON (1963) confirmed PARFITT's findings and demonstrated a gradual increase in the amount of tooth mobility with successive thrusts if the intervals between the application of force were greater than 2 minutes.

SHIMIZU (1967) investigated the action potential of the anterior superior alveolar nerve following the application of force to maxillary canines in cats. He found that ADRIAN-BRONK's law (which sta es that the intensity of excitation of a nerve is directly related to the frequency of discharge of the individual neurons and the number of neurons) could be applied to the relationship between the force exerted and the frequency of action potential with forces of from 50 to 200 gm.

BOWMAN and NAKFOOR (1968) and SOLTIS *et al.* (1971) studied patients' ability to differentiate the intensity of force applied to teeth and found that the most accurate discrimination was when the forces applied were between 50 and 500 gm. On the other hand, SOLTIS *et al.* applied forces of from 40 to 150 gm and found that there was a significant reduction of the pain threshold after the application of orthodontic forces for four days.

HANNAM (1969) employed a strain gauge to record the responses of periodontal mechanoreceptor units by applying cyclic forces to dog teeth at intervals of 30 seconds. The behaviour of the 60 units studied could be divided into a rapidly adapting set which only responded when the rate of mechanical stimulation exceeded a certain value and in which the latency of response decreased when the rate of stimulus was increased and the thresholds were consistently high. A set of slowly adapting units were least sensitive to dynamic forces and in them spontaneous activity was consistently low.

LEWIN (1970a, b) used an omnidirectional transducer to measure the direction of tooth movement within the periodontal space. The direction of tooth displacement in response to applied forces was governed by such factors as the shape and length of the root, the characteristics of the fluid content of the periodontal space, volume, composition and orientation of the periodontal fibres and the surrounding alveolar bone.

PICTON and SATTER (1972) found a significant increase in tooth mobility after the periodontal membrane had been traumatized.

WILLS *et al.* (1972) concluded that the periodontal membrane was viscoelastic in nature and may be represented by three, and possibly five, of the VOIGT models which characterized viso-elastic stress/strain constitutive relations. It is generally

agreed that these models represent the response of a viscoelastic solid.

The effects of tooth movement on the periodontal membrane vasculature have been investigated also. ZAKI and VAN HUYSEN (1963) found numerous small blood vessels on the pressure side, which they thought to be closely related to the bone surface undergoing resorption.

CASTELLI and DEMPSTER (1965) applied forces to monkey teeth, then injected india ink to investigate the changes in the periodontal vasculature due to the compressive forces. They found that certain teeth developed ischaemic regions in the periodontal membrane whilst others showed different responses or retained the normal vascular patterns. They suggested that these alterations in the periodontal blood supply resulting from responses to applied forces would play a role in long-term changes involving bone-building and resorption in tooth movement.

KUFTINEC (1968) reported the almost complete obliteration of the blood vessels of the periodontal membrane on the pressure side and a significant increase in vascularity on the tension side at 30 minutes and one hour after application of force. At 90 minutes and 2 hours, the tension side showed re-establishment of the initial vascularity and after 4 hours, compensatory changes and reorganization of vascularity were seen on the pressure side.

GIANELLY (1969) observed vascular changes in dog periodontal membranes resulting from the application of forces ranging from 50 to 150 gm.

KHOUW and GOLDHABER (1970) applied forces of 50 gm in monkeys and 250 gm in dogs to investigate the vascular changes of the periodontal membrane associated with tooth movement. After 24 hours, there was partial or complete occlusion of the vessels in the periodontal membrane on the pressure side and considerably extended vessels on the tension side. Two days later the differences were less pronounced and seven days after the application of force, on the pressure side, where bone resorption was evident, the vasculature was re-established. New bone formation and increased vascularity was still present within the periodontal membrane on the tension side. The density of the vessels exceeded that in control regions. These vascular alterations were similar to those found in apposition and resorption sites associated with the physiologic mesial migration of the posterior teeth of monkeys, and were considered typical for active formation and resorption of bone.

(iii) Attempts to Elucidate the Biological Basis of Tooth Movement

In the previous sections a number of approaches to various aspects in this problem have been reviewed, but to date the underlying mechanism governing the tissue changes incident to tooth

movement, i.e. the effects of mechanical stress on the tissues, remains unknown.

BIEN and AYERS (1965) and BIEN (1966a, b) have attempted one explanation. BIEN (1966b) proposed that the viscoelastic periodontal membrane, which could be expressed in terms of a combination of MAXWELL and VOIGT models, played an important role governing tooth movements. He argued that the responses of the supporting tissues to orthodontic forces could be divided into two phases. In the first phase, that of intrusion, the periodontal membrane acts in the manner of a squeeze film. The intrusive force during this phase operates as a pumping mechanism for maintaining blood flow. This is followed in the second phase, by partial stenosis of the capillaries in the periodontal membrane. The randomly oriented periodontal fibres, which surround the blood vessels, tighten, compress and constrict the vessels that run between them, causing stenosis. Due to the change of blood flow in the stenosed vessels, gas bubble production occurs in the vascular zones of low pressure. During the prolonged application of force on a tooth, to which must be added masticatory forces, these gas bubbles diffuse through the vessel wall into the interstitial tissues and are finally lodged under and between spicules of bone. The existence of gas bubbles creates a favourable local climate for bone resorption. Rapid decalcification in the pressure areas results in an increased calcium ion concentration and pH in the local interstitial oedema fluids. In this way a favourable milieu for

the formation of a calcification front is established in the tension area. Although the hypothesis has several interesting aspects, there remains the need for further substantiation of the precise roles played by vascular stasis and local gas tensions in bone resorptive processes.

On the basis of BASSETT and BECKER's results (1962), EPKER and FROST (1965, 1966) advanced a hypothesis based on the differential alteration of the surface curvature of the alveolar bone depending on whether one considers the pressure or tension surface. As a load is applied to a bone, surface resorption or formation occurs in those locations where the surface curvature changes. At those bone surfaces which increase in convexity as an external load is applied, net resorption tends to occur, whilst at the concave surface new bone formation occurs.

BASSETT (1966, 1971) explained the underlying mechanisms governing tooth replacement due to force on the basis of piezoelectric effects. Application of a tipping orthodontic force deforms the adjacent alveolar walls in the same manner as cantilevers fixed at their base. At the leading edge, the pressure of the tooth compels the alveolar bone to become convex (electropositive) towards the long axis of the root. At the trailing edge, the tension exerted by the periodontal membrane deforms the alveolar bone to become concave (electronegative) with respect to the long axis of the root. BASSETT (1971)

pointed out that the orthodontic terms "compression" and "tension" must be examined as they apply to teeth subjected to force, which, in most cases was a tipping movement forcing the contiguous alveolar bone of the socket to deform as cantilevers. The ultimate deformation of the alveolar walls is responsible for the piezoelectric potentials and these, added to the predictable electric polarity created by the different streaming potentials between the blood vessels of the compression and tension sides, would account for the characteristic cellular responses. However, his explanations of tipping orthodontic tooth movement are somewhat vague and fail to explain various tooth movements such as extrusion, intrusion, rotation and bodily tooth movement. Nor does he satisfactorily explain the histological responses recorded following tipping tooth movement, such as bone resorption at the trailing edge and bone formation at the leading edge in the apical alveolar bone regions.

Explanations of bone resorptive and deposition mechanisms based on electrical effects may be attractive, but far from decisive. The data applies mainly to experimental results obtained on simple long bones, or pieces of bone, whereas alveolar bone, in which the teeth are embedded is a more complex anatomical structure and therefore when stressed one could expect a more complicated electrical charge distribution than found in long bones.

On the other hand, JUSTUS and LUFT (1970) presented a mechano-chemical hypothesis for bone remodelling. Changes induced in extracellular calcium concentrations by the mechanical stress act as the triggering stimulus for the bone changes due to modification of the solubility of the hydroxyapatite crystals under stress. This in turn alters the local extracellular calcium concentration within the bone. The bone cells respond to this locally altered calcium concentration by producing the appropriate structural bone changes required to resist the stress.

D. Summary of Literature Survey

In the foregoing sections the literature on the effects of mechanical stress on tissues and its relation to the immediate problem, i.e. the mechanism of orthodontic tooth movement has been reviewed.

In the field of general biology a variety of biological constituents have been subjected to pressure, the effects of which are well documented. In the field of medicine the effects of mechanical stresses on calcified tissues deservedly have been investigated, particular attention having been paid to the possibility that the electric effects generated when hard tissues are subjected to stress may act as a triggering mechanism for bone remodelling.

In the orthodontic field, the morphological sequences of tooth movement when force has been applied to teeth using a variety of techniques, have been described fully. However, the key mechanism governing tooth movement, which in fact is a form of natural bone remodelling, remains unknown.

There is an apparent discrepancy between observations in the orthodontic field and those in the biological and medical fields. In sundry biological systems hydrostatic pressures of more than 1,000 psi (1,033.227 Kg/cm^2) quite often have little effect. But in orthodontics, in general, forces of less than 100 grammes per square centimetre have been considered as optimal for orthodontic tooth movement. There is agreement that above this figure unfavourable tissue changes will occur.

There is another apparent discrepancy. In medicine, particularly in the field of orthopaedic surgery, it is accepted without question that bone formation occurs at the compressed side of a bone subjected to pressure. On the other hand, orthodontists believe that bone resorption occurs at the side of compression when a tooth is moved through bone.

Although no ready explanation is available to account for these apparent discrepancies in bone responses it is unlikely that the basic mechanism in each case will be different.

At this stage, elucidation of the precise nature of the cellular

responses to the direct effects of mechanical stress in a situation closely simulating the immediate field more than ever seems to have become an important and urgent problem requiring solution.

3. THE USE OF CELL CULTURE TECHNIQUES

It is accepted scientific method, providing it is possible to retain reasonably meaningful responses, that complex systems can be broken down to more fundamental units in order to understand basic cellular events. The whole organism consists of vast numbers of cells, systematically integrated to maintain life. This integrity is so complex and inter-related that it is not always easy to analyse functions of individual components of the organism *in situ*. Tissue culture methods, first used in 1907 by HARRISON seem feasible means whereby analysis of individual cell systems can be placed in an environment which is significantly simplified and controlled. The *in vivo* experimental situation is essential to provide an understanding of the overall phenomena, but the elucidation of the basic mechanisms of cell behaviour are still optimally studied under *in vitro* conditions because in many instances, related individual factors can be studied *seriatim*, or in sequence, as desired. This is not to say that such techniques will provide the ultimate answers to understanding cellular phenomena, but at least they are a logical, efficient means of achieving the ultimate goal.

There remains the difficulty of knowing to what extent the behaviour of any type of cell *in vitro* mirrors its behaviour in the intact animal. It is almost impossible to bring a part of an organism into an *in vitro* environment without impairing or altering its true nature. This limitation should always be borne in mind. Nevertheless, tissue culture methods are now widely used in experimental biology and medicine, particularly in the fields of cellular biology, histology, embryology, physiology, pathology, virology, microbiology immunology and cancer research.

In order to clarify the direct effects of pressure on cells which are considered to play an important role in alveolar bone remodelling incident to tooth movement, one approach is to utilize *in vitro* methods. Accordingly, NAKAMURA (1968) employed mouse fibroblast L strain cells in tissue culture and studied the effects of compressive forces ranging from 6.28 gm/cm^2 to 109.59 gm/cm^2 by phase contrast. He observed various degrees and aspects of cellular breakdown and irreversible cellular degenerative changes with forces above 26.94 gm/cm^2.

NAKAMURA and THONARD (1972) obtained similar results utilizing an identical experimental set-up and monitoring the uptake of H^3-thymidine, H^3-uridine and H^3-proline by autoradiography.

In the present study the established cell line, mouse L-929 fibroblasts derived from C3H mouse subcutaneous tissue was used

Cells were subjected to varying compressive forces under experimental conditions modified from those previously described (NAKAMURA and THONARD, 1972).

MATERIALS AND METHODS — CHAPTER II

1. CELL CULTURE SYSTEM

All experiments were performed on L-929 fibroblast (EARLE *et al.*, 1943, 1948) monolayer cultures grown in EAGLE's basal medium supplemented with 10 per cent bovine serum (see Appendix).

The cells, received from Grand Island Biological Co., New York, in 16 x 125 mm test tubes containing EAGLE's minimum essential medium supplemented with 5 to 10 per cent foetal calf serum, were incubated for several hours. This was followed by exchanging half the volume of the medium with EAGLE's basal medium and 20 per cent bovine serum. When growth of the cells in the conditioned medium was observed, the ratio of the original medium was reduced until completely replaced with EAGLE's basal medium and 20 per cent bovine serum at consecutive medium exchanges. Subculture was made into 160 ml square bottles with EAGLE's basal medium and 20 per cent bovine serum. After several passages the concentration of bovine serum was lowered to 10 per cent and this level was maintained through subsequent subcultures and replicate cultures.

The procedures for subculturing and replicate culturing were identical except that 160 ml square culture bottles were used for

the former and petri dishes containing coverslips were used for the latter. The culture procedure was as follows: old medium was replaced with new medium. Cell monolayers in the culture bottles were scraped off with a rubber policeman and suspended in the medium. To make a homogeneous cell suspension, this was followed by pipetting to avoid the possibility of cell damage by trypsin or ethylene-diaminetetra-acetic acid (EDTA). Fifteen ml of the cell suspension supplemented with 10 per cent bovine serum, was poured into double the number of culture bottles and cultured at 37°C. By this means the same number of bottles was available for subsequent subculture and replicate culture. The number of cells transferred at any time was never less that 5×10^6 cells per ml. The presence of mycoplasmas was periodically tested by using mycoplasma medium (see Appendix) and/or staining (FOGH and FOGH, 1964).

The replicate culturing was performed after two days incubation. The final cell suspension made up with EAGLE's medium and 10 per cent bovine serum, was seeded into 90 mm diameter petri dishes containing 22 x 22 mm coverslips on the bottom. Fifteen ml of the cell suspension containing 5×10^6 cells/ml was poured into the petri dishes which were incubated in a 5 per cent carbon dioxide - 95 per cent - air environment in Brewers' anaerobic jars at 37°C in an incubator. After 1 to 2 days cultivation the coverslips and their attached cell monolayers were used for the experiments.

2. APPLICATION OF COMPRESSIVE FORCES

Experiments were undertaken by transferring the cells which had been cultured for 1 to 2 days on 22 x 22 mm coverslips to the apparatus shown in Figures 1a, b, c. The coverslips,with their attached cell monolayers, were picked from the petri dishes and placed with the cell monolayer facing upwards over a 1 mm thick glass slide measuring 22 x 22 mm, located on the bottom of 45 mm diameter glass petri dishes. The glass slide held the coverslip 1 mm above the bottom of the petri dish so that compressive forces produced by weights placed on the device could be applied to the cells covering an area of 22 x 22 mm. Culture medium covered the glass slide and coverslip.

The special apparatus was placed on the cells and a given weight was added.

The apparatus (Figures 1a, b) consists of: a polystyrene tube (diameter 34 mm, height 15 mm); a polystyrene disc (diameter 34 mm, thickness 1.5 mm) into which 49 x 1 mm diameter holes, 3 mm apart had been drilled; a holding ring made of methylmethacrylate resin; a porous cellulose triacetate membrane (pore size, 0.2μ; diameter 47 mm) (see Appendix).

In a preliminary study, the membrane was stretched and kept extremely tight on the outer surface of the tube by the holding ring (NAKAMURA and THONARD, 1972) and the plastic disc was omitted,

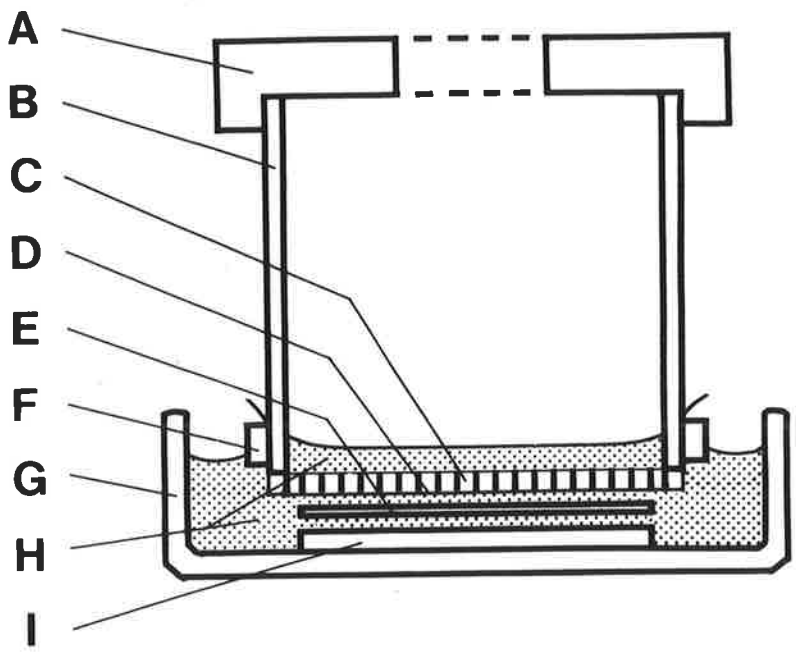

Figure 1a Diagrammatic section of assembled apparatus (improved model and petri dish

A. Lead weight
B. Polystyrene tube
C. Polystyrene disk with holes
D. Cellulose triacetate permeable membrane
E. Coverslip with attached cell monolayer
F. Plastic ring for holding membrane
G. Petri dish
H. Medium
I. 22 x 22 mm glass slide

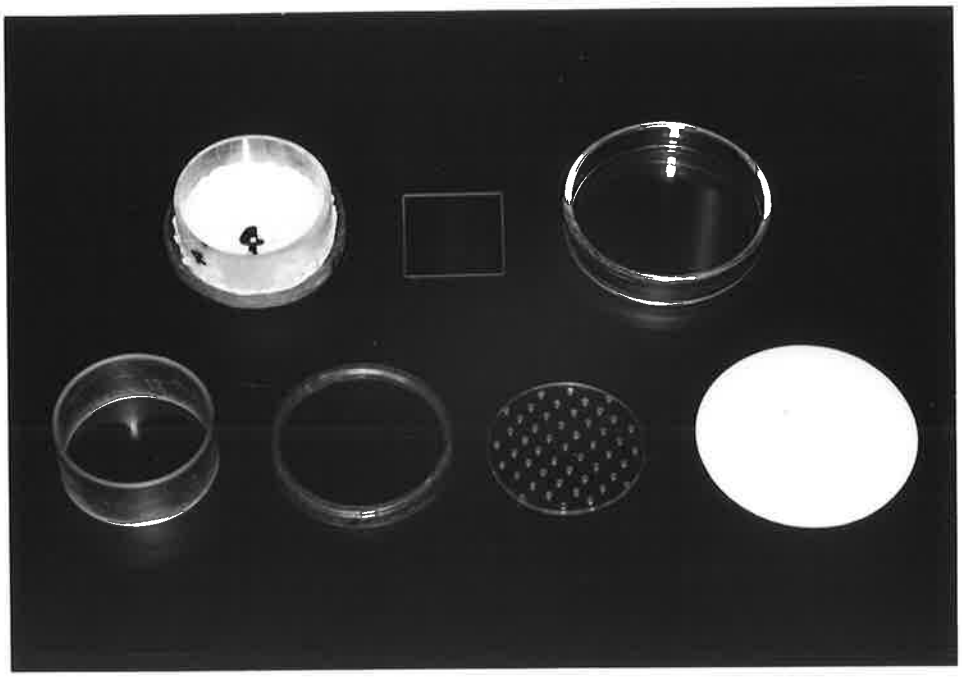

Figure 1b Overall view of the apparatus (improved model) and petri dish

Front row (from left to right):

Polystyrene tube, Plastic ring, Polystyrene disk and Cellulose triacetate permeable membrane

Back row (from left to right):

Assembled apparatus, 22 x 22 mm glass slide and experimental petri dish.

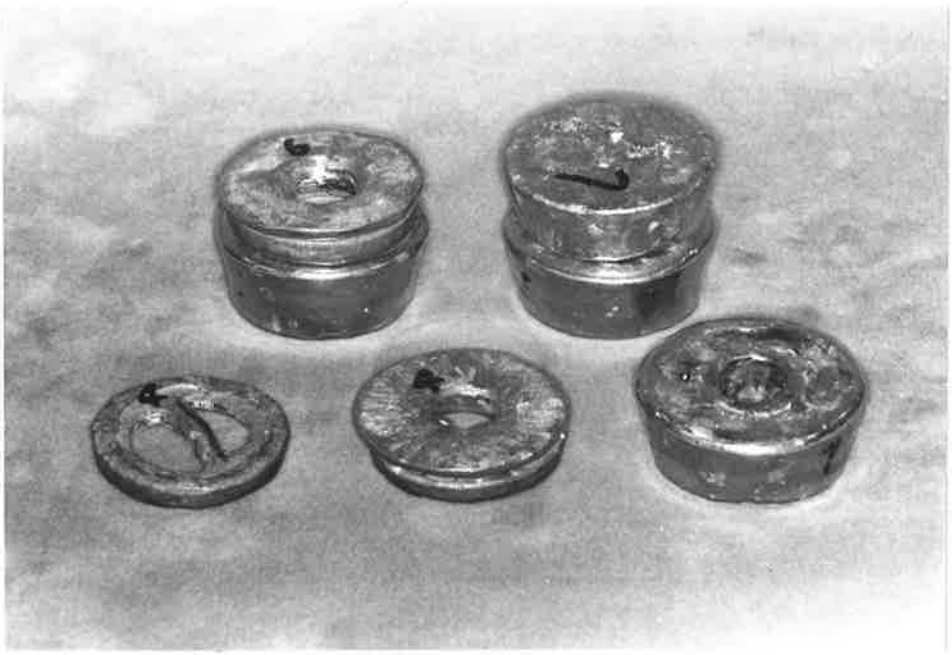

Figure 1c Overall view of the weight

Front row (from left to right):

The weights for 10 gm/cm^2, 20 gm/cm^2 and 40 gm/cm^2

Back row (from left to right):

The weights for 60 gm/cm^2 and 80 gm/cm^2

as shown in Figure 2. The use of the permeable membrane permitted the necessary gas exchange and nutrient supply to the cells under compression. Unfortunately, the force distribution to the cells beneath the membrane was found to be uneven, mainly due to the elastic nature of the membrane. The actual force applied to the cells, as determined by strain gauges, was 25 per cent less than the force calculated to be produced by the device and a given weight.

To overcome this problem, it was considered that a material which supported the membrane and transmitted the calculated forces to the cells was essential. At the same time it was necessary that the material should retain the advantages of the permeable membrane. For those reasons, the previously described plastic disc with holes was selected. The disc was trimmed to 34 mm in diameter so as to fit to the bottom end of the tube. It seemed that the more holes there were in the disc the less would be the impairment of the functions of the membrane. On the other hand, the disc had to be of sufficient strength to support the applied forces.

Preliminary experiments were carried out to determine the viability of cells growing under a glass slide or coverslip. It was found that cells were capable of growing to within 5 to 6 mm of the periphery under a glass slide or coverslip. From these observations the 1 mm diameter holes were drilled at intersections

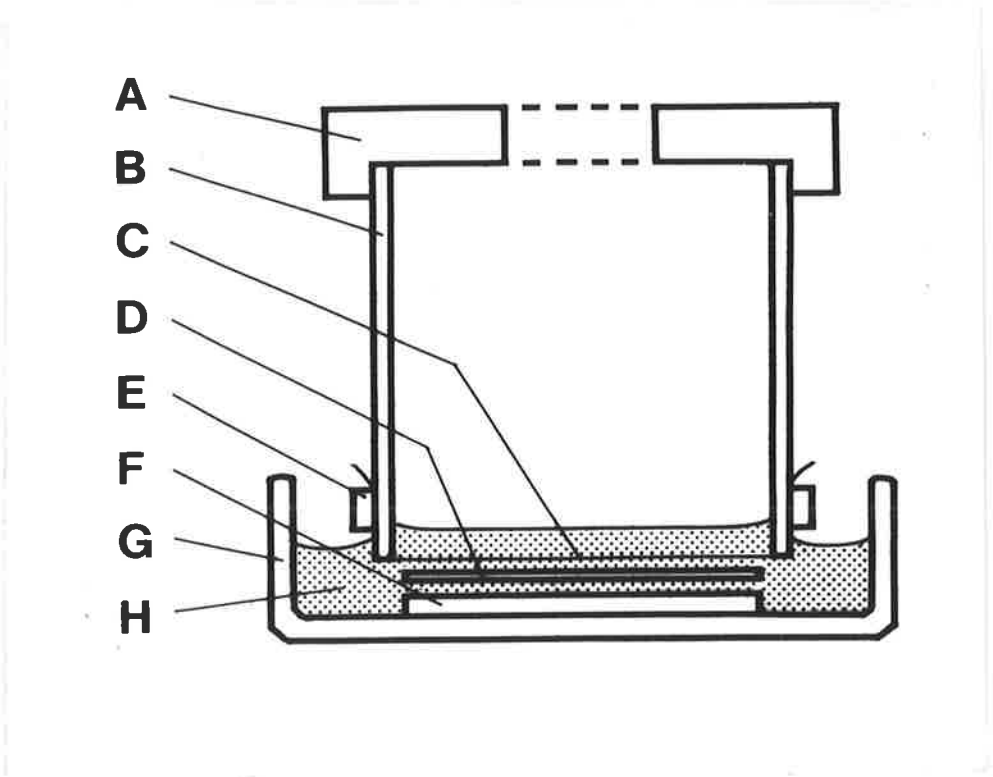

Figure 2 Diagrammatic section of assembled apparatus (prototype) and experimental petri dish.

A. Lead weight

B. Polystyrene tube

C. Cellulose triacetate permeable membrane

D. Coverslip with attached cell monolayer

E. Plastic ring for holding membrane

F. 22 x 22 mm glass slide

G. Petri dish

H. Medium

on the disc formed by horizontal and vertical lines drawn 4 mm apart. The number of holes was 49 altogether. Beneath the disc the most remote distance from the nearest hole was 2 mm, this being the midpoint of the diagonal lines of the square which was formed by 4 holes.

When the apparatus was assembled (Figure 1a), the plastic disc was placed on the bottom end of the tube. The wet permeable membrane was then placed beneath the disc and then the holding ring was passed over the membrane from the bottom end of the tube in order to hold the membrane on the outer surface of the tube. So as to ensure an adequate number of cells for subsequent observation, the permeable membrane was not stretched tautly over the plastic disc, because under these conditions it was found that a number of cells became detached from the glass coverslip surface when the apparatus was lifted off the cells at the end of the experimental period. The apparatus was kept immersed in HANKS' balanced salt solution (see Appendix) prior to the experiments. During the experiments, sufficient medium was placed in the apparatus so that the medium could reach the cells through the holes of the disc and the permeable membrane at the bottom of the apparatus. After a given period the apparatus was taken from the petri dish, rinsed in distilled water and air-dried. It was kept in a dry condition until next used.

The lead weights which were placed on top of the apparatus were moulded into a ring shape so as to fit on to the top end of the

plastic tube (Figure 1c). Total experimental weights placed on the cells were calculated to be 10 gm/cm^2, 20 gm/cm^2, 40 gm/cm^2, 60 gm/cm^2 and 80 gm/cm^2. Each load was applied to the cells for periods of 30 minutes 1, 2 and 4 hours respectively.

At the end of a given experimental period, the coverslips were collected and washed twice in HANKS' balanced salt solution. The cells were then processed for cytochemistry or autoradiography.

This experimental system made investigations on the effects of compressive forces on the cells possible. It was designed to provide regulated experimental conditions, such as constant environment and constant compressive forces on the cells.

3. CYTOCHEMISTRY

The cytochemical techniques employed, generally were those described by BARKA and ANDERSON (1965), BURSTONE (1962), LILLIE (1965) and PEARSE (1960, 1968). The original methods were followed as much as possible, but various modifications were made in order to better demonstrate specific cytochemical activities in the cell culture samples. These modifications will be described in detail in the respective sections which follow.

Fixation of the cells was performed in the appropriate fixing solution immediately after the termination of the period of applied force.

Haematoxylin

Haematoxylin staining was used to observe the general morphology of the compressed cells. The stain was prepared after the method of HARRIS (cited by LILLIE, 1965). The cells were fixed in 4 per cent cacodylate buffered glutaraldehyde solution (see Appendix) for 15 minutes as recommended by HOPWOOD (1967), rinsed and stained in diluted HARRIS Haematoxylin (see Appendix) solution (2 or 3 drops of the staining solution in 10 ml of distilled water) overnight. They were washed in tap water, dehydrated through ethanol and acetone, cleared in xylol, and mounted in a synthetic mounting medium(DPX) (see Appendix).

Feulgen reaction

The Feulgen reaction was performed to demonstrate nucleoprotein, particularly desoxyribonucleic acids (DNA). The method used was first recommended by FEULGEN and ROSSENBECK (1924), modified by BARKA and ANDERSON (1965).

The cells were fixed in 10 per cent neutral formalin solution (see Appendix) for 15 minutes, rinsed in distilled water followed by 1 N HCl at room temperature before transfer into 1 N HCl at 60^{o}C for 10 minutes for hydrolysis. They were then placed into SCHIFF's reagent (after DeTOMASI, 1963; see Appendix) for 1 hour, followed by rinsing for 2 minutes in each of three changes of freshly pre-

pared bisulfite solution (see Appendix). After washing in tap water, they were dehydrated through ethanol, cleared in xylol, and mounted in DPX.

A critical stage was the duration of hydrolysis in 1 N HCl at 60°C. Various hydrolyzing periods, ranging from 8 to 30 minutes were examined; hydrolysis for 10 minutes was finally selected as producing optimal results.

Periodic acid-SCHIFF (PAS) reaction

The periodic acid-SCHIFF reaction was performed to demonstrate carbohydrates. The method used was as recommended by HOTCHKISS (1948) and McMANUS (1948).

The cells were fixed in 4 per cent cacodylate buffered glutaraldehyde solution for 15 minutes, rinsed in distilled water and then oxidized with an 0.5 per cent aqueous solution of periodic acid for 10 minutes. This was followed by brief washing in distilled water and staining in SCHIFF's reagent for 1 hour, followed by rinsing in three changes of freshly prepared bisulfite solution for 2 minutes each. Finally they were washed briefly and dehydrated through ethanol, cleared in xylol, and mounted in DPX.

Sudan black B

Sudan black B staining was performed as a general lipid stain according to the method of LISON (1953).

The cells, which were fixed in 4 per cent cacodylate buffered glutaraldehyde solution for 15 minutes and rinsed in water, were brought into 70 per cent ethanol and rinsed briefly. They were stained with Sudan black B solution (see Appendix) for 30 minutes, followed by brief rinsing in 70 per cent ethanol, washing in tap water, and finally mounting in glycerin jelly (see Appendix) and sealing with paraffin wax.

Aldehyde fuchsin-alcian blue

To demonstrate mucopolysaccharides, aldehyde fuchsin-alcian blue staining was performed as recommended by SPICER and MEYER (1960).

The cells were fixed in 10 per cent neutral formalin solution containing 0.5 per cent cetyltrimethyl ammonium bromide for 30 minutes (BARKA and ANDERSON, 1965), followed by staining in HALMI's (1953) aldehyde-fuchsin solution for 1 hour. The cells were rinsed then in 70 per cent ethanol and in 3 per cent acetic acid for 3 minutes before transfer into 0.3 per cent alcian blue (see Appendix) in a 3 per cent acetic acid solution for 30 minutes. Finally, they were rinsed in distilled water briefly, dehydrated through ethanol, cleared in xylol, and mounted in DPX.

Alkaline phosphatase

Either the calcium-cobalt method of GOMORI (1939) and TAKAMATSU (1939), or the naphthol AS phosphate method of BURSTONE (1958a)were performed to demonstrate alkaline phosphatase activity.

In the method of GOMORI and TAKAMATSU, the cells were fixed in cold formol-calcium solution (pH 7.4) (see Appendix) for 10 minutes, followed by rinsing in distilled water. They were then incubated in the incubating mixture at 37°C for 1 hour, followed by rinsing in 2 per cent calcium chloride solution for 2 minutes and immersing in 2 per cent cobaltous acetate solution for 5 minutes. After rinsing in four changes of distilled water within 1 minute, the cells were placed in 1 per cent ammonium sulfide solution for 5 minutes. Finally, they were washed in tap water for 30 minutes, mounted in glycerin jelly and sealed with paraffin wax.

In the method of BURSTONE, the cells were fixed in cold acetone for 1 minute, incubated in naphthol AS-BI phosphate solution (see Appendix) at room temperature for 1 hour, washed in distilled water, mounted in glycerin jelly, and sealed with paraffin wax.

For the staining controls, prior to staining the cells were incubated either in the staining solution without the naphthol AS-BI phosphate, or in the complete solution after inactivation in 15 per cent acetic acid for 3 minutes. Otherwise they were treated in the same manner.

Acid phosphatase

The naphthol AS phosphate method was used to demonstrate acid phosphatase activity (BURSTONE, 1958a, b, 1961b). Naphthol AS-BI phosphate was utilized as the substrate and fast red violet LB salt (see Appendix) as the coupler.

Cells were fixed in cold acetone for 1 minute, then incubated in naphthol AS-BI phosphate solution for 1 hour at room temperature. This was followed by washing in tap water, mounting in glycerine jelly and sealing with paraffin wax.

As a staining control the cells were incubated in the staining solution without the substrate. Otherwise they were treated in the same manner.

Succinic dehydrogenase

Succinic dehydrogenase activity was examined to observe mitochondrial function, utilizing nitro blue tetrazolium (see Appendix). The technique used was that of NACHLAS *et al.* (1957), modified by BARKA and ANDERSON (1965). In both these methods fixation occurs after incubation with substrate, but because a significant proportion of cells rounded up and became detached when incubated unfixed in the presence of substrate, the samples were fixed in 8 per cent unneutralized, aqueous formalin solution at room temperature for 1 minute prior to incubation. As recommended by WALKER and

SELIGMAN (1961), the formalin fixed samples were incubated with the substrate for an extended period of up to 2 hours to offset the possible inhibition of enzymatic activity. The procedure adopted was as follows: the cells were fixed in 8 per cent unneutralized, aqueous formalin solution at room temperature for 1 minute followed by brief rinsing in distilled water. They were then incubated with substrate at 37°C for 2 hours. Finally, they were rinsed in distilled water, mounted in glycerin jelly and sealed with paraffin wax.

For a staining control, cells were incubated in a mixture without the substrate, otherwise they were treated by the same procedure.

Cytochrome oxidase

Cytochrome oxidase activity was performed to demonstrate the oxidative metabolism of the cells. The technique employed was that recommended by BURSTONE (1960, 1961a). As in the case of succinic dehydrogenase, originally fixation was recommended after incubation, but for similar reasons, fixation prior to incubation was carried out.

Accordingly, cells were fixed in 8 per cent unneutralized, aqueous formalin solution at room temperature for 1 minute. After a brief rinse in distilled water they were incubated in the presence of substrate (p-amino-diphenylamine) and the coupler (1-hydroxy-2-naphthoic acid)

at room temperature for 2 hours. This was followed by transferring the cells into 10 per cent cobaltous acetate solution for 1 hour. Then they were washed in tap water, mounted in glycerin jelly and sealed with paraffin wax.

For a staining control the cells were either boiled in water for 20 minutes prior to incubation with substrate, or incubated in the substrate mixture containing 0.001 M solution of sodium azide. Otherwise they were treated in the same manner.

Diphosphopyridine nucleotide (DPN)-linked dehydrogenases

Glutamic acid dehydrogenase

Malic dehydrogenase

α-glycerophosphate dehydrogenase

Lactic dehydrogenase

To observe oxidative enzyme activities other than succinic dehydrogenase and cytochrome oxidase, DPN-linked dehydrogenases, i.e. glutamic acid, malic, α-glycerophosphate and lactic dehydrogenase activities were examined. The methods used were those recommended by NACHLAS *et al.* (1958) and HESS *et al.* (1958), modified by BARKA and ANDERSON (1965).

As in the cases of succinic dehydrogenase and cytochrome oxidase, the cells were fixed prior to an extended incubation period with substrate.

The cells were fixed in 8 per cent unneutralized, aqueous formalin solution at room temperature for 1 minute prior to incubation with substrate followed by a brief rinsing in distilled water. They were then incubated in the presence of the appropriate substrate and DPN solution (see Appendix) at 37°C for 2 hours. Following incubation, they were rinsed in tap water, mounted in glycerin jelly and sealed with paraffin wax.

For the staining controls, the cells were incubated in the incubating solution without the respective substrates, otherwise the same procedure was followed.

4. AUTORADIOGRAPHY

The autoradiographic techniques used in the present work were those described by BASERGA and MALAMUD (1969), FEINENDEGEN (1967) and ROGERS (1967) with various modifications which will be described hereunder.

The radioisotopes used were tritiated thymidine (H^3-thymidine, 5,000 mC/m mol), tritiated uridine (H^3-uridine, 740 mC/m mol) and tritiated proline (H^3-proline, 6,000 mC/m mol) (see Appendix).

H^3-thymidine and H^3-uridine were used as specific precursors for studying deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis respectively, and H^3-proline for protein (parti-

cularly collagen) synthesis. The concentrations used were:

H^3-thymidine	-	0.05 µC per ml
H^3-uridine	-	0.1 µC per ml
H^3-proline	-	1.0 µC per ml

For pulse labelling, the compressed cells on the coverslips were brought into 90 mm plastic petri dishes containing pre-warmed medium to 37°C and one of the radioisotopes described above. They were kept in contact with the radioisotope for 20 minutes in the case of H^3-thymidine and for 1 hour in the cases of H^3-uridine and H^3-proline.

Following pulse labelling with the radioisotope, the coverslips were picked up from the plastic petri dishes and rinsed in HANKS' balanced salt solution several times. This was followed by incubation with medium containing 10 µg/ml of non-labelled thymidine, uridine or proline, as appropriate for 1 hour at 37°C ("cold" chaser). The cells were then rinsed in HANKS' balanced salt solution several times and fixed with acetic alcohol (glacial acetic acid : ethanol, 1:3) for 30 minutes. The coverslips were rinsed in 70 per cent ethanol and washed by tap water for 1 hour, followed by rinsing in distilled water several times. Coverslips and cells were then air-dried and attached, cells up, to 1 x 3 inch glass slides with paraffin wax.

Coverslips with attached cell monolayers which were not compressed were simultaneously treated for autoradiography as controls. Those which were labelled with radioisotopes were used as a "hot" standard. Cells grown under normal tissue culture conditions in the absence of isotopes served as a "cold" standard.

The glass slides with the fixed coverslips were then coated with nuclear tracking emulsion (see Appendix) which was melted at 44°C and diluted with an equal volume of distilled water. After two hours the dried glass slides were placed into light proof black bakelite boxes (see Appendix) in which an appropriate amount of silica gel was placed. In each box a few slides of "hot" standard and of "cold" standard were kept with the experimental slides in order to check unpredictable effects upon the slides from extraneous radiation during exposure. The light proof boxes were sealed with black pressure sensitive tape (see Appendix), and exposed at 4°C for four weeks, at the end of which time they were processed photographically. They were developed for 5 minutes (see Appendix) with occasional agitation at 18°C, rinsed briefly in water, fixed for 8 minutes (see Appendix) with occasional agitation at 18°C and rinsed in three changes of distilled water for 30 minutes. The processed slides were then allowed to air-dry. New developer and fixer were used after processing every 20 slides.

After the photographic processing, staining was performed with

methyl green-pyronin Y (see Appendix) (BRACHET, 1942) for 20 minutes.

Finally the slides and attached coverslips were rinsed in distilled water for a few seconds, followed by dehydration through acetone and acetone - xylol, clearing in xylol and mounting in DPX.

RESULTS CHAPTER III

1. CYTOCHEMISTRY

Haematoxylin

Normally, nuclei, cellular processes and cytoplasm were well delineated by Haematoxylin staining (Fig. 3a). Most cells were generally mononuclear, but some were multinuclear and in various stages of mitotic sequence.

Following the application of 10 gm/cm^2 to the cells for various periods, the cellular morphology in general appeared to be normal but there were some cells which formed a round cytoplasmic blister which was easily identified as early as the 30 minute period. The number of cells with blister formation was slightly increased in the 4 hour group, but the vast majority of cells remained unaltered.

In the 20 gm/cm^2 group, after various loading periods of up to 4 hours, the vast majority of cells remained normal as in the 10 gm/cm^2 group and only a very small percentage formed blisters.

Following the application of 40 gm/cm^2, the number of blister forming cells increased slightly in each experimental period. compared with the corresponding periods in the 10 gm/cm^2 and 20 gm/cm^2 groups, but the majority of the cells appeared to be normal (Fig. 3b). However, after 4 hours an increased number of cells, assumed a round

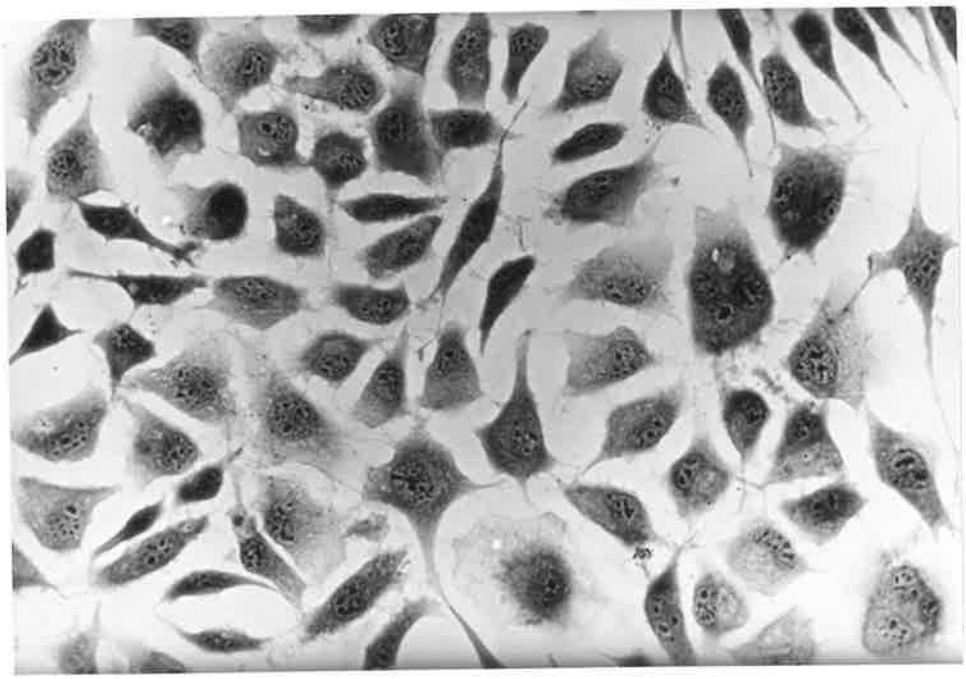

Figure 3a Haematoxylin staining (after Harris) - The cells cytoplasm and nucleus stained with haematoxylin. The cell shape varied ranging from a spindle to a wide-spread shape with many cellular processes. 4% cacodylate buffered glutaraldehyde fixed. Phase contrast. 250x.

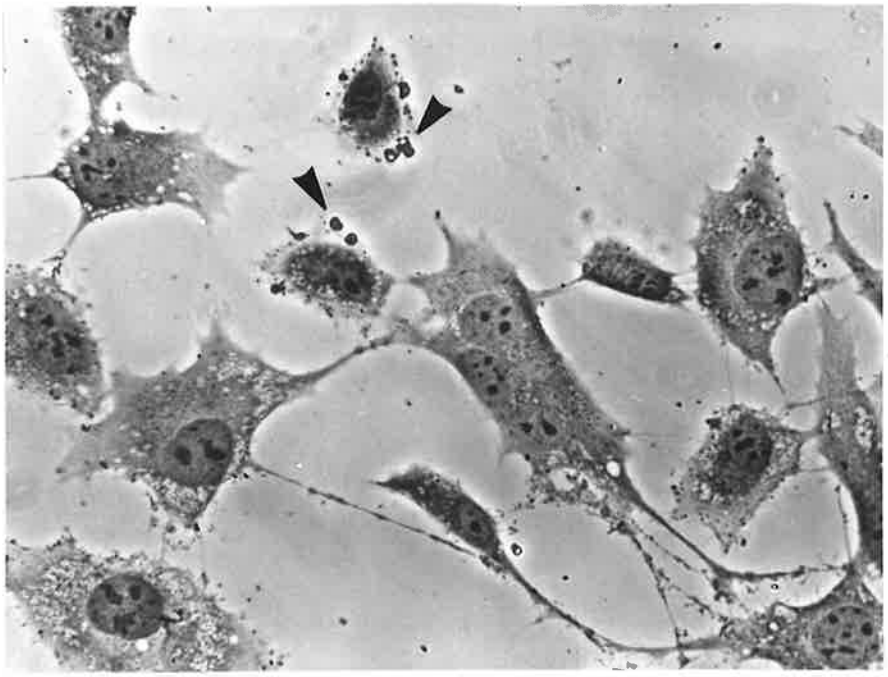

Figure 3b Haematoxylin staining (after Harris) - The cytoplasmic blister formation in cells is shown (arrows). The number and size of blisters increased with time during the experiment. Phase contrast. 600x.

or oval appearance without blister formation.

In the 60 gm/cm^2 group an increasing number of cells became round or oval in shape after 2 hours and 4 hours of compression compared with the 40 gm/cm^2 group. The number of cells with blister formation also increased after each experimental period compared with the corresponding experimental periods in the 10 gm/cm^2, 20 gm/cm^2 and 40 gm/cm^2 groups.

Following the application of compressive forces of 80 gm/cm^2, which was the maximum force applied in the present experiments, nearly half the population of cells were round or oval in shape after the longer experimental periods of 2 hours and 4 hours, but these morphologically altered cells did not form blisters. The number of blister forming cells reached a maximum of slightly over 50 per cent in this group. Generally, the longer experimental periods increased the degree of blister formation observed. The ramainder of the cells were morphologically normal.

Feulgen reaction

The nuclei of all normal cells stained positively with the Feulgen reaction but the cytoplasm remained negative (Fig. 4).

There were no striking differences in the intensity of the Feulgen reaction in any of the experimental groups to which compressive forces ranging from 10 gm/cm^2 to 80 gm/cm^2 for periods of from 30 minutes to 4 hours had been applied. The cells showed

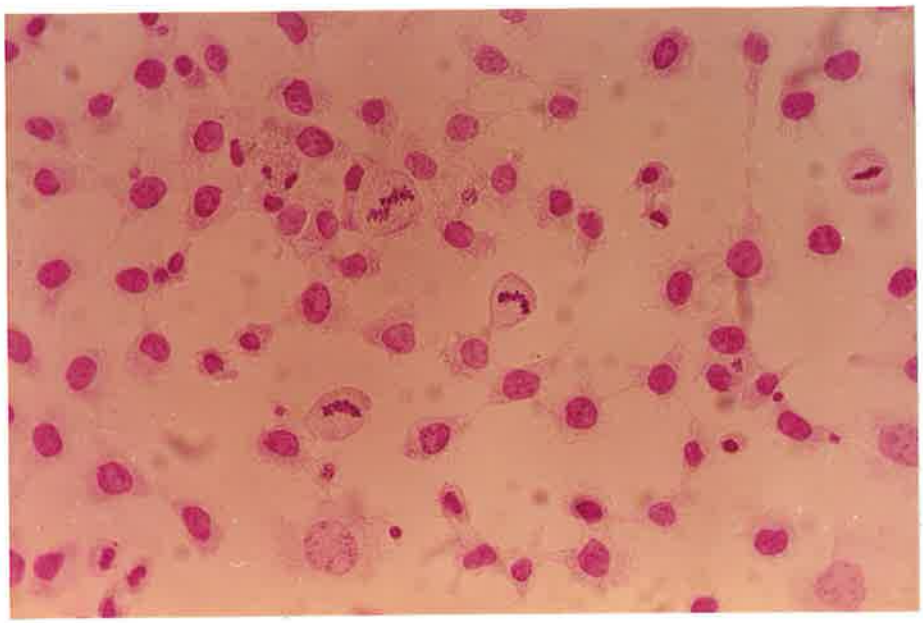

Figure 4 Feulgen reaction (after Feulgen and Rossenbeck) - The cell nucleus bound the basic fuchsin. The cytoplasm did not stain and remained clear. 10% neutral formalin fixed. 250x

the same intensity of staining as in the control groups.

The morphological features paralleled those observed with haematoxylin staining, except that blister formation was hard to discern in the negative stained cytoplasm.

Periodic acid-Schiff (PAS) reaction

Both the nuclei and cytoplasm stained regularly in normal cells with the PAS reaction (Fig. 5).

As in the case of the Feulgen reaction, no unequivocal changes in staining intensity or distribution were discernible when compressive forces ranging from 10 gm/cm^2 to 80 gm/cm^2 for from 30 minutes to 4 hours were applied to the cells.

The cellular morphology was very similar to that seen with haematoxylin and blister formation was easily observed as a structureless ovoid body against a slightly darker basic fuchsin background in the cytoplasm.

Aldehyde fuchsin - alcian blue

It was not possible to discern any changes in compressed compared with normal cells using this stain, due to the low intensity of staining of either intra or extra cellular components.

Sudan black B

Normal cells stained with varying degrees of intensity with Sudan black B and in many the nucleus was clearly outlined by an

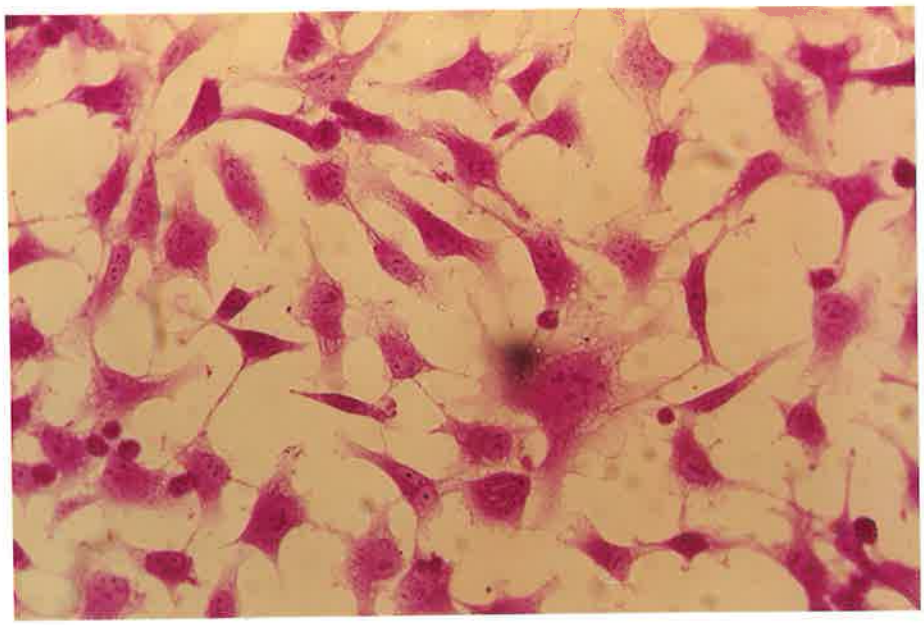

Figure 5 Periodic acid - Schiff reaction (after Hotchkiss and McManus) - The cells stained overall with basic fuchsin. 4% cacodylate buffered glutaraldehyde fixed 250x.

intensely stained ring of cytoplasm (Fig. 6). Numerous, quite intense staining, Sudan black B positive granules or vacuoles of various sizes were observed in the cytoplasm.

However, the experimental conditions to which cells were subjected had little effect on the Sudan black B staining.

Morphologically the cells resembled those observed with haematoxylin staining, and blisters were clearly observed.

Alkaline phosphatase

With either Gomori and Takamatsu's calcium cobalt method or Burstone's naphthol AS phosphate method, alkaline phosphatase activity was not successfully demonstrated at significant levels. The metal salt or fast red violet LB salt did not precipitate in any cellular structures.

Therefore, comment on the effects of compressive forces on cells is of little value using the activity of this enzyme as an assay of cellular activity - at least under the conditions of these experiments.

Acid phosphatase

Acid phosphatase activity was marked by the deposition of the azo dye as fine red granules which were confined to a location in the cytoplasm adjacent to the nucleus corresponding with the Golgi complex (Fig. 7). In multinuclear cells the enzyme activity

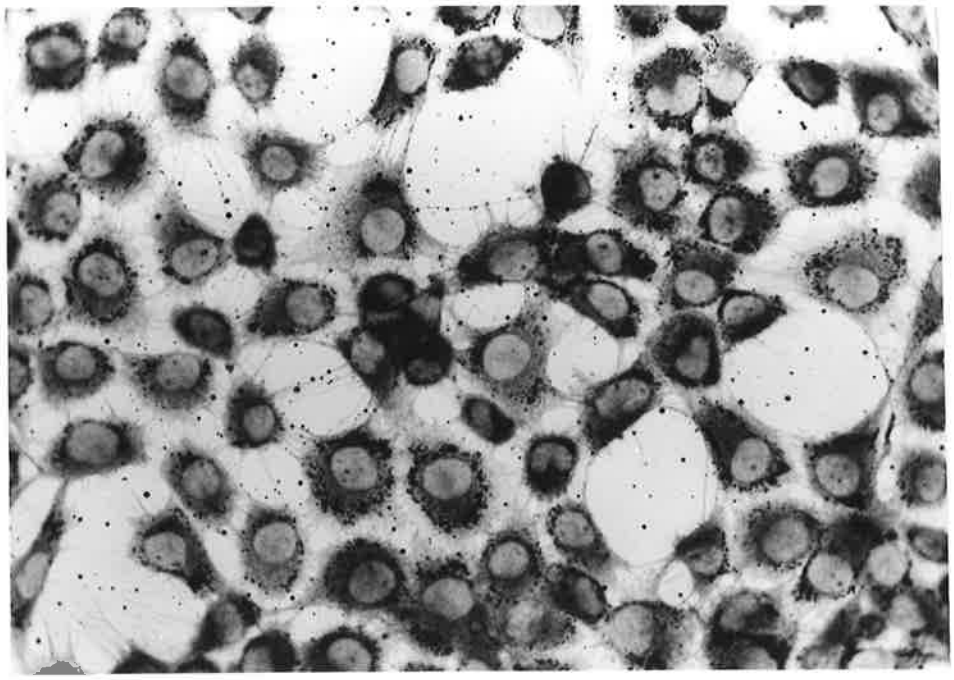

Figure 6 Sudan black B (after Lison) - The cells varied in staining reactions from light blue, grey or orange. A concentration of Sudan black B positive granules is observed around the nucleus. 4% cacodylate buffered glutaraldehyde fixed. 250x.

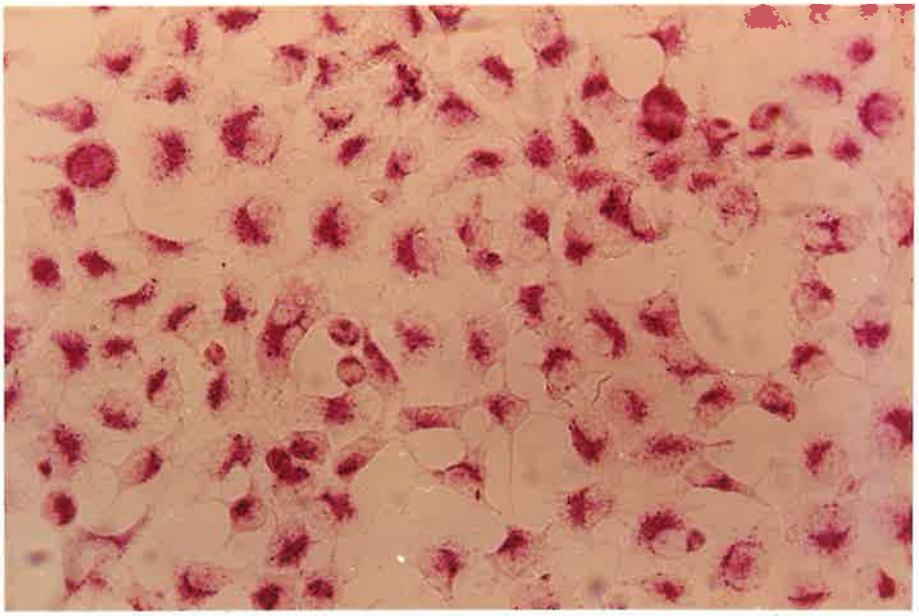

Figure 7 Acid phosphatase (after Burstone) - with the naphthol AS-BI phosphate method, the acid phosphatase activity was marked by the vivid red azo dye staining at a region in the cytoplasm corresponding to the Golgi complex adjacent to the nucleus. Cold acetone fixed. 250x.

could be observed usually in the cytoplasm between the nuclei.

Acid phosphatase activity was little affected by compressive forces of 10 gm/cm^2, 20 gm/cm^2 and 40 gm/cm^2 applied for periods of up to 4 hours. The enzyme activity appeared as intense as in the control group.

In cells subjected to either 60 gm/cm^2 or 80 gm/cm^2, however, the enzyme activity was reduced to moderate-to-weak levels after the experimental periods. The amount of inhibition increased with time of compression.

Cytoplasmic blisters were observed far less frequently compared with haematoxylin staining because they were not stained with the fast red violet LB salt and remained clear against an unstained background.

Succinic dehydrogenase

Succinic dehydrogenase activity was observed throughout the cytoplasm (Fig. 8a). The nucleus was clearly delineated from the cytoplasm.

Cells compressed by 10 gm/cm^2 were little affected after the application of the forces for any of the experimental periods used.

In both the 20 gm/cm^2 and 40 gm/cm^2 groups, however, the enzyme activity was reduced from intense to moderate reactivity after each experimental period; the amount of inhibition increased with the

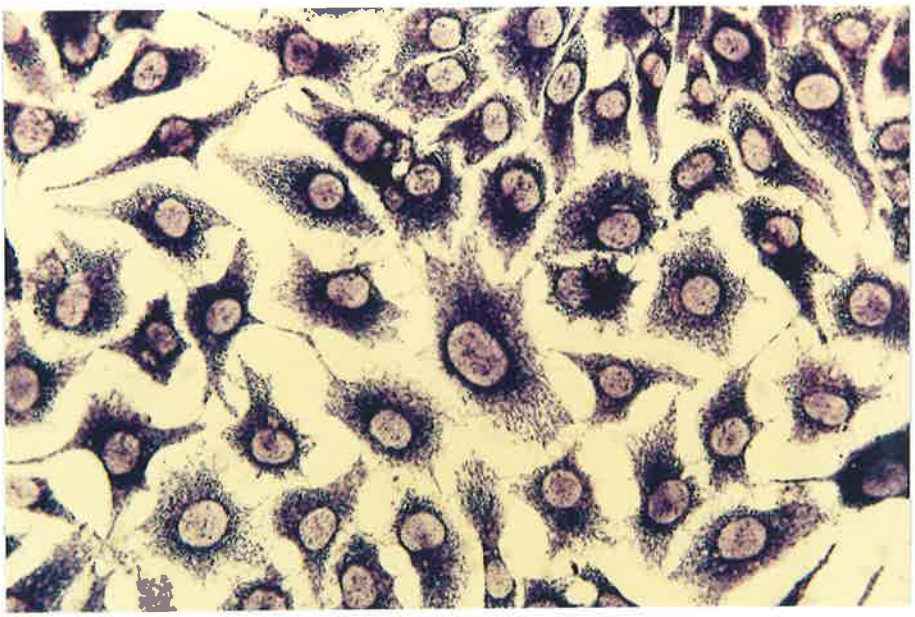

Figure 8a Succinic dehydrogenase (after Nachlas *et al.*) - With the nitro-blue tetrazolium method, the enzyme activity was marked by the cytoplasmic deposition of formazan, whose colour ranged from dark purple to dark blue to almost black, or a combination of these. 8% un-neutralized, aqueous formalin fixed. 250x.

time of compression.

In the 60 gm/cm^2 and 80 gm/cm^2 groups, the enzyme activity was reduced markedly (Fig. 8b). After 30 minutes compression the activity was moderate, but after longer experimental periods the level of activity fell dramatically to very weak or zero. In all cases the amount of inhibition increased with the time of compression. Among the cell monolayers which contained little observable enzyme activity there remained some cells which stained with moderate intensity, but the number of these cells was limited. Differences in responses between the 60 gm/cm^2 and 80 gm/cm^2 groups were not apparent. An interesting finding in these two experimental groups was the difference of the enzyme activity between clearly monolayered cells and a small proportion of cells aggregated into scattered clones. These latter invariably showed moderate to intense enzyme activity, even when the monolayered cells showed a considerably reduced activity.

Cell blisters did not stain with the nitro-blue tetrazolium and therefore were not observable.

Cytochrome oxidase

Cytochrome oxidase activity was evenly distributed throughout the cytoplasm (Fig. 9a) as indicated by the deposition of a reddish-brown to blue-black precipitate.

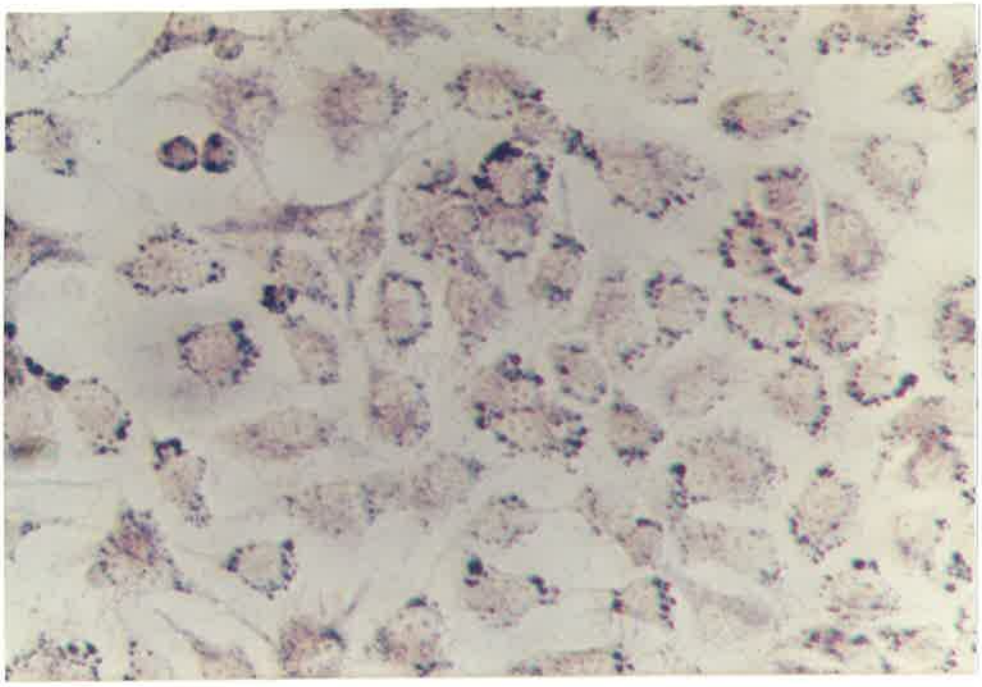

Figure 8b Succinic dehydrogenase (after Nachlas *et al.*) - The cells were subject to a compressive force of 60 gm/cm^2 for 4 hours. A clear cut distinction between normal cells (Fig. 8a) and damaged cells was observed in their enzyme activity. It is apparent that the level of enzyme activity fell dramatically to very weak or zero. A large number of vacuoles can be observed around the nucleus. 250x.

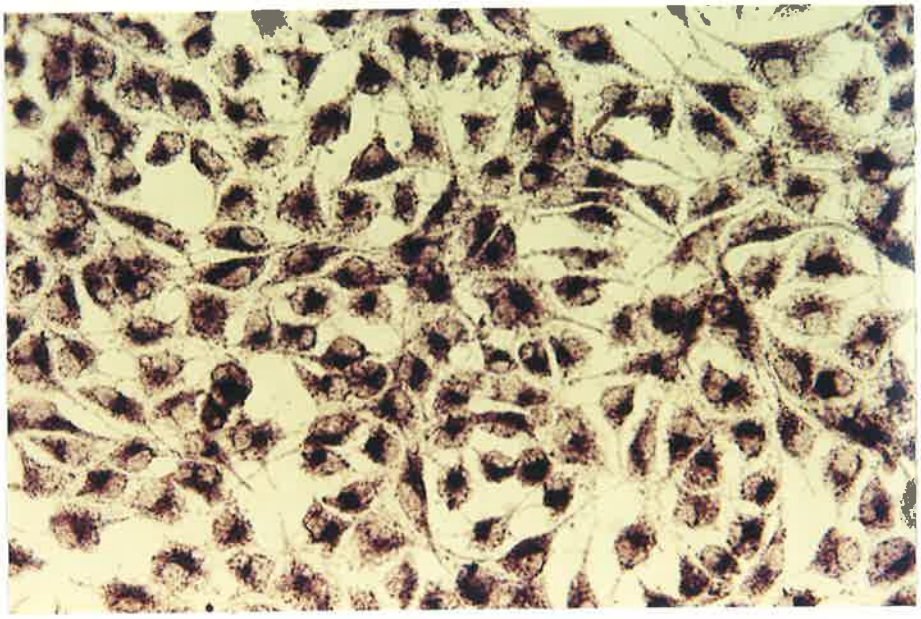

Figure 9a Cytochrome oxidase (after Burstone) - With the p-amino-diphenylamine method, the enzyme activity was marked by the cytoplasmic deposition of a reddish brown to blue-black precipitate. 8% unneutralized, aqueous formalin fixed. 158x.

The effect of the experimental procedures on cytochrome oxidase activity was identical to that observed with succinic dehydrogenase activity. Thus, with compressive forces of 10 gm/cm^2 the activity was little affected. In the 20 gm/cm^2 and 40 gm/cm^2 groups the enzyme activity was reduced by degrees rated as intense to moderate depending on the experimental periods, with the more intensely reduced activity following the longer periods of force application. With compressive forces of 60 gm/cm^2 and 80 gm/cm^2 the enzyme activity was considerably disturbed. After 30 minutes compression the activity was moderate, but after longer experimental periods the level of activity fell dramatically to very weak or zero, with the amount of inhibition increasing with the time of compression (Fig. 9b). Similar findings with respect to the intensity of the enzyme activity in cloned cells compared with monolayered cells was observed as in the case of succinic dehydrogenase.

As with the succinic dehydrogenase, blister formation was not apparent due to inability of the metal salt to precipitate in the blisters.

Diphosphopyridine nucleotide (DPN) - linked dehydrogenases

Glutamic acid dehydrogenase

Malic dehydrogenase

α-glycerophosphate dehydrogenase

Lactic dehydrogenase

The four selected dehydrogenase enzyme activities appeared

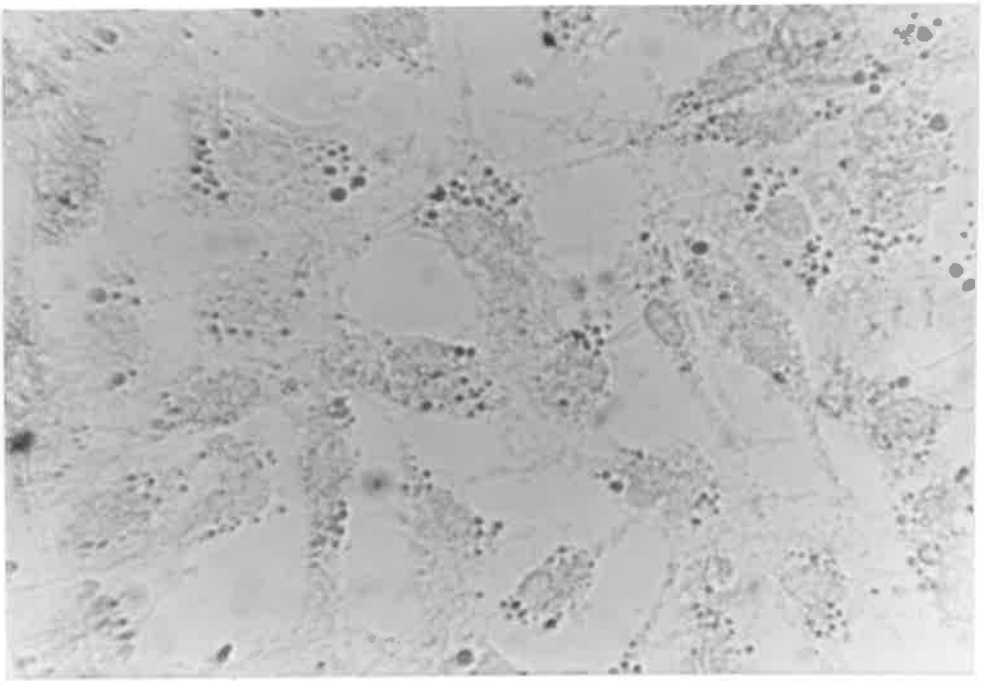

Figure 9b Cytochrome oxidase (after Burstone) - Cells subjected to a compressive force of 60 gm/cm^2 for 4 hours. As with the succinic dehydrogenase activity, a clear cut distinction between normal cells (Fig. 9a) and damaged cells was observed in their enzyme activity, which fell dramatically to very weak or zero. A large number of vacuoles can be observed around the nucleus. 8% unneutralized, aqueous formalin fixed. 250x.

identical in their normal cellcular distribution to that seen with succinic dehydrogenase. Therefore, the findings for the four dehydrogenases will be considered together. The formazan granules were deposited evenly throughout the intracellular area except the nucleus (Fig. 10). Occasionally larger size deeper staining granules were observed in the cytoplasm adjacent to the nucleus.

The findings revealed that, unlike succinic dehydrogenase, the activities of the four enzymes were little affected by the experimental conditions. By and large the staining reactions could not be discriminated from those of the control groups. Because all blisters reacted negatively with the nitro-blue tetrazolium they were not distinguishable.

AUTORADIOGRAPHY

In the autoradiographic preparations the silver grains were observed at specific sites in the cells, depending on which radioisotope was used. The silver grains could best be observed under high magnifications.

A "cold" standard in which the cells were not exposed to the radioisotopes but otherwise treated in the same manner as the experimental specimens, was used as one control for labelling. Any easily detectable increase in background grain counts was compared with counts in the corresponding "hot" standard slides.

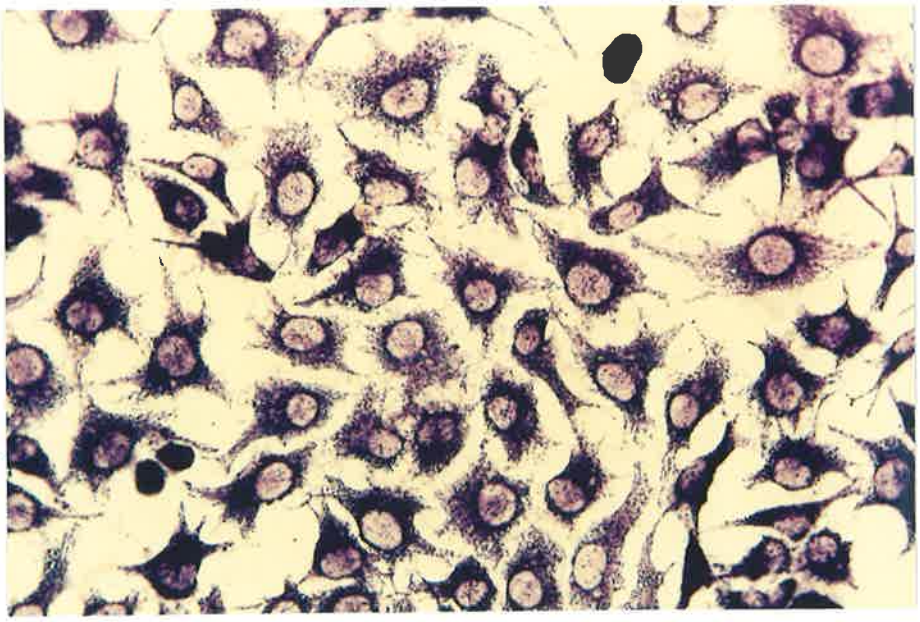

Figure 10 DPN-linked dehydrogenases (after Nachlas *et al.* and Hess *et al.*) - The enzyme activity was identical to that of succinic dehydrogenase. The formazan colour, which ranged from dark purple to dark blue to almost black or a combination of these was observed throughout the cytoplasm. 8% unneutralized, aqueous formalin fixed. 250x.

A "hot" standard, in which cells were exposed to the radioisotopes in the same way as was the experimental specimen, was used as another control. These cells were not subjected to compressive forces.

Such information, obtained from the two standards, was utilized as a basis for determining the effects of the exogenous agents, i.e. compressive forces, upon the incorporation of the labelled precursors. Comparisons were made on the basis of differences in the total number of labelled cells per 1000 cells per experiment; no cell was scored as being labelled unless the grain count exceeded 10 grains.

H^3-thymidine

In a "hot" standard, approximately 30 per cent of the cells were labelled. Labelled cells were marked by fine silver grains on their nucleus (Fig. 11).

The number of labelled cells was little different from the "hot" standard following the application of from 10 gm/cm^2 to 60 gm/cm^2 for periods of 30 minutes to 2 hours. But after 4 hours of compression, even with 10 gm/cm^2, the number of labelled cells decreased slightly compared with the "hot" standard. With compressive forces of 80 gm/cm^2 the number of the labelled cells dropped slightly after each experimental period to the same levels as the cells which received 10 gm/cm^2 to 60 gm/cm^2 for 4 hours.

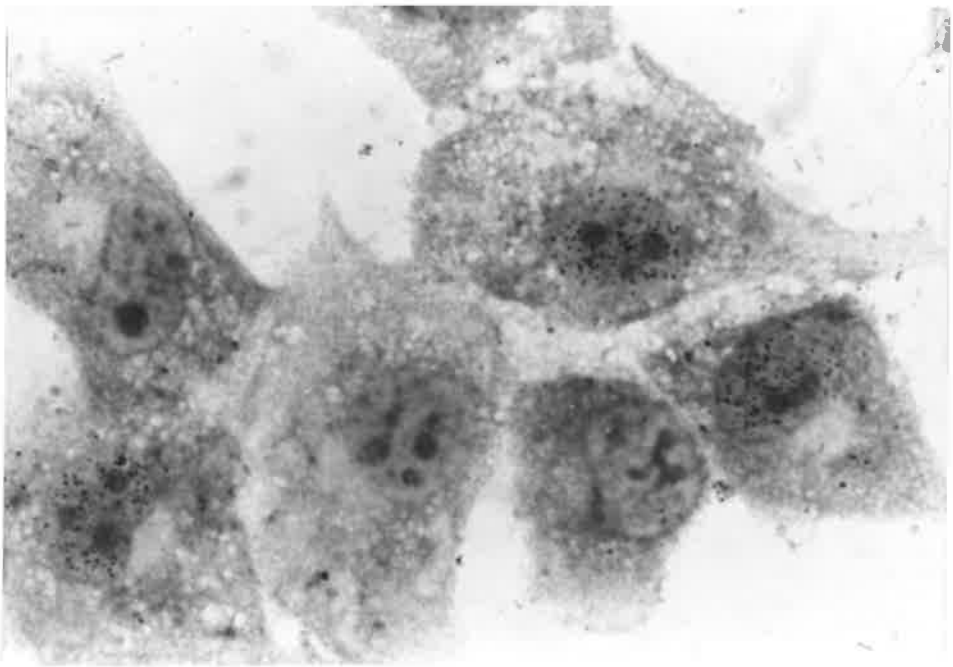

Figure 11 Autoradiography (H^3-thymidine) - Silver grains are observed on the nucleus, but not on the cytoplasm. Three labelled cells can be seen. Methyl green and pyronin Y staining. Acetic alcohol fixed. 630x.

H^3-uridine

In the "hot" standards, approximately 95 per cent of the cells were labelled. Labelled cells were quite heavily marked by silver grains over their cytoplasm as well as the nucleolus (Fig. 12).

Following the application of 10 gm/cm^2, 20 gm/cm^2, 40 gm/cm^2 and 60 gm/cm^2 for 30 minutes to 2 hours the number of labelled cells was little different from the controls. As in the case of H^3-thymidine incorporation, after 4 hours the number of labelled cells was slightly reduced compared with the "hot" standards, and this trend was continued with cells receiving 80 gm/cm^2 for times ranging from 30 minutes on.

H^3-proline

In the "hot" standards controls approximately 98 per cent of the cells were labelled by fine silver grains over their cytoplasm (Fig. 13).

Following the application of 10 gm/cm^2 to 80 gm/cm^2 for 30 minutes to 2 hours the number of labelled cells was little different from the controls. But after 4 hours the number of labelled cells dropped in all compressive force groups.

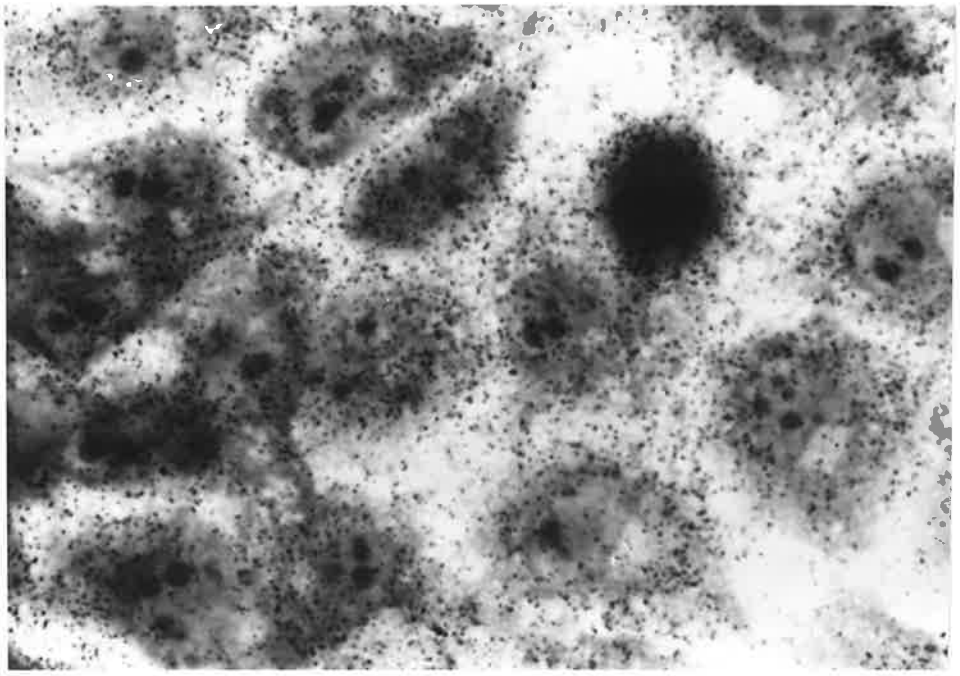

Figure 12 Autoradiography (H^3-uridine) - Silver grains are observed covering the cytoplasm and nucleus. Quite heavy labelling on all the cells can be seen. Methyl green and pyronin Y staining. Acetic alcohol fixed. 630x.

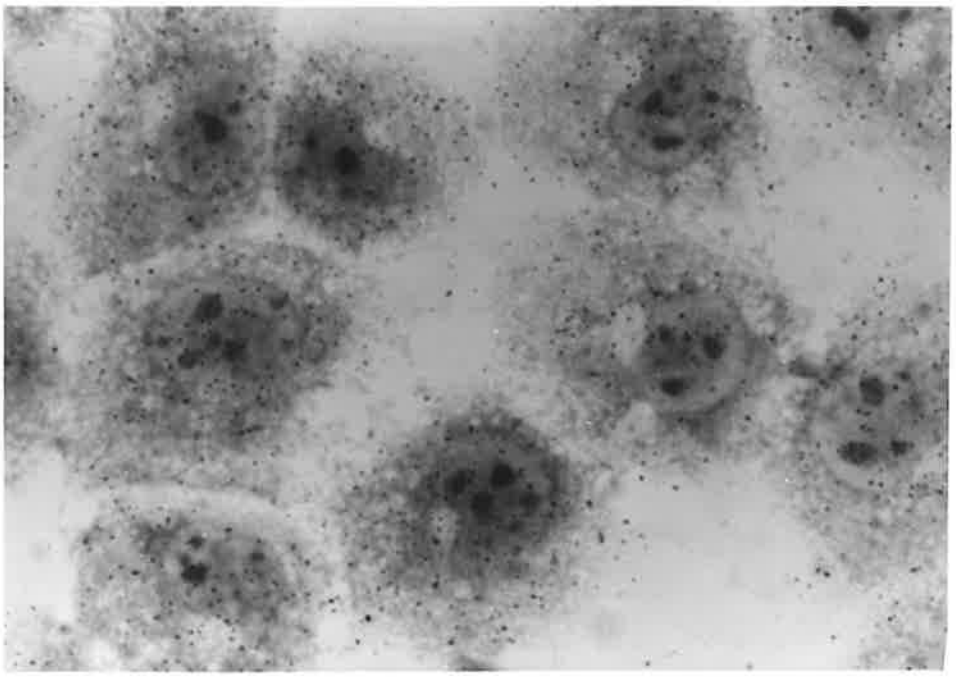

Figure 13 Autoradiography (H^3-proline) - Silver grains are observed on the cytoplasm. Methyl green and pyronin Y staining. Acetic alcohol fixed. 630x.

DISCUSSION OF PRESENT RESULTS — CHAPTER IV

1. CYTOCHEMISTRY

The present findings can be summarised as follows: Morphological observations revealed cytoplasmic blister formation as early as 30 minutes following the application of a compressive force of 10 gm/cm^2 or greater. The number of cells which formed blisters remained small in the 10 gm/cm^2, 20 gm/cm^2 and 40 gm/cm^2 groups, but increased in proportion to the force and with time of compression in the 60 gm/cm^2 and 80 gm/cm^2 groups. In addition, an increasing number of cells became ovoid after the application of forces in excess of 40 gm/cm^2 for 2 hours or 4 hours. Nevertheless, normal cells were observed after the application of forces of up to 80 gm/cm^2 for the maximum period of 4 hours.

Cytochemical study with the Feulgen and PAS reactions, Sudan Black B, and the four dehydrogenases, i.e. glutamic acid, malic, α-glycerophosphate and lactic dehydrogenases, revealed little change in the intracellular cytochemical or enzyme activity under the present experimental conditions.

However, cytochemical observations of acid phosphatase, succinic dehydrogenase and cytochrome oxidase enzyme activities presented different results.

The acid phosphatase activity remained unchanged in the 10 gm/cm^2, 20 gm/cm^2 and 40 gm/cm^2 groups after the various experimental periods. But in the 60 gm/cm^2 and 80 gm/cm^2 groups the enzyme activity dropped to comparatively moderate to weak levels, the amount of inhibition increasing with the duration of compression.

Succinic dehydrogenase and cytochrome oxidase activities appeared to parallel one another and more dramatic changes were quite apparent. With forces of 10 gm/cm^2 the enzyme activity was not noticeably changed, and with 20 gm/cm^2 and 40 gm/cm^2 forces the activities were reduced to levels recorded as intense to moderate after 30 minutes to 4 hours of compression. In the 60 gm/cm^2 and 80 gm/cm^2 groups the two enzyme activities were reduced moderately even after 30 minutes, and fell sharply to very weak or no staining with longer experimental periods. The amount of inhibition increased with the time of compression.

How can the present findings on cells to which compressive forces of 10 gm/cm^2 to 80 gm/cm^2 were applied for periods of 30 minutes to 4 hours be related to the *in vivo* situation?

The Feulgen reaction is a method for the histochemical demonstration of DNA, whose specificity and chemistry is now generally accepted. The findings of the present experiments indicate that compressive forces ranging from 10 gm/cm^2 to 80 gm/cm^2 for

periods ranging from 30 minutes to 4 hours had little effects on these aspects of DNA metabolism which the Feulgen reaction could demonstrate qualitatively. For this reason the further study of metabolism using autoradiography of H^3-thymidine was undertaken as described in the following section.

For the histochemical demonstration of glycogen, neutral mucopolysaccharides, mucoproteins, glycoproteins and certain lipids the periodic acid-Schiff reaction is routinely used. The findings from the present experiments indicate that the compressive forces used had little effect on carbohydrate metabolism as assessed by PAS reaction.

Sudan black B is a general lipid stain. The method utilizes a lipid-soluble dye, Sudan black B, to stain intracellular lipids. The findings obtained indicate that the present experimental conditions had little effects on localization or accumulation of lipids.

Failure to discern changes in the intensity of the Feulgen or PAS reaction and Sudan black B staining might mean that DNA, carbohydrate and lipid metabolism of the cells were hardly affected by compressive forces of up to 80 gm/cm^2 for 4 hours. However, the limitation of the sensitivity of the techniques employed for their respective substrates must be borne in mind and in each case, the assessments in these experiments were qualitative only in nature.

For a more precise estimation of changes in the metabolism of the above compounds a biochemical analysis of intra and extra cellular changes of each macromolecule would be necessary.

Although acid phosphatase histochemistry is of recent origin compared with alkaline phosphatase or succinic dehydrogenase, it is generally agreed to be one of the more reliable enzyme histochemical methods. The technique employed in the present experiments was the naphthol AS phosphate method which demonstrates acid phosphomonoesterases. The method utilizes the release by enzymatic hydrolysis of substituted naphthols to bind with diazonium salts. The binding is believed to be partly due to the hydrogen bonding between the peptide groupings of the protein and the azo dye (BURSTONE, 1962, p.144). Because of the low solubility and the affinity for protein of the substituted naphthols, an accurate localization of the enzyme usually can be obtained. Acid phosphatases are widely distributed in animal tissues and are considered to be important lysosomal enzymes (de DUVE, 1959), whose function is related to phagocytosis, pinocytosis and exudation in conjunction with the Golgi complex. As such the presence of the enzyme is a good indicator of lysosomal function as distinct from other aspects of cellular function such as energy production, protein synthesis or DNA replication. Of greater interest is the demonstration that the enzyme is present in considerable amounts in osteoclasts actively engaged in bone remodelling (SCHAJAWICZ and CABRINI, 1958; HANDELMAN *et al.*, 1964).

The present findings showed that compressive forces of 10 gm/cm^2 to 40 gm/cm^2 had little effect on acid phosphatase activity in the cells, but in the 60 gm/cm^2 and 80 gm/cm^2 groups the enzyme activity decreased from moderate to weak with time of compression. The results are interesting with respect to remodelling when bone is subjected to stress.

The technique for the demonstration of succinic dehydrogenase activity is well established histochemically and is considered to represent biological oxidations at the intracellular level, i.e. in the organelles most intimately involved, the mitochondria. The findings indicate that the compressive forces used for the various experimental periods affected the biological oxidation system represented by succinic dehydrogenase. The compressive forces employed, particularly 60 gm/cm^2 and 80 gm/cm^2, seriously reduced the activity of the enzyme.

Cytochrome oxidase is an iron-porphyrin enzyme which catalyzes the reduction of oxygen utilized by mammalian tissues (BURSTONE, 1962, p.429). The enzyme is thought to be entirely intramitochondrial and closely associated with the ultrastructure of the mitochondria. The histochemical technique employed is based on the "nadi" reaction, derived from the first two letters of naphthol and diamine, from which the indophenol colour is formed as a result of the action of oxidase on naphthol and diamine. The findings closely parallel those obtained in assessing succinic dehydrogenase activity. The compressive forces

employed, particularly 60 gm/cm^2 and 80 gm/cm^2, seriously reduce the enzyme activity.

The histochemical demonstration of the four dehydrogenases, glutamic, malic, α-glycerophosphate and lactic dehydrogenase, utilizes the same principle as that for succinic dehydrogenase. One can observe the formazan colour as a result of reduction of tetrazolium salt. From the histochemical point of view, according to PEARSE (1960, p.574), malic and succinic dehydrogenase activities are the best indicators of TCA cycle activity. Glutamic dehydrogenase is related to the synthesis of glutamic acid; α-glycerophosphate and lactic dehydrogenases are related to glycolysis. Under the present experimental conditions little effect on the activities of these dehydrogenases was observed as a result of the applied forces.

The effects of the compressive forces on the different oxidative enzymes seem to conflict, especially between those observed with succinic dehydrogenase and cytochrome oxidase, *viz à viz* the four dehydrogenases. Although PEARSE (1960, p.572) considers it is possible to histochemically localize ten, and possibly eleven, of the specific coenzyme-linked dehydrogenases with a high degree of sensitivity, BARKA and ANDERSON (1965, p.318) maintain that the problem of demonstrating specific dehydrogenases is not resolved and that these enzymes are not exclusively mitochondrial (BARKA and ANDERSON, 1965, p.309).

With these comments in mind, it seems more logical to place greater

emphasis on the results demonstrating succinic dehydrogenase and cytochrome oxidase activities which are more reliable. These oxidative enzymes, (succinic dehydrogenase and cytochrome oxidase) are intimately mitochondrial and are good indicators of the maintenance of vital processes in aerobic organisms. Without the proper functioning of mitochondria there is no protein synthesis, DNA replication, and other cellular functions. The present findings indicate that with compressive force in excess of 20 gm/cm^2, particularly with 60 gm/cm^2 and 80 gm/cm^2, inhibition of mitochondrial function increased with time of compression. This may indicate the presence of pressure-sensitive components in the complex structure of mitochondria.

In assessing the succinic dehydrogenase and cytochrome oxidase cytochemistry an interesting finding was that cells which grew into clones retained moderate to intense enzyme activity even when the activities of the surrounding monolayered cells were considerably less. This finding could indicate the relative resistance to compressive forces of cloned cells, which may more closely resemble the *in vivo* situation.

Cytoplasmic blister formation was observed as early as 30 minutes after the application of compressive forces of 10 gm/cm^2 or greater. Interestingly enough, the blisters were observed even in cells which showed intense cytochemical activity or autoradiographic labelling. What the biological significance of blister formation is is conjectural at present. It is well known that blisters can result from

several different treatments. For example, following contact with chemicals (HOGUE, 1919; ZOLLINGER, 1948; CRAWFORD and BARER, 1951; LATTA and KUTSAKIS, 1957; KAWAHARA *et al.*, 1968), after stimulation with electric currents (BUCKLEY, 1960a,b) and during mitosis (BOSS, 1955; ROBBINS and GONATAS, 1964). It is considered that with the exception of mitosis, blister formation is a pathological phenomenon.

The increasing numbers of rounded-up cells which were observed following experimental periods of 2 hours and 4 hours with compressive forces of 40 gm/cm^2, 60 gm/cm^2 and 80 gm/cm^2 is considered to be due to a sudden "decompression" effect when the load on the cells was removed. As fixation of the cells followed immediately in the cytochemical studies, the cells would not have had time to establish their normal cytoplasmic extensions. In order to test the recovery potential of these cells they were allowed to equilibrate with the culture medium for varying time periods following the removal of the compressive forces. It was observed (NAKAMURA and THONARD, unpublished data) that most cells assumed their normal cytoplasmic extension after approximately five hours and they appeared to be viable up to 24 hours, the limit of the observation period.

Unfortunately, the effects of pressure on the various enzymes have not been investigated to any great extent. In the biological field, MORITA and his co-workers (MORITA and ZOBELL, 1956; MORITA, 1957; MORITA and HOWE, 1957; HILL and MORITA, 1964) investigated the effects of high hydrostatic pressure on the activities of some dehydrogenases

and phosphatases in *E.coli* and marine bacteria. They observed progressive enzyme inactivation at pressures of 600 atmospheres or greater, which they postulated was due to disturbances induced in the TCA cycle by the pressure.

In the orthodontic field, DEGUCHI and his co-workers (TAKIMOTO *et al.*, 1966; DEGUCHI and MORI, 1968; TAKIMOTO *et al.*, 1968; DEGUCHI, 1969a,b) observed the effects of experimental tooth movement on various phosphatases. Unfortunately they inserted rubber dam between the teeth to produce displacement. The results of these investigators provide no information on the relationships between known forces and their effects on tissues. This information seems to be essential, since orthodontic tooth movement can be effective only with the application of a minimum degree of force. It is therefore not feasible to relate the above quoted results to the present findings; they will be discussed shortly as they relate to general biology.

The present findings and the aforementioned discussion should be accepted with certain reservations. Firstly, the results were obtained in an *in vitro* system in which it is always difficult to know to what extent the behaviour of any cell type mirrors its behaviour in the intact animal. At the same time it could be maintained that the results provide information which could lead to the formulation of ideas directed towards reaching the goal of optimal orthodontic treatment. No one approach to the subject can be expected to solve

the problem.

Secondly, the techniques employed were cytochemical methods which are qualitative in nature, at least in the present study. Biochemical and/or electron microscopic approaches could provide more precise information.

2. AUTORADIOGRAPHY

The present findings can be summarized as follows: in the H^3-thymidine series the labelling was observed exclusively on the nucleus. Approximately 30 per cent of control cells incubated with H^3-thymidine became labelled. Following the application of 10 gm/cm^2, 20 gm/cm^2, 40 gm/cm^2 and 60 gm/cm^2 for periods of from 30 minutes to 2 hours the number of the labelled cells remained about the same as in the controls, and after 4 hours the number of labelled cells was slightly reduced. With compressive forces of 80 gm/cm^2 the number of labelled cells decreased after each experimental period to the same level as recorded for cells to which 10 gm/cm^2, 20 gm/cm^2, 40 gm/cm^2 and 60 gm/cm^2 forces had been applied for 4 hours.

In the H^3-uridine series, labelling was observed uniformly over the whole cell. Approximately 95 per cent of control cells incubated with H^3-uridine were labelled. The effects of the experimental conditions followed an identical pattern to the H^3-thymidine series. Thus, with compressive forces of up to 60 gm/cm^2 no effects on H^3-uridine incorporation were observed until the weights had been

applied for 4 hours. However with a force of 80 gm/cm^2 decreased H^3-uridine uptake was noted after 30 minutes progressively decreasing to about the same level as observed for the lesser forces after 4 hours of application.

In the H^3-proline series, labelling was observed over the cytoplasm. In the "hot" standard approximately 98 per cent of cells were labelled. The findings were somewhat different from those with H^3-thymidine and H^3-uridine. Following the application of 10 gm/cm^2 to 80 gm/cm^2 for from 30 minutes to 2 hours the number of labelled cells was little different to that seen in the "hot" standard. But after 4 hours of compression, the number of labelled cells dropped in all groups.

Each radioisotope selected represents a specific precursor of an essential biological entity. Thymidine is only incorporated into cells undergoing DNA synthesis at the time of exposure to the radioisotope. The self replicating role of DNA ensures the synthesis of new DNA chains in response to genetically coded directions. The role of DNA in protein synthesis is to direct cellular protein synthesis in accordance with the genetic code which the DNA carries. It is generally believed that this information is carried by messenger RNA from the nucleus to the cytoplasm where protein synthesis is mediated through other types of RNA and appropriate amino acids on the ribosomes.

RNA, one constituent of which is uridine, is a nucleic acid located

in the nucleolus and cytoplasm. It is classified into three types, i.e. messenger RNA, which carried the information of DNA to the ribosomes and becomes the template for protein synthesis, ribosomal RNA, which is associated with the ribosomes of the endoplasmic reticulum in the cytoplasm, and transfer RNA, which transfers amino acids to the proper sites on the RNA template of the messenger RNA. Acting in proper sequence, these three types of RNA achieve protein synthesis.

The amino acid proline is an essential component of collagen the most important and abundant structural protein of mammals. It is synthesised by fibroblasts, which "deposit" the protein in the form of extracellular fibres. The present findings indicate that the experimental conditions employed had no apparent effect on the three vital biological functions monitored from the point of inhibiting cellular activity. At the same time, it is also apparent that with longer applications of the compressive forces, inhibitory effects on cell protein synthesis will result.

The resistance of the nucleic acids to the effects of high compressive forces has already been mentioned. ZIMMERMAN (1963) and ZIMMERMAN and SILBERMAN (1964) found that incorporation of H^3-thymidine into fertilized *Arbacia* eggs continued at pressures as high as 5,000 psi. But at pressures of 7,500 psi and greater the incorporation of the radioisotope was inhibited.

In the orthodontic field, DALE *et al.* (1964), CRUMLEY (1964), STALLARD (1967), BAUMRIND (1969), KVAM (1969), KOUMAS and MATTHEWS

(1969), DeANGELIS (1970), and BAUMRIND and BUCK (1970) reported similar results using H^3-thymidine incorporation to assess the effects of forces on cell metabolism in experimental animals. Unfortunately, these authors did not record the relationship between the forces utilized and the resultant effects on cell metabolism. Therefore, it is difficult to relate these studies to the present findings.

The reservations regarding the cytochemical findings must also apply to the autoradiographic findings. Firstly, the results were obtained under *in vitro* conditions. The advantages and disadvantages have been described in the previous sections, and are not repeated again.

Secondly, although autoradiography is one of the optimal methods for observing the localization of radioactive tagged metabolites in single cells, as BASERGA and MALAMUD (1969, p.129) have pointed out, the technique is deficient as a quantitative method compared with radiochemical methods. For this reason, an extensive quantitative expression of grain counts per cell was not undertaken.

3. RATIONALIZATION OF PRESENT FINDINGS

The cytochemical and autoradiographic findings have been discussed separately in the foregoing sections. An attempt to correlate and reconcile the results will be made in this section.

As already described, the cytochemical findings revealed that acid phosphatase, succinic dehydrogenase and cytochrome oxidase

activities were affected by the compressive forces employed. In particular, the latter two oxidative enzymes were severely affected when compressive forces of 60 gm/cm^2 and 80 gm/cm^2 were applied for periods of 1 hour and longer.

On the other hand, the autoradiographic studies monitoring nucleic acid and collagen metabolism showed that the effects of the compressive forces were minimal even after the application of forces for 4 hours; the changes were certainly small compared to the decreased activities of the succinic dehydrogenase and cytochrome oxidise enzymes.

These results appear anomalous, because it is not unreasonable to expect that changes in the activity of any one of the enzyme systems investigated should be reflected by parallel changes in related metabolic pathways. Severe reduction of oxidative enzyme activities should lead to an impairment of DNA, RNA and protein metabolism, particularly if these have a mitochondrial association. Several explanations for the apparent anomaly are possible, but it is unlikely that any one factor can account for the observed differences in results.

In the first place, differences between the activities of the oxidative enzymes and acid phosphatase as assessed by cytochemical means and of metabolic activities of nucleic acids and structural proteins measured by incorporation of isotopically-labelled precursors may be related simply to differences in the two methods of assessment.

For the cytochemical studies the cells were fixed immediately following the removal of the particular compressive force. On the other hand, in the precursor incorporation studies, fixation of cells for autoradiographic processing did not occur until the cells had been incubated with non-labelled precursor for one hour following removal of the compressive forces.

Under these conditions the cytochemical findings reflect the immediate effects of compressive forces on the cells whereas the autoradiographic studies reflect the effects of the forces on cells which are in a phase of recovery from physical force. If this is so, then the results indicate that the majority of cells can recover from the effects of compressive forces, if these act for up to two hours only, and it is only when forces are applied for periods of at least four hours that reduced metabolic activity is more obvious.

Secondly, it is possible that the differences in the results of the experiments are due to intrinsically different susceptibilities of functional enzymes associated with the cell membrane, cytoplasm or mitochondria, *viz à viz* enzymes associated with structural protein metabolism, DNA replication or RNA translation. In these experiments, the extent of any mitochondrial damage is not known; the fact that RNA transcription and protein synthesis has continued indicates that either mitochondrial damage did not occur, or if so, its effects were minimal.

Nevertheless, assuming reasonably equal sensitivity of the cyto-

chemical stains employed, the fact that the activities of three enzymes were markedly decreased by compressive forces whereas several DPN-linked enzymes were not, lends some support to the concept of differential susceptibility to compressive forces between enzyme systems.

A further possibility is that a sequential disarrangement of cell metabolism may be expected when "non specific" insults are applied to cells. Certainly physical force, as applied in these experiments, must be regarded in this category and its effects could be imagined to initiate a series of metabolic events which may terminate in cell death. Before reaching this state the compressive forces may in the first place affect cell membranes, leading to an impairment of ion transport and oxygen exchange which could be observed by changes in oxidative enzyme activities.

Although anaerobic glycolytic pathways may continue to supply energy for cell metabolism and lactic acid accumulation be neutralized by sufficient media buffering capacity, the mammalian cell retains the need for electron transport through the cytochrome system to cytochrome oxidase. In the absence of oxidative metabolism, in this case due to insufficiency of cytochrome oxidase, eventually nucleic acid and structural protein synthesis will be affected as secondary phenomena.

Whatever explanation(s) may be accepted or rejected to explain the different results of the cytochemical and autoradiographic techniques there can be little doubt that the experiments do indicate the need for studying the effects of forces on cells for longer time periods; biochemical and electron microscopic studies could yield further valuable information on these matters.

Certainly, the fact that cells subjected to reasonably high compressive forces for significant periods of time retain the ability to recover when the forces are removed, is information which points up still further areas of experimentation.

GENERAL DISCUSSION CHAPTER V

A. ON THE EFFECTS OF MECHANICAL STRESS ON CELLS

It is quite apparent from the literature review that the reported effects of stress on biological material differ depending on whether one is considering the orthodontic literature or that encompassing the wider biological literature. In the latter, for example, the effects of hydrostatic pressures greater than 1,000 psi have been investigated; in fact, there is general agreement that there are few effects on biological materials at lesser forces. But in the field of orthodontics on the other hand, optimum orthodontic tooth movement is effected with forces measured in terms of a few grammes per square centimeter. With forces much in excess of these levels, unfavourable tissue reactions occur. What is the explanation for these differences, which seem to be real, despite the fact that similar biological sources are utilised?

To understand the difference, it is necessary to review the experimental methods employed in both fields. In the general biology field, with some exceptions almost all the experiments were performed in specially designed, closed chambers in which various biological materials were maintained in a medium. Hydrostatic pressures were applied either by high-pressure hydraulic pumps or by centrifugal force. Thus, the medium compresses the biological

materials uniformly from all directions. In marine biology, hydrostatic pressures are, in fact, one of the main environmental parameters and must not be neglected in considering the relationship between life and the sea depth.

The situation in orthodontics is quite different from the above. The compressive forces are produced by direct application to the tissues. The solid material, in this case the calcified tooth root, exerts its compressive forces directly on the tissues. The different ways of force transmission employed in the two systems seems to provide an ample reason for differing results.

In the current experiments direct compression was applied to cells with a special apparatus which attempted to simulate the situation pertaining to that found in orthodontic tooth movement.

As revealed by the present study, mitochondrial function, represented by succinic dehydrogenase and cytochrome oxidase activities, was the most vulnerable part of the cellular functions to the compressive forces; and to a lesser degree, lysosomal function, represented by acid phosphatase activity. Since the experimental design presumably provided the necessary nutrient and oxygen supply to the cells during the compressive stage, the findings can be regarded as representing the effects of the compressive forces. It could be expected that loss or reduction of even one of the above key cell functions could lead to serious disturbances in general cell function and metabolism since these functions are delicately interrelated.

The observation of cytoplasmic blister formation only confirms that the effects of the compressive forces resulted in an overstress of cellular integrity.

From the viewpoint of cell kinetics, a somewhat different approach can be applied to the above problem. Biological tissue is always in a dynamic balance in which metabolic activity continues and cell components are constantly renewed. If one considers the tissue as a compartment, the number of cells in the compartment depends on cell influx following mitosis on the one hand, and on cell outflux as cells leave the compartment on the other hand. In the normal tissue, these cell movements are well balanced so as to maintain the structure of the tissue. When an extraneous force acts on the compartment, recovery and continuance of homeostasis would depend largely upon the population of damaged cells. If the number of damaged or otherwise affected cells is small, the compartments would be restored soon by rapid cell influx to the original level. If the number of damaged cells is large, restoration of the compartment would require a longer period of time and permanent damage could result because the cell influx cannot replace the large loss of cells.

This reasoning could be applied to the present problem. The findings showed that application of compressive forces ranging from 10 gm/cm^2 to 40 gm/cm^2 resulted in blister formation of a small percentage of cells and correspondingly slight decreases in succinic

dehydrogenase and cytochrome oxidase activities, but with 60 and 80 gm/cm^2 forces, an increasing number of cells developed cytoplasmic blisters and greater enzyme inhibition with increasing time of compression.

If the compressive force is appropriate and large enough to induce bone remodelling in a local area and small enough to minimize local tissue damage, the use of force could be acceptable in a field where teeth are to be moved and the only available method of inducing the necessary bone remodelling is to apply force. In so doing, the application of minimal but optimal forces is essential. There would most certainly be a force, or more likely a range of forces, over which bone remodelling can be induced locally with minimum tissue damage.

B. ON THE MECHANISM OF ORTHODONTIC TOOTH MOVEMENT

The literature reviewed so far has clearly indicated that orthodontic tooth movement is in fact bone remodelling which is induced by mechanical stress. GOTTLIEB's comment (1946) to the effect that in the absence of bone remodelling no forces or techniques could achieve tooth movement greater than the distance between tooth and alveolar bone assumes great importance. The only way to move a tooth over greater distances is to produce the necessary favourable local tissue changes for bone remodelling to occur, in addition to creating enough space for the tooth movement, thus:

Mechanical stress -⇒ bone remodelling

However, the mechanism responsible for bone remodelling when mechanical stress is applied, is not known. It seems likely that this could conform to a general principle of biological control as pointed out by some investigators. The term "negative feedback" system was originally used in the electronics industry; upon receiving an incoming stimulus of a certain intensity, the system works towards the direction of reducing the stimulus and finally restoring its original state. Such an explanation could provide at least a working hypothesis to explain the mechanism of bone remodelling. Orthodontic force is a foreign mechanical stress whcih has no connection with the original functions of the integrity of the tooth and its supporting tissues. Accordingly, the supporting tissues of the alveolus should attempt to restore tissue integrity by a reduction of the stimulus to regain homeostasis. If a positive feedback occurred, the initial stress would increase with time. HOOROBIN (1970) maintained that in a positive feedback system any applied force would increase in intensity so rapidly as to create a completely unstable system and this was extremely rare in biology.

It seems that the most critical stage, so far as tooth movement under orthodontic stress is concerned, is at the initial stage of application of force to the tooth crown, followed by transmission of

the force to the local tissues, and tooth movement; because once a negative feedback system is established, bone resorption, remodelling and tissue reactions would continue until the stress fell below the threshold level.

From our knowledge of calcified tissue research, we know that bone resorption is not necessarily carried out solely by osteoclasts, but sometimes by other cells. Therefore in the ensuing discussion the possible ways of bone resorption will be reviewed and related to the immediate problem. For convenience these may be considered as:

(a) The direct effect of stress, or

(b) the secondary effect of stress in either case to -

  (i) stimulate already existing cells which contain the genetic potential for bone resorption, or

  (ii) stimulate precursor cells to differentiate into cells which are capable of bone resorption

(a) (i) The direct effect of stress to stimulate already existing cells, which contain the genetic potential, for bone resorption.

In the first place, it seems appropriate to discuss the cells which are capable of bone resorption of which the osteoclast is thought to play a key role. Osteoclasts were first identified by KÖLLIKER (1873). At one time the function of osteoclasts was under debate (HANCOX, 1949), but the bone resorbing ability of the cells has now been demonstrated by a number of techniques, such as

autoradiography (ARNOLD and JEE, 1957, cinemicrography (GAILLARD, 1955; GOLDHABER, 1960; HANCOX, 1963) and electron microscopy (SCOTT and PEASE, 1956; CAMERON and ROBINSON, 1958; HANCOX and BOOTHROYD, 1963). According to HANCOX (1972), the cells are large and generally multinucleated; the largest contains several hundred nuclei, the smallest, but one or two, with an average of ten or twenty. The cells are highly mobile (GOLDHAVER, 1963; HANCOX, 1963) and are often found in Howship's lacunae. According to GOLDHABER (1963) osteoclasts seem to be involved actively in the resorptive process, where the bone literally appeared to "melt away" as a result of the "bubbling and boiling" activity at the surface of the osteoclasts. Similar types of bone resorbing cells, multinucleated giant cells have been described by others (IRVING and HANDELMAN, 1963; IRVING and HEELEY, 1970).

Osteocytes also have been reported to effect bone resorption (BAUD, 1962; BÉLANGER *et al.*, 1963). This has been confirmed by several reports (BÉLANGER *et al.*, 1966; HEUCK, 1970; NICHOLS, 1970; BAYLINK and WERGEDAL, 1971; BÉLANGER, 1971), although CAMERON (1969) is not in agreement with these findings. However, osteocytic activity may account for only about 1.3% of the bone resorption mediated by osteoclasts (BAYLINK and WERGEDAL, 1971) so that the role of osteocytes in bone resorption seems to be somewhat minor and limited.

Other cells are believed capable of taking part in bone resorption. Small round cells (IRVING and BOND, 1968; IRVING and HEELEY, 1970), mononuclear giant cells (WEINMANN and SICHER, 1955; GAILLARD, 1959; TONNA, 1960), endothelial cells (JAFFE, 1933; CAMERON, 1961) and macrophages (McLEAN and BLOOM, 1941; GOLDHABER, 1961) have all been implicated in the resorptive process.

These reports reveal that bone resorption is not an exclusive function of one type of cell with a specific morphology, but rather is a function of many cells which retain the capacity for resorption and can be activated under suitable environmental conditions. In this discussion the term "osteoclast" will be used in its broadest sense to describe a cell capable of bone resorption.

As for the mechanism of bone resorption, it has long been debated whether the primary change is the removal of hydroxyapatite crystals or of collagen. Based on ultrastructural findings, HANCOX and BOOTHROYD (1961, 1963) and CAMERON (1972) suggested that the removal of the crystals was the first step effected by the osteoclasts. On the other hand, SCOTT and PEASE (1956), DUDLEY and SPIRO (1961) and GONZALES and KARNOVSKY (1961), also using ultrastructural techniques, maintained that the primary change was removal of collagen, because the presence of crystals around the brush border area of the osteoclasts indicated prior removal of collagen. CAMERON (1972) explained the existence of the apatite crystals as the result of precipitation

from a high local concentration of calcium and phosphate ions followed by subsequent use of an alkaline fixative employed during the processing of the tissues for electron microscopy. In contrast, based on enzymatic investigations, VAES (1965, 1968) proposed a concomitant removal of apatite crystals and collagen.

Whichever of these two components of bone is removed first, lysosomal enzymes are known to be involved in both phases of bone resorption. VAES (1965, 1966, 1968, 1970) and VAES and JACQUES (1965) demonstrated six lysosomal acid hydrolases concerned with bone resorption. VAES (1968, 1970) has observed bone resorption of embryonic mouse calvaria *in vitro*, characterized by solubilization of both mineral and organic components under the action of parathyroid hormone (PTH). During the progress of resorption he found increased amounts of acid hydrolases in the medium. He presumed that the release of the enzymes was not due to a direct disruption of the lysosomal membrane by PTH, but rather to an exocytosis of the whole lysosomal content and that the excreted enzymes were replaced by new intracellular synthesis. Moreover, lactate and citrate which, he thought, originated from increased glycolysis, performed concomitant solubilization of bone mineral. Other enzymes, such as hyaluronidase (VAES, 1966) and collagenase (WOODS and NICHOLS, 1965) might play a role in resorbing bone. The possible role of mitochondria in controlling the level of Ca^{++} within osteoclasts has been reported recently (MATTHEWS *et al.*, 1971).

In addition to PTH other factors influence calcium transport and homeostasis. Vitamin A is considered to be intimately related to bone resorption (FELL and MELLANBY, 1950; REYNOLDS, 1968; BARNICOT and DATTA, 1972). Changing levels of oestrogen (BLOOM *et al.*, 1958; TAYLOR and BÉLANGER, 1969) and high levels of oxygen in tissue culture (GOLDHABER, 1961) are also known to act as stimuli.

More relevant to the present subject, it has been demonstrated in many animal experiments that compressive forces on bone can act as an initial stimulus for bone resorption (SANDSTEDT, 1904, 1905; REITAN, 1951; STOREY and SMITH, 1952). The efficiency of this type of stimulus, is largely dependent on the magnitude of the force and duration of application.

The present findings showed that prolonged compression of cells with heavier forces resulted in reduction of succinic dehydrogenase and cytochrome oxidase activities and to a lesser degree, of acid phosphatase. It is quite likely that more severe effects may be expected after longer compression periods than the maximum experimental period employed of 4 hours.

Although the cells used in the present experiments were fibroblasts, the findings may be applicable to osteoclasts, since the basic cell machinery and its functions no doubt have fundamental common characteristics. The major difference is that in the osteo-

clast the machinery may be arranged in the most efficient form to carry on a specialized function - resorption of hard tissues. However, should the function of an organelle, e.g. nucleus or mitochondria or lysosome or endoplasmic reticulum be impaired, there would be no bone resorption.

With compressive forces, some or all of the cell machinery might be stimulated by the mechanical stress. But it is as yet unknown how the cells are activated to assume a resorptive order. Before such mechanisms can be understood, more information on the milieu surrounding the locus of bone resorption, in particular osteoclasts, is required. An observation by BÉLANGER *et al.*, (1966) seems pertinent. During investigations of the mechanisms of bone resorption they concluded the appearance of osteoclasts was associated with features common to such systemic or local conditions as hyperparathyroidism, hypervitaminosisA, dietary calcium deficiency, bone fractures or ectopic bones and teeth for example.

This might indicate that the osteoclasts are capable of carrying out their functions in "subnormal" environments compared with other cell types.

Equally important is the preservation of an adequate vascular system to ensure maximum supply and disposal of essential metabolites and waste products. The importance of vascularity has been stressed

many times (TRUETA and AMATO, 1960; TRUETA and TRIAS, 1961; TRUETA, 1962, 1963; VAES and NICHOLS, 1962; HOLDEN, 1972).

Based on electron microscopic observations of osteoclasts, LUCHT (1972) suggested that the localization of osteoclasts in close proximity to vessels might facilitate the transportation of absorbed products to the blood. MATTHEWS (1970) and MATTHEWS *et al.* (1970) reported an intracellular gradient of mitochondrial granules which consisted primarily of mineral. They considered these to be a result of a regulatory mechanism aimed at maintaining the level of ionic calcium within the cell (MARTIN and MATTHEWS, 1970). Osteoclasts adjacent to the resorbing surface exhibited high levels of granules in the mitochondria while those on the vascular surface had none, thus suggesting a transport of mineral across the cells from bone to blood vessel.

The importance of maintaining an adequate blood supply to the local tissues during orthodontic treatment has been emphasized often (SANDSTEDT, 1904, 1905; SCHWARZ, 1932; CASTELLI and DEMPSTER, 1965; KHOUW and GOLDHAVER, 1970). Occlusion of the periodontal vasculature due to the compressive forces acting between tooth and alveolar bone is a common event in orthodontic tooth movement.

(a) (ii) The direct effect of stress to stimulate precursor cells to differentiate into cells capable of bone resorption.

HANCOX (1972) considered that osteoclasts originated by the fusion of either one or two precursor cell types. One form is probably involved in the production of collagen and morphologically resembles (or are) fibroblasts, reticular cells, mesenchyme cells, true osteoblasts or vascular endothelium. The other form is more freely mobile and resembles macrophages, mononuclear cells, wandering cells, and blood monocytes. But his experiments do not delineate the origin of the cells, nor where the postulated site of fusion is located.

On the other hand, WEINMANN and SICHER (1955) nominate the possible source of osteoclasts as being osteocytes whose vitality for one reason or another was decreased. However, they too were not able to definitely determine the origin.

It is possible that compressive forces might stimulate precursor cells to differentiate into osteoclasts which play the main role in bone resorption. This could be important, since the number of existing osteoclasts might be very small under normal conditions; in addition the resorbing ability of the osteocytes and other cell types may be rather limited. In order to effect sufficient bone resorption to provide enough space for tooth movement, it is necessary to generate still more osteoclasts. Furthermore, the number of osteoclasts produced should be quite high, since the life-span of the cells seems to be fairly short, according to HANCOX (1972). Although OWEN (1971 found a lower number of osteoclasts compared with osteo-

blasts in most cases of bone resorption and maintained that the reason for this was the greater functional efficiency of the osteoclasts, nevertheless a population of active osteoclasts is necessary to perform bone resorption while new cells are "prepared" for assuming a functional role.

The key mechanism of differentiation under the influence of compressive forces, remains a matter of speculation. WEINMANN and SICHER (1955) considered compressive force as one stimulus for differentiation, but they did not provide an explanation for the mechanism.

(b) The secondary effect of stress to (i) stimulate the already existing cells which contain the genetic potential for bone resorption, or (ii) stimulate precursor cells to differentiate into the cells which are capable of bone resorption.

In this section, the two cell parameters will be discussed together. The secondary effects of stress are other possible activators of osteoclastic bone resorption. In this case the force itself has no direct activation role, but secondary effects through some form of signal or message produced by the primary force might play a role in stimulating already existing osteoclasts or the precursor cells to differentiate into osteoclasts to resorb bone.

An increasing volume of literature deals with these possibilities

of bone remodelling under mechanical stress. One possibility to consider is the piezo electric effect. As already reviewed in the introduction, a hypothesis has been proposed which depends on the findings that electric potentials are generated on either side of compressed bone. Another finding, related to the above is that significant amounts of bone formation were observed *in vivo* when the bone was stimulated by electric currents (YASUDA *et al.*, 1955). As for the origin of the electric effect, agreement has yet to be reached. Possible origins are peizo electricity (FUKADA and Yasuda, 1957), semiconductor (BASSET, 1965, 1966; BECKER, 1966) and streaming potential (CERQUIGLINI *et al.*, 1967; ANDERSON and ERIKSSON, 1968, 1970).

Whether electric charges stimulate bone formation or resorption is uncertain; bone formation in negatively charged fields has been confirmed by some investigators (BASSETT *et al.*, 1964; PAWLUK and BASSETT, 1970) but challenged by others (FRIENDENBERG and KOHANIM, 1968; O'CONNOR *et al.*, 1969; HAMBURY *et al.*, 1971; WILLIAMS AND PERLETZ, 1971). Definite data concerning bone resorption in positively charged fields, with activation of osteoclasts, is lacking.

The application of force causes alveolar bone distortion which is considered to be a source of electric currents (PICTON and DAVIES, 1967; GRIMM, 1972). There might be some relationship between the alveolar bone distortion, electric effects and bone remodelling, but at the moment the hypothesis seems to be far from proven.

Another possibility is stimulation by calcium ions (JUSTUS and LUFT, 1970). Alteration of the local levels of calcium ions which may arise from adjacent hydroxyapatite crystals, depending on the amount of mechanical stress, activates bone cells or precursor cells for bone remodelling. Mechanochemistry, on which this hypothesis relies, is a relatively new field and needs more substantiation.

Still another possibility is neural stimulation by those signals, or messages, which are produced by the stress (SMITH, 1967; CURREY, 1968). In this case there will be two intermediate steps; first, transformation into signals or messages due to the application of the stress and secondly, nerve stimulation by those signals or messages, prior to the final activation of the local cells by the nerves. Adaptive bone growth in response to stress could be influenced either by nerve contribution or by the electric effect which has already been discussed above.

Obviously, there are many aspects of bone resorption; at this stage neither the direct nor secondary effects can be overlooked, because the whole phenomenon of bone resorption, particularly the role of the osteoclast, has to be clarified in more detail.

C. ON THE MAGNITUDE OF ORTHODONTIC FORCE

As mentioned in the introduction, present methods for orthodontic tooth movement depend on the application of force. ORBAN

(1936) pointed out from histological observations that tooth movement by orthodontic appliances imposes an overstress in the biologic sense. The present findings seem to confirm his findings at the cellular level.

The orthodontist faces a dilemma. On the one hand he has to correct malocclusions in patients; on the other, experimental results indicate that the tooth movement which he will initiate may not be biologically acceptable. The preceding discussion in this section seems to be appropriate to the problem. The crucial question is how much force is sufficient to cause favourable tissue changes to permit tooth movement taking into account individual biological differences? Although it is impossible to define a precise optimal force it does seem possible to define an optimum force range; it is mandatory that the orthodontist ensures that the forces he utilizes for tooth movement are within this range. If not, the intended tooth movement is not achieved, is delayed, or, more importantly, unfavourable tissue damages will occur.

An empirical way to assess the suitability of the force employed, is indirectly *ex post facto* i.e. by a recognizable amount of tooth movement, or lack of pain, or lack of abnormal tooth mobility, or lack of severe reaction to percussion testing, etc. But it is far better to know the magnitude of the force in advance, not indirectly or *ex post facto*.

Currently there is no reliable, easily applied means of assessing forces in use on patients but the development of methods for so doing no doubt will evolve eventually through research.

D. ON ORTHODONTIC TOOTH MOVEMENT

It is pertinment to enquire if there are any better alternatives to induce bone remodelling other than the application of force, which is an essential component of today's orthodontic treatment. Application of force might be only one of several stimuli which can bring about bone remodelling for tooth movement. Other possibilities are chemical, electrical, magnetic force, heat, vibration, biological, or as yet unknown, stimuli.

It is apparent that the orthodontist can hardly discard the present system but a cursory consideration of some of the above possibilities may be of interest. For instance, the use of heat as a stimulus for bone remodelling. TWEEDLE and BUNDY (1965) reported an increased rate of tooth movement by applying local heat. Literature is available which reports a positive effect of heat upon bone growth (RING and lEE, 1958; RICHARDS and STOFER, 1959; SPAGNOLI *et al.*, 1964; BUNDY *et al.*, 1965).

Moving teeth with high frequency oscillations has also been attempted (MUMAW, 1972).

Biological stimulation, such as achieved with hormones has also been attempted. The influence of cortisone acetate and adrenocorti-

cotropic hormones in rabbits and guinea pigs (STOREY, 1958), parathyroid hormone in the rats (GIANELLY and SCHNUR, 1969), somatotropin in the rats (DECKER, 1971) and parathyroid hormone and cortisone acetate in cats (DAVIDOVITCH *et al.*, 1972) on tooth movement has been investigated. It is apparent that cortisone effects a marked influence on tooth movement in experimental animals.

These attempts are interesting but only experimentally at this stage. It seems that the practical application of these methods is premature and that further substantiation is needed before taking any further steps with respect to the treatment of patients.

CONCLUSIONS CHAPTER VI

It has been clearly shown by other workers that orthodontic tooth movement is, in effect, a result of bone remodelling under mechanical stress. This poses the question - how do the involved cells react to the mechanical stress? The relationship between the stress and the response of the cell, which is a basic unit of the tissue, must be resolved if the mechanism of orthodontic tooth movement is to be understood. Solution of these problems will provide a basis for the further understanding of control mechanisms governing orthodontic tooth movement.

An *in vitro* approach in which the responses of isolated cell systems were analysed following the application of known forces ranging from 10 gm/cm^2 to 80 gm/cm^2 for the periods of 30 minutes to 4 hours has been made in this study. Compressive forces were provided by a specially fabricated apparatus and directly applied to mouse fibroblast strain L-929 cells. The effects of the compressive forces on cells were assessed using several standard cytochemical methods and stains such as the Feulgen reaction, the periodic acid-Schiff reaction, alcian blue, sudan black B, alkaline phosphatase, acid phosphatase, succinic dehydrogenase, cytochrome oxidase, glutamic acid-, malic-, α-glycerophosphate- and lactic dehydrogenases and tritium autoradiography with H^3-thymidine,

H^3-uridine and H^3-proline.

Cytoplasmic blisters were observed following compression of the cells with forces of 10 gm/cm^2 for 30 minutes, increasing in proportion to the magnitude of the forces and to time. Succinic dehydrogenase and cytochrome oxidase activities were severely reduced by increasing compressive forces and over longer periods of time. Acid phosphatase activity was markedly reduced by 60 and 80 gm/cm^2 forces with increasing time of compression. But all metabolism governing the Feulgen reaction, periodic acid-Schiff reaction, sudan black B, glutamic acid-, malic-, α-glycerophosphate- and lactic dehydrogenases showed little disturbance under the present experimental conditions.

Autoradiography with H^3-thymidine, H^3-uridine and H^3-proline showed little effects except a slight drop in the incorporation rate following the longest experimental period.

The results were discussed with respect to the biological meaning to each cytochemical and autoradiographic tagging technique employed. It is apparent that the effect on cellular reactions was dependent on the time and the magnitude of the stress, i.e. increased time of application or heavier forces resulted in more inhibitory effects on the cells.

It is considered that loss of or reduction in even a single

cellular function could result in a disruption of other functions finally terminating with cell death, since all cell functions are delicately interrelated. On this basis, it could be concluded that any force used may be an overstress to the integrity of the cell in the strictest sense.

The present findings show that application of compressive forces ranging from 10 gm/cm^2 to 40 gm/cm^2 results in blister formation in a small percentage of the cell population, accompanied by slight falls in succinic dehydrogenase and cytochrome oxidase activities. With forces of 60 gm/cm^2 and 80 gm/cm^2 an increasing number of cells develop cytoplasmic blisters and further inhibition of these enzymes occurred with increasing time of compression.

If the compressive force is appropriate and large enough to induce bone remodelling and small enough to minimize damage to the local tissues, the use of force could be acceptable.

Certainly there would be a point, or more likely a range of forces, where both requirements were satisfied, i.e. forces of sufficient magnitude to induce bone remodelling whilst at the same time inducing minimal damage in the local tissues. These considerations may be supported by cell kinetics governing cell recovery and have quite practical applications to orthodontics. An attempt was made to explain the mechanism of orthodontic tooth movement as a negative feedback system whose ultimate goal is to

retain homeostasis. Orthodontic forces are foreign stresses which have no connection with the original functions of the integrity of tooth and its supporting tissues. In order to maintain the integrity of the tissue the response must be directed towards a reduction or removal of the extraneous stimulus.

Possible mechanisms of bone resorption under the influence of mechanical stress have been discussed. Bone resorption, which is needed to provide space for the teeth to move into, may be induced either by direct or secondary effects of compressive forces on cells of the local tissues.

These cells are probably no different to other types of cells with regard to their basic cellular machinery and function. It seems that the only difference may be that this machinery is geared to the most efficient means to carry out a specialised function, namely resorption of hard tissues. Considering the present findings, bone resorption under mechanical stress is dependent on a delicate tripod relationship between force magnitude, duration of force application and local cellular kinetics.

Finally, one may conclude with an abbreviated and somewhat conjectural proposal of the mechanisms governing orthodontic tooth movement in the light of our present knowledge of this problem. At the outset, one must regard tooth movement as the result of bone remodelling under mechanical stress in which bone cells or precursor cells play a vital and necessary role. This is well

supported by an accumulation of experimental evidence in the orthodontic field.

The primary effect is due to either the direct effects of the compressive forces on the cells of the local tissues under stress or to the secondary effects which are transformed into some physical or chemical form following the application of the compressive force.

This could be followed by activation of the cells although it is unknown how these cells are driven to the state of active bone resorption.

As revealed in the present study, the effects of compressive forces are not necessarily directed towards an acceleration of cellular functions, but rather are inhibiting. Specific bone resorption cells might be activated under somewhat "subnormal" conditions and remain capable of performing their specific function, as BÉLANGER *et al.* (1966) have pointed out.

Once the local climate is built up for bone resorption, and as long as the stress continues, the resorptive mechanisms may continue to function until the stress falls below the threshold level through homeostatic adjustment. Resorption will continue without interruption whenever stress is optimal, but if the magnitude of the stress is too large or too small delayed or unwanted side effects emerge.

However, before a final mechanism of bone resorption can be proposed, more information on the immediate resorptive milieu, particularly the osteoclasts, is required.

APPENDIX

Parenthetic notes to all items, phrases or sentences designated "(see Appendix)" are listed below according to pagination -

Page

CHAPTER I

5 Conversion for various units of mechanical forces. One atmosphere is equivalent to 14.696 pounds/inch^2(psi), 1.033227 kg/cm^2, 1.01325 bars, 1.01325 x 10^6 dynes/cm^2, 760 mmHg at 0^{o}C, or 1.01325 x 10^5 newtons/meter^2 (N/m^2).

CHAPTER II

83 L-929 fibroblasts. Obtained from Grand Island Biological Co., New York, U.S.A.

83 Eagle's basal medium. Supplied by Commonwealth Serum Laboratories (C.S.L.), Victoria, Australia. The concentration of antibiotics was as recommended by Eagle (Science, 122:501, 1955). Antibiotics used were penicillin, 50 units/ml, streptomycin, 50 µg/ml as supplied with the medium.

83 Bovine serum. Supplied by C.S.L., Victoria.

84 Mycoplasma medium. Supplied by C.S.L., Victoria. The medium was issued as a sterile digest broth containing 15% horse serum and 0.2% yeast extract. Phenol red had beed added as a pH indicator adjusted to pH 7.6.

85 Cellulose triacetate membrane. Metricel$^{(R)}$, Type GA-8, Gelman Instrument Co., Michigan, U.S.A.

Page

87 Hanks' balanced salt solution. Supplied by C.S.L., Victoria. The concentration of all components was as recommended by Hanks, J.H. and Wallace R.E. (Proc.Soc.Exptl.Biol.Med., 71:196, 1949).

89 4% cacodylate buffered glutaraldehyde solution. A 4% solution of glutaraldehyde buffered to pH 7.4 by 0.2M cacodylate buffer formulated as follows:

0.2M cacodylate buffer (pH 7.4)

Sodium cacodylate solution (0.2M)	25 ml
Hydrochloric acid (0.2M)	1.35 ml
Distilled water made up to	100 ml

89 Haematoxylin. Supplied by G.T. Gurr, London, England.

89 DPX. Synthetic mounting media. Supplied by G.T. Gurr.

89 10% neutral formalin solution:

Formaldehyde solution which was buffered to approximately neutral pH with calcium carbonate	10 ml
Balanced salt solution	90 ml

89 Basic fuchsin. Supplied by G.T. Gurr

90 Bisulfite solution:

Hydrochloric acid (HCl) I N	10 ml
Sodium metabisulfite solution (10%)	10 ml
Distilled water	200 ml

91 Sudan Black B. Supplied by G.T. Gurr.

91 Glycerin Jelly. Supplied by G.T. Gurr.

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91 Alcian blue. Supplied by G.T. Gurr.

92 Formol-calcium solution (10%)

Formaldehyde solution which was buffered to approximately neutral pH with calcium carbonate	10 ml
Calcium chloride	1 g
Distilled water made up to	100 ml

pH of the solution was adjusted with IN NaOH before use.

92 Naphthol AS-BI phosphate. Supplied by Sigma Chemicals, St. Louis, U.S.A.

93 Red violet LB salt. Supplied by Sigma Chemicals.

93 Nitro blue terazolium. Supplied by Sigma Chemicals.

96 Diphosphopyridine nucleotide (reduced form). Supplied by Sigma Chemicals.

96 Radioisotopes. All the radioisotopes were obtained from the Radiochemical Centre, Amersham, England.

98 Nuclear tracking emulsion. K2 type. Supplied by Ilford, Ilford, England.

98 Light proof black bakelite exposing box. Supplied by Clay adams, New York, U.S.A.,

98 Black pressure sensitive tape. Type 235. Supplied by Minnesota Mining and Mfg. Co., Minnesota, U.S.A.

98 Developer. Phen-X$^{(R)}$. Supplied by Ilford.

98 Fixer, Hypam$^{(R)}$. Supplied by Ilford.
One part of concentrated solution was diluted with five parts of distilled water.

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99 Pyronin Y. Supplied by G.T. Gurr.

99 Methyl green. Supplied by G.T. Gurr.

FIGURES

Figure 1a Diagrammatic section of assembled apparatus (improved model) and petri dish.

A. Lead weight
B. Polystyrene tube
C. Polystyrene disk with holes
D. Cellulose triacetate permeable membrane
E. Coverslip with attached cell monolayer
F. Plastic ring for holding membrane
G. Petri dish
H. Medium
I. 22 x 22 mm glass slide

Figure 1b Overall view of the apparatus (improved model) and petri dish.

Front row (from left to right):
Polystyrene tube, Plastic ring, Polystyrene disk and Cellulose triacetate permeable membrane

Back row (from left to right):
Assembled apparatus, 22 x 22 mm glass slide and experimental petri dish.

Figure 1c Overall view of the weight.

Front row (from left to right):
The weights for 10 gm/cm^2, 20 gm/cm^2 and 40 gm/cm^2

Back row (from left to right):
The weights for 60 gm/cm^2 and 80 gm/cm^2

Figure 2 Diagrammatic section of assembled apparatus (prototype) and experimental petri dish.

A. Lead weight
B. Polystyrene tube
C. Cellulose triacetate permeable membrane
D. Coverslip with attached cell monolayer
E. Plastic ring for holding membrane
F. 22 x 22 mm glass slide
G. Petri dish
H. Medium

Figure 3a Haematoxylin staining (after Harris) --- The cells cytoplasm and nucleus stained with haematoxylin. The cell shape varied ranging from a spindle to a wide-spread shape with many cellular processes. 4% cacodylate buffered glutaraldehyde fixed. Phase contrast. 250x.

Figure 3b Haematoxylin staining (after Harris) --- The cytoplasmic blister formation in cells is shown (arrows). The number and size of blisters increased with time during the experiment. Phase contrast. 600x.

Figure 4 Feulgen reaction (after Feulgen and Rossenbeck) --- The cell nucleus bound the basic fuchsin. The cytoplasm did not stain and remained clear. 10% neutral formalin fixed. 250x.

Figure 5 Periodic acid - Schiff reaction (after Hotchkiss and McManus) --- The cells stained overall with basic fuchsin. 4% cacodylate buffered glutaraldehyde fixed 250x.

Figure 6 Sudan black B (after Lison) --- The cells varied in staining reactions from light blue, grey or orange. A concentration of Sudan black B positive granules is observed around the nucleus. 4% cacodylate buffered glutaraldehyde fixed. 250x.

Figure 7 Acid phosphatase (after Burstone) --- with the naphthol AS-BI phosphate method, the acid phosphatase activity was marked by the vivid red azo dye staining at a region in the cytoplasm corresponding to the Golgi complex adjacent to the nucleus. Cold acetone fixed. 250x.

Figure 8a Succinic dehydrogenase (after Nachlas *et al.*) --- With the nitro-blue tetrazolium method, the enzyme activity was marked by the cytoplasmic deposition of formazan, whose colour ranged from dark purple to dark blue to almost black, or a combination of these. 8% un-neutralized, aqueous formalin fixed. 250x.

Figure 8b Succinic dehydrogenase (after Nachlas *et al.*) --- The cells were subject to a compressive force of 60 gm/cm^2 for 4 hours. A clear cut distinction between normal cells (Fig. 8a) and damaged cells was observed in their enzyme activity. It is apparent that the level of enzyme activity fell dramatically to very weak or zero. A large number of vacuoles can be observed around the nucleus. 250x.

Figure 9a Cytochrome oxidase (after Burstone) --- With the p-amino-diphenylamine method, the enzyme activity was marked by the cytoplasmic deposition of a reddish brown to blue-black precipitate. 8% unneutralized, aqueous formalin fixed. 158x.

Figure 9b Cytochrome oxidase (after Burstone) --- Cells subjected to a compressive force of 60 gm/cm^2 for 4 hours. As with the succinic dehydrogenase activity, a clear cut distinction between normal cells (Fig. 9a) and damaged cells was observed in their enzyme activity which fell dramatically to very weak or zero. A large number of vacuoles can be observed around the nucleus. 8% unneutralized, aqueous formalin fixed. 250x.

Figure 10 DPN-linked dehydrogenases (after Nachlas *et al.* and Hess *et al.*) --- The enzyme activity was identical to that of succinic dehydrogenase. The formazan colour, which ranged from dark purple to dark blue to almost black or a combination of these was observed throughout the cytoplasm. 8% unneutralized, aqueous formalin fixed. 250x.

Figure 11 Autoradiography (H^3-thymidine) --- Silver grains are observed on the nucleus, but not on the cytoplasm. Three labelled cells can be seen. Methyl green and pyronin Y staining. Acetic alcohol fixed. 630x.

Figure 12 Autoradiography (H^3-uridine) --- Silver grains are observed covering the cytoplasm and nucleus. Quite heavy labelling on all the cells can be seen. Methyl green and pyronin Y staining. Acetic alcohol fixed. 630x.

Figure 13 Autoradiography (H^3-proline) --- Silver grains are observed on the cytoplasm. Methyl green and pyronin Y staining. Acetic alcohol fixed. 630x

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