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# AUTORADIOGRAPHIC STUDY ON THE EFFECTS OF COMPRESSIVE FORCES ON CELLS IN VITRO – A PRELIMINARY REPORT*

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## Introduction

A major object of orthodontic treatment is the movement of teeth in relation to the alveolar bone of the maxilla or mandible. When the inherent forces of an orthodontic appliance are transmitted to the teeth so as to change their relationship to various points in the skull anatomy, certain reactions take place in the tissues surrounding the teeth. There is a tendency, in present day clinical practice, to become obsessed with the mechanical ease with which tooth movement can be accomplished and to overlook the biologic limitations of the affected tissues. These limitations must eventually be related to the effects of appliances upon the metabolism of individual cells. A knowledge of the responses of living tissues and cells to orthodontic forces would seem mandatory in planning proper orthodontic treatment. Confirmation of this viewpoint is provided by a recent conference on research related to malocclusion.[1] Some information on the size of forces applied through orthodontic appliances is available[2, 3], but there has been little study of cell response to different mechanical forces. Obviously, enough force must be applied in order to accomplish the required treatment, but if cell death or irreversible necrosis is the result, the treatment will fail.

In this respect two experimental approaches are feasible; (i) in vivo analysis of overall tissue responses following orthodontic forces, or (ii) analysis of isolated cell systems after the application of known forces.

Many publications are available which describe the in vivo approach. Orthodontic texts 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 as a result of osteoblastic activity[4]. It is, however, impossible to investigate cell or tissue responses to compressive force using this approach and the results of in vivo experiments show wide variation due to the large numbers of uncontrollable parameters involved.

Glücksmann studied the role of compressive and tensile forces on chondrogenesis and bone formation using organ cultures of foetal long bone[5, 6, 7]. These experiments were of a morphological and histological type and provided no information at the cellular and subcellular levels from the functional viewpoint. On the other hand, current texts in orthopaedics maintain that compressive forces inhibit osteoclasts and encourage osteoblastic activity while absence of compression inhibits osteoblasts and permits osteoclastic activity[8]. Obviously, there is a need for further experimental data to resolve these apparently conflicting viewpoints.

It is necessary to know how the cell, which is a single unit of the tissue, reacts towards compressive forces and this can only be controlled under in vitro conditions. It is the purpose of the present study to provide some data concerning the response of cells of connective tissue origin to compressive forces by means of autoradiographic techniques.

## Materials and Methods

### Materials

All experiments were performed on mouse L-929 fibroblasts[9] (obtained from Grand Island Biological Co., New York, U.S.A.). Monolayer cultures grown

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in Medium 199 (Hanks' base, supplied from Commonwealth Serum Laboratories, Victoria) were supplemented with 10 per cent bovine serum (supplied from C.S.L., Victoria), 100 units/ml of Penicillin (Crystapen$^R$, Glaxo-Allenbury Ltd., Victoria) and 100 $\mu$g/ml of Streptomycin (Glaxo-Allenbury Ltd., Victoria).

Following two days of subculture, replicate cultures were made. Homogenous cell suspensions (2 x $10^5$ cells/ml) were plated into 90 mm petri dishes with 22 x 22 mm coverslips located on the bottom. Cells were incubated and allowed to attach to the coverslips for two days at 37°C in an environment of 5 per cent $CO_2$ in air. Experiments were followed after two days incubation in exactly the same temperature and environment as with the replicate culture.

**Experimental design**

Experiments were undertaken by applying a device (shown in *Figure 1*) to cells which had been cultured for two days on 22 x 22 mm coverslips. The coverslips with their attached cell monolayers were picked up from the petri dishes and placed, cells up, over a glass slide (22 x 22 mm, 1 mm thick) located on the bottom of 45 mm experimental petri dishes. The glass slide held the coverslip 1 mm above the bottom of the experimental petri dish so that compressive forces produced by the device and a given weight could be applied to the cells covering an area of 22 x 22 mm square. The culture medium was kept in the experimental petri dish so as to cover the glass slide and coverslip.

Following the initial equilibration, the special device was placed on the cells. On top of the device a given weight was added and the experiments started. The apparatus consists of a simple polystyrene tube (Diameter 34 mm, Height 15 mm) whose bottom end has a porous cellulose triacetate membrane (Metricel$^R$, Type GA-8, Pore size 0.2$\mu$, Diameter 47 mm, Gelman Instrument Co., Michigan, U.S.A.). The membrane was stretched and kept extremely tight on the outer surface of the tube by a holding ring made of methylmethacrylate resin. The membrane was changed frequently to keep the tension constant. The use of a permeable membrane permitted the cells under compression to undergo their necessary gas exchange. The weights were moulded into a ring shape with lead to fit on to the top end of the tube.

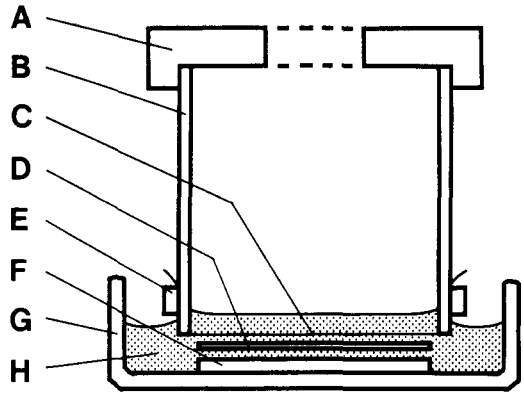



Fig. 1 — Diagrammatic section of assembled device and experimental petri dish.
A: Weight made of lead
B: Polystyrene tube
C: Cellulose triacetate permeable membrane
D: Coverslip with attached cell monolayer
E: Plastic ring for holding membrane
F: 22 x 22 mm glass slide
G: Experimental petri dish
H: Medium

Total experimental weights placed on the cells were calculated to be 10 g/cm$^2$, 20 g/cm$^2$ and 40 g/cm$^2$. The experiments were performed for periods of 30 minutes, one, two and four hours respectively.

Subsequent testing of the actual compressive forces exerted on the cells via the elastic acetate membrane, utilizing a force displacement transducer (Model FT 03B, working range adjusted at maximum weight of 200g; Grass Instrument Co. Ltd., Quincy, Mass., U.S.A.) coupled to a chart recorder (Model B-24, Rikadenki Kogyo Co. Ltd., Tokyo, Japan) revealed that only about 75% of the calculated compressive forces were exerted on the cells under the conditions described above. For example, cells calculated to receive compressive forces in the range of 40 g/cm$^2$, in fact received forces probably less than 30 g/cm$^2$, 20 g/cm$^2$ to 15 g/cm$^2$ and 10 g/cm$^2$ to 7.5 g/cm$^2$, 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 treated for autoradiography. Coverslips which were not compressed were simultaneously treated for autoradiography as controls. Those which were labelled with radioisotopes were used as a hot standard, while those which were not labelled were used as a cold standard.

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

**Autoradiography**

Radioisotopes used were $H^3$-thymidine (5,000 mC/m mol), $H^3$-uridine (740 mC/m mol) and $H^3$-proline (6,000 mC/m mol). All of the radioisotopes were obtained from Radiochemical Centre, Amersham, England.

$H^3$-thymidine and $H^3$-uridine were used as specific precursors for studying DNA and RNA synthesis respectively and $H^3$-proline for amino acid synthesis. The concentrations used were:—

$H^3$-thymidine — 0.05 $\mu$C per ml
$H^3$-uridine — 0.1 $\mu$C per ml
$H^3$-proline — 1.0 $\mu$C per ml

The compressed cells on the coverslips were brought into 90 mm Falcon plastic petri dishes (Falcon Plastics, Los Angeles, U.S.A.) containing 37°C pre-warmed medium and a given concentration of one of the radioisotopes described above. They were kept in contact with the radioisotope for a given period. The contact period for $H^3$-thymidine and $H^3$-proline was one hour. With $H^3$-uridine the contact period was four hours.

Following incubation 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 fixation with acetic alcohol (glacial acetic acid:ethyl alcohol, 1:3) for 30 minutes. Then the coverslips were rinsed in 70 per cent ethyl alcohol and washed by tap water for one hour. This subsequent washing removed the water-soluble or acid-soluble substances. After rinsing the coverslips in distilled water several times they were allowed to air-dry and were then fixed, cells up, on 1" x 3" size glass slides with paraffin wax.

The glass slides with the fixed and dried coverslips were then coated by nuclear tracking emulsion (K2 type, Ilford, England) which was melted at a temperature of 50°C and diluted with an equal volume of distilled water. After dipping the glass slides in the emulsion they were held in the vertical plane for draining on to tissue paper and drying at room temperature. After two hours the glass slides were dried completely and were then placed into light proof black bakelite exposing boxes (Clay Adams, New York, U.S.A.) in which an appropriate supply of silica gel was kept. 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 boxes from outside sources during exposure. The light proof boxes were sealed with black pressure sensitive tape (Type 235, Minnesota Mining and Manufacturing Co., Minnesota, U.S.A.), and exposed at 4°C in a refrigerator for five weeks, at the end of which time the glass slides were processed photographically. They were developed for five minutes in Phen-X (Ilford, England) with occasional agitation, rinsed briefly in water, fixed for eight minutes in diluted fixer (Hypam, Ilford, England) with occasional agitation and rinsed in three changes of water for 30 minutes. The processed glass slides were then allowed to air-dry. 200 ml of new developer and fixing reagent were used after processing every 20 glass slides.

Staining after photographic processing was performed with methyl green pyronin Y (after Brachet)[10]. The stained preparation was then observed under a microscope. For the general observation of cells, Haematoxylin-eosin staining was performed.

## Findings

The morphologic and autoradiographic changes found in the compressed cells did not differ substantially from those found in the control cells.

The compressed cells were observed to have intact cytoplasmic and nuclear outlines with no cytoplasmic blister formation and evenly stained organelles as in the control cells (*Figures 2 and 3*).

Following autoradiography in the $H^3$-thymidine series, the experimental cell groups which received compressive forces of ca. 30 g/cm$^2$ showed exclusive nuclear labelling in half of the cell population (*Figures 4 and 5*). The number of labelled cells in the control groups were the same as in the experimental groups. In the $H^3$-uridine and $H^3$-proline series both experimental and control cells showed the overall incorporation of these two labelled precursors into the cytoplasmic regions (*Figures 6, 7, 8 and 9*). Silver grains were distributed on most of the cells, but there were some unlabelled cells in the experimental groups.

These changes were observed equally in the cells at the centre of the coverslips and at the peripheral sites.

Varying experimental periods and weights were investigated. Cells in the same compressive force groups were observed equally labelled in experiments of short duration to those of long duration. Comparing different compressive forces, the groups of cells with heavier forces applied for shorter experimental periods did not differ from the cells with lighter forces applied for longer experimental periods. Unfortunately, under the conditions employed to date, cells subjected to the heavier compressive forces for maximum times also showed no overt signs of impaired function.

## Discussion

It can be seen from the present qualitative autoradiographic studies that the metabolism of the three labelled precursors was not disturbed in the majority of cells by the compressive forces. Many workers have shown that these three isotopes are immediate and essential precursors for the synthesis of the following substances; thymidine to desoxyribonucleic acid (DNA), uridine to ribonucleic acid (RNA) and proline to collagen[11–15]. Energy production, self-duplication and transformation are essential processes to maintain life. While energy accumulates as adenosine triphosphate in mitochondria, self-duplication and genetic mechanisms are carried on with DNA utilizing the energy. The present results indicate that protein synthesis, which is of vital importance to maintain life, will continue in the majority of cells after the application of actual compressive forces ranging up to 30 g/cm$^2$ for a maximum period of four hours.

The life cycle of a cell can be divided into two main stages; these include the mitotic phases and interphases. The stage in which the cells incorporate a precursor, such as thymidine, and synthesize DNA (DNA synthesis phase or S phase), occurs during interphase. The interphases of the pre- and

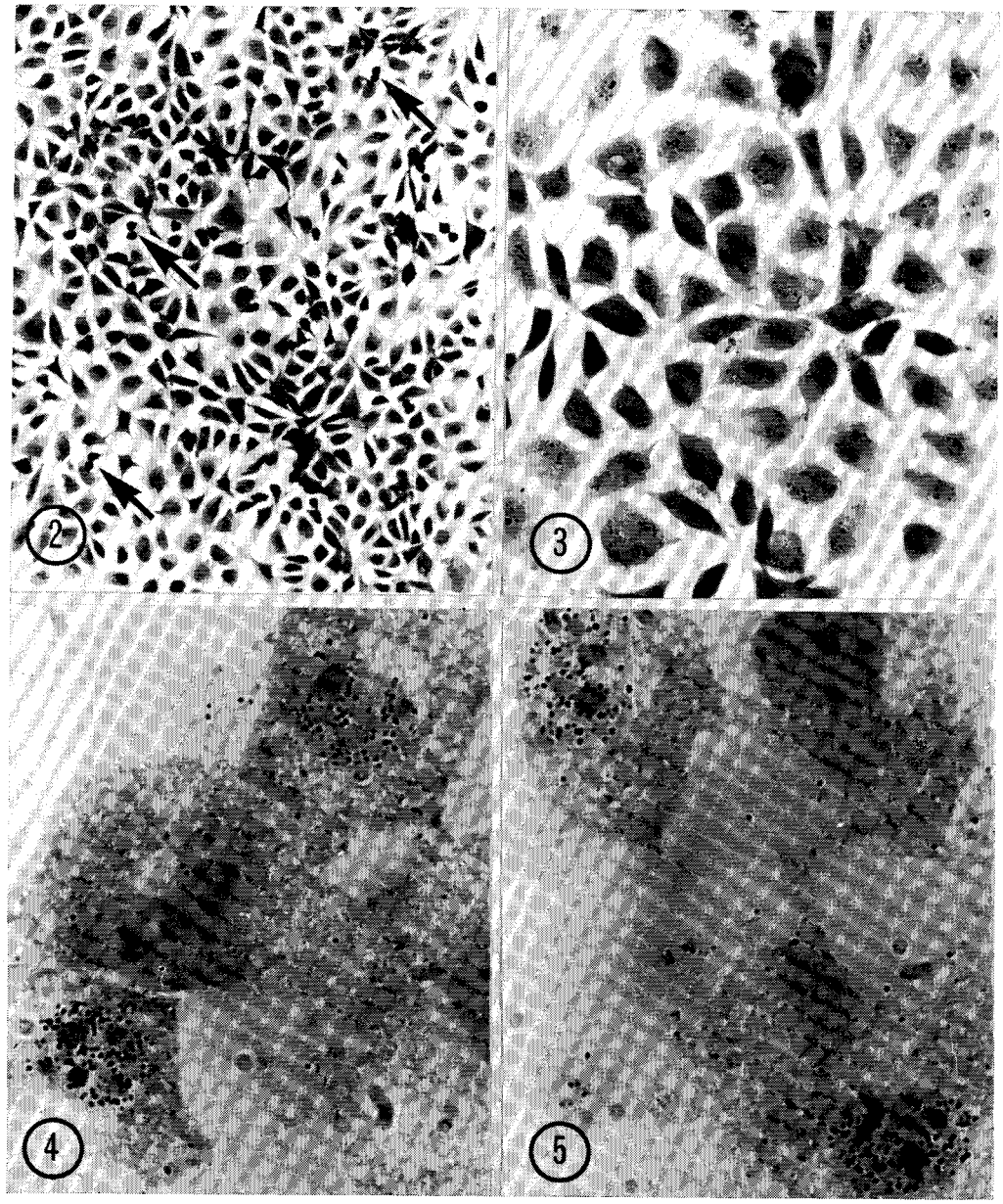
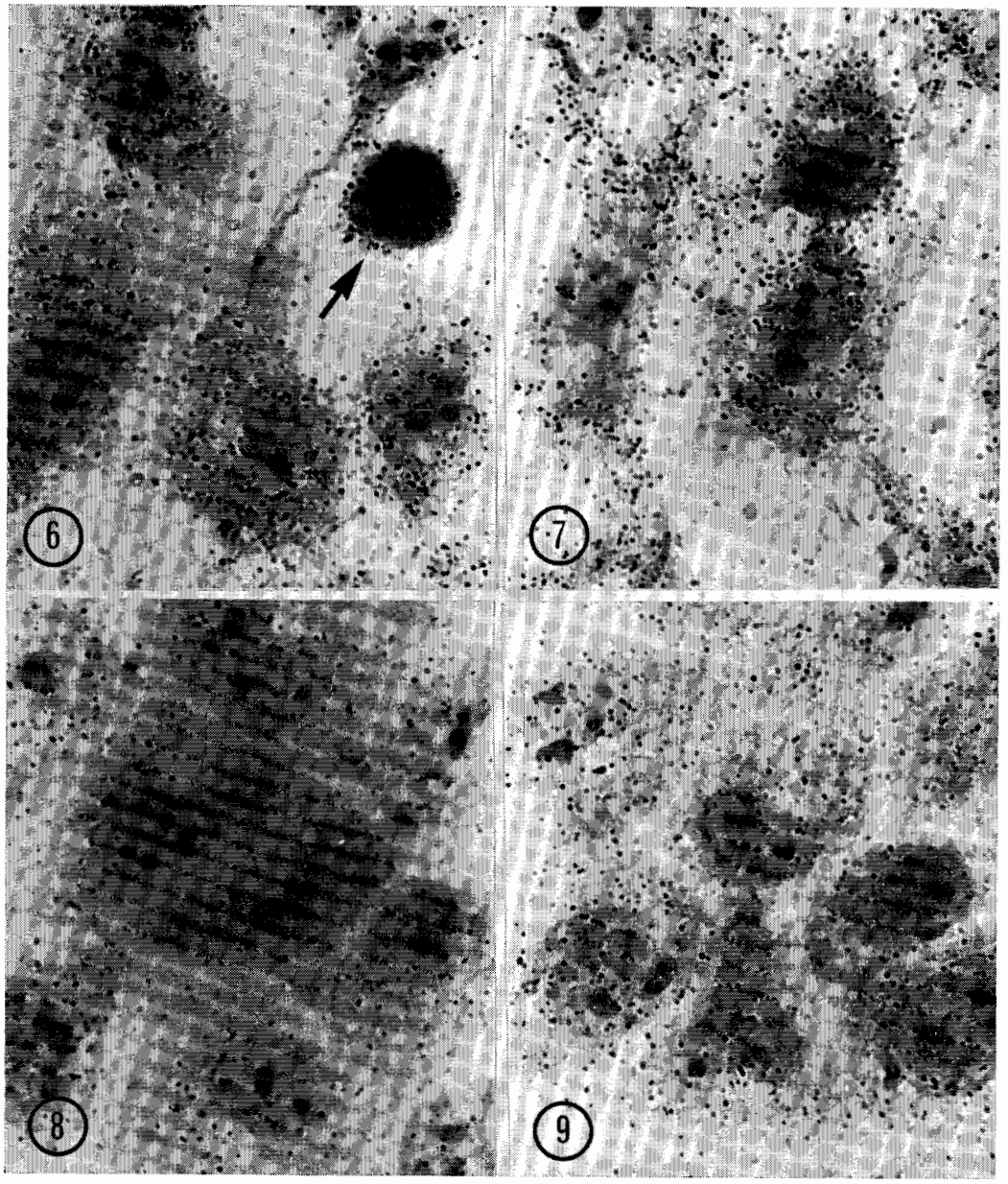



Fig. 2 — Haematoxylin-eosin staining. Cells at their mitotic stage can be observed (arrows). x 125.0
Fig. 3 — Haematoxylin-eosin staining. x 312.5
Figs. 4 and 5 — Typical autoradiographs of $H^3$-thymidine. x 1250




Figs. 6 and 7 — Typical autoradiographs of $H^3$-uridine. A cell at its mitotic stage can be observed (arrow). x 1250
Figs. 8 and 9 — Typical autoradiographs of $H^3$-proline. Note the even labelling. x 1250

post- DNA synthesis phase are called the pre DNA synthesis phase, or $G_1$ phase and post DNA synthesis phase or $G_2$ phase, respectively. When a cell system is homogenous and the cells are distributed in a random phase of their life cycle, a percentage of the cells can be considered to be in S phase. If labelled thymidine is applied to the cell system at a random phase for a given period, S phase cells, as well as some $G_1$ phase cells which are due to reach the S phase during the radioisotope period, would incorporate the labelled precursor. Subsequent autoradiographic processing permits the observation of DNA synthesis sites as silver grains *(Figures 4 and 5)*. In our experiments, due to the short isotope contact period of 1 hour, cells in other stages, i.e. $G_2$ phase, mitotic phases and a percentage of cells in the $G_1$ phase would not synthesize DNA and be labelled. This is because the radioisotope contact periods were immediately followed by fixation in the present study. It seems logical that the reason for the unlabelled cells in the thymidine series of the present study can be explained on the basis of the phases described, since the study had a homogenous cell system whose life cycle was considered to be in a random phase and not, therefore, synchronized.

The results reported here do not determine the critical forces governing irreversible cell necrosis. An explanation is required as to why approximately half of the cells in the $H^3$-thymidine series, and the majority of the cells in the $H^3$-uridine and $H^3$-proline series were labelled. Concerning the $H^3$-thymidine, the reason was presumed to be that stated above. But the reason for the other finding is not clear. In cells metabolizing normally, all cells except those few actually in mitosis should be labelled with uridine and proline, since the radioisotope contact periods were long enough. The cells which were not labelled might be so extensively damaged that they could not incorporate the labelled precursors. It is of interest to speculate whether the cell necrosis occurs at a definite compressive force level. It was thought that the number of cells undergoing necrosis would gradually increase in proportion to the increase of compressive forces to eventually involve the total cell population, rather than suddenly taking place at a definite force level. Previous results seemed to support this postulate[16], but further studies are needed on the magnitude of the compressive force necessary to determine initial cell damage *vis a vis* irreversible necrosis in the total cell population. It is not unreasonable to believe that cell necrosis might have already started with compressive forces under 30 g/cm$^2$ because some cells were not labelled. To clarify these points, quantitative autoradiography —i.e. a labelling index and cell kinetics following compression of the cells — or histochemistry, which will demonstrate other aspects of cell function, are of value. Such studies are still in progress, and the detailed data will be reported in subsequent publications.

The present results differ from the previous results[16], in which it was found that forces in excess of 26.94 g/cm$^2$ caused irreversible and degenerative changes to the cells. On the other hand, in the present study the majority of cells which received compressive forces of up to 30 g/cm$^2$ could incorporate the labelled precursors. It seems that the difference partly arises from different criteria; morphologic observations with phase contrast microscopy for the previous study and autoradiography for the present study. It is much more likely that the divergence arose from the difference in materials and design used to construct the loading device. Both studies utilized principally the same loading system, i.e. a specially fabricated loading device which compressed cells. In the previous study a transparent but rigid material, i.e. glass, was used as the base of the device because the aim of the study was to observe the responses of compressed cells under living conditions. Gas exchange and nutrient supply to the cells under compression was not possible through the glass, but only from the periphery of the device.

In the present study, on the other hand, gas exchange as well as nutrient supply were freely available through the cellulose triacetate membrane.

In so doing, however, the actual forces acting on the cells was found to be less than those calculated by virtue of previous experience. The actual maximum compressive forces reached of *ca.* 30 g/cm$^2$ closely correspond to those previously reported for cell necrosis to occur (26.94 g/cm$^2$). Current experiments aim to rectify this deficiency in our present system, whilst simultaneously retaining the advantages of free gaseous and nutrient exchange.

In effect, the previous findings probably resulted from the combined effects of compressive forces and insufficient gas exchange and nutrient supply, while the present findings are based more upon the effects of compressive forces. It seems appropriate that the differences emphasize the importance of gas exchange and nutrient supply to the cells under compression in order to maintain cellular function.

## Summary

Experiments were performed to demonstrate the response of cells of connective tissue origin to various compressive forces by means of qualitative autoradiography. A special device was fabricated to compress the cells. The radioisotopes $H^3$-thymidine, $H^3$-uridine and $H^3$-proline were used. Autoradiographs were made by routine procedures.

Morphologic and autoradiographic changes found in the compressed cells did not differ substantially from those found in the control cells. The compressed cells were observed to retain their intact cytoplasmic and nuclear outline without evidence of cytoplasmic blister formation. Their

organelles were evenly stained as in the control cells. With $H^3$-thymidine half of the experimental cells were observed to have exclusive nuclear labelling. With $H^3$-uridine and $H^3$-proline the majority of experimental cells were observed to have overall labelling of the cytoplasm. Some unlabelled cells were observed in the experimental groups. These changes were equally observed in the cells which were located at the centre of the coverslips as well as at the peripheral margins. Prolonged experimental times and increased compressive forces did not affect the findings.

Present results indicate that protein synthesis, which is of vital importance to maintain life, will be maintained in the majority of cells following the application of compressive forces of up to 30 $g/cm^2$ for a maximum period of four hours. Possible reasons for these results are discussed.

To clarify the effects of mechanical stress upon tissues it is emphasized that further data must be accumulated in related areas. The problem is not exclusively orthodontic, but rather is it related to many other biological fields.

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## REFERENCES

1. Moorrees, C. F. A., Burstone, C. J., Christiansen, R. L., Hixon, E. H., and Weinstein, S., Research related to malocclusion, *Amer. J. Orthodont.*, **59:** 1-18, 1971.
2. Storey, E., and Smith, R., Force in orthodontics and its relation to tooth movement, *Aust. J. Dent.*, **56:** 11-18, 1952.
3. Lee, B. W., Relationship between tooth-movement rate and estimated pressure applied. *J. Dent. Res.*, **44:** 1053, 1965.
4. Graber, T. M., Biomechanical principles of orthodontic tooth movement, in Orthodontics. Principles and practice, W. B. Saunders Co., Philadelphia & London, 2nd ed., 1966, pp. 489-528.
5. Glückmann, A., Studies on bone mechanisms in vitro. I. Influence of pressure on orientation of structure *Anat. Rec.*, **72:** 97-113, 1938.
6. Glücksmann, A., Studies on bone mechanics in vitro. II. The role of tension and pressure in chondrogenesis, *Anat. Rec.*, **73:** 39-55, 1939.
7. Glücksmann, A., The role of mechanical stresses in bone formation in vitro, *J. Anat.*, **76:** 231-239, 1942.
8. McLean, F. C., and Urist, M. R., Bone. Fundamentals of the physiology of skeletal tissue, The University of Chicago Press, Chicago & London, 3rd ed., 1968, pp. 43-44.
9. Sanford, K. K., Earle, W. R., and Likely, G. D., The growth in vitro of single isolated tissue cells, *J. Nat. Cancer Inst.*, **9:** 229-246, 1948.
10. Brachet, J., La localisation des acides pentose nucléiques dans les tisses animaux et les oeufs d'Amphibiens en voie de développement, *Arch. de biol.*, Paris, **53:** 207-257, 1942 (cited by Pearse, A. G. E., Hitsochemistry. Theoretical and applied, J. & A. Churchill Ltd., London, 3rd ed., 1968, pp. 651-652).
11. Reichard, P., and Estborn, B., Utilization of desoxyribosides in the synthesis of polynucleotides, *J. Biol. Chem.*, **188:** 839-846, 1951.
12. Friedkin, M., and Wood, H., Utilization of thymidine-$C^{14}$ by bone marrow cells and isolated thymus nuclei, *J. Biol. Chem.*, **220:** 639-651, 1956.
13. Smith, R. H., and Jackson, S. F., Studies on the biosynthesis of collagen. II. The conversion of $^{14}C$-hydroxyproline by fowl osteoblasts in tissue culture, *J. Biophys. & Biochem. Cytol.*, **3:** 913-922, 1957.
14. Ross, R., and Benditt, E. P., Wound healing and collagen formation. III. A quantitative radioautographic study of the utilization of proline-$H^3$ in wounds from normal and corbutic guinea pigs, *J. Cell Biol.*, **15:** 99-108, 1962.
15. Prescott, D. M., and Bender, M. A., Synthesis of RNA and protein during mitosis in mammalian tissue culture cells, *Exptl. Cell Res.*, **26:** 260-268, 1962.
16. Nakamura, M., In vitro study on the cellular responses to the compressive force in orthodontics, *J. Osaka Dent. Univ.*, **2:** 1-22, 1968.

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