AN EVALUATION OF *IN VITRO* CELL RESPONSES TO HYDRON ROOT CANAL FILLING MATERIAL

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Research Report sumitted in part fulfilment of the requirements for the Degree of Master of Dental Surgery

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The University of Adelaide

October 1984

PRECIS

The aims of this study were to develop an in vitro toxicity testing model suitable for the assessment of endodontic filling materials, and by means of the regime developed, gather incidental data on the effects of HYDRON root canal filling material on cellular metabolism. The data generated would thus provide more information on the biocompatibility or otherwise of HYDRON, and help to give a clearer indication of the material's suitability for continued clinical use or not.

HYDRON, a poly-2-hydroxyethyl methacrylate, has been recently (1978) marketed as a root canal filling material. To assist in clarification of its putative biological effects, biosynthetic activities of cells exposed to HYDRON in vitro were assessed, and compared with those of cells not exposed to HYDRON. Both pre-polymerized and freshly mixed HYDRON (hereafter called unpolymerized HYDRON) were tested. In addition, the effects on cell metabolism of two other commonly used endontic filling materials - AH26* and Tubliseal+ - were recorded.

An in vitro testing system was specifically developed for the assessment of the materials' effects on two cellular (L-cells) activities - cell division, and synthesis and secretion of extracellular proteoglycan matrix material. The method had several advantages including the ability to detect the reversibility of cell responses to biomaterials, the adaptability of the system to assess the effects of solid "insoluble" materials, liquids, or extracts of soluble or semi-

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* AH26 Root Canal Sealer - De Trey® - Zurich, Switzerland.
\+ Tubliseal Root Canal Sealer - Kerr® - Romulus, Michigan, U.S.A.

soluble substances. The nature of the data generated (real numbers, not ranked values) allowed ready statistical analysis of results.

The results indicated that both pre-polymerized HYDRON and unpolymerized HYDRON depressed both the rates of cell division, and the synthesis and secretion of proteoglycan matrix material. However, pre-polymerized HYDRON affected these cell functions to a far lesser extent.

Some recovery of cultures of L-cells (fibroblasts) following removal of the HYDRON from the in vitro system was noted after 24 hours - evidenced by increases in the rate of biosynthetic incorporation of the radiolabelled precursors [^{3}H] thymidine and [^{35}S] sulphate, when compared with cells where HYDRON was present in the system over the entire experimental period.

Extracts of unpolymerized HYDRON - up to 14 days extraction - significantly depressed the measured cellular activities, indicating an ongoing toxic effect.

In general, the results indicated that HYDRON - particularly in its freshly mixed state, significantly depressed the rates of cell division and extracellular matrix synthesis in vitro. Further in vivo and in vitro tests would be indicated to assess the extent of the effects of HYDRON on tissue healing, and to ascertain the mechanisms of action and toxic components of the material.

DECLARATION

This report is submitted in part fulfilment of the requirements for the Degree of Master of Dental Surgery in The University of Adelaide. Candidature for the Degree was satisfied by a Qualifying Examination in 1982.

This report contains no material which, except where due reference is made, has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge, this thesis contains no material previously published or written by another person, except where due reference has been made in the text.

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October 1984

ACKNOWLEDGEMENTS

I would like to thank the Chairmen of Departments of Restorative Dentistry - Professor T. Brown, Oral Biology - Professor J. Thonard and Dr. A. Rogers, and Pathology - Professor B. Vernon Roberts at The University of Adelaide for making available such materials and facilities as have been required for this project.

My thanks are also extended to Dr. Robert Hirsch for his assistance with preparation of Scanning Electron Micrographs.

To Dr. Geoffrey Heithersay and Dr. Rory Hume I extend sincere thanks for my introduction to this subject area of research.

To Dr. Ole Wiebkin I would like to express my sincere appreciation of the time and encouragement given to me during the period of my course. His guidanance has been invaluable and his scientific approach an example to follow.

Finally, I wish to dearly thank my wife, Jane, who through her continuous love and support, gave me the opportunity for further academic pursuit.

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AIMS AND OBJECTIVES

The aims of this study were twofold: to develop an *in vitro* toxicity testing model suitable for the assessment of endodontic filling materials, and by means of the regime developed, gather incidental data on the effects of HYDRON root canal filling material on cellular metabolism.

Metabolic pathways studied were cell division (measured by [^{3}H] thymidine uptake), and biosynthesis of proteoglycans (measured by [^{35}S] sulphate uptake).

Both of these metabolic processes are important in restitution of tissues following injury, and thus were used as parameters for assessment of toxicity in the cell culture system developed.

CHAPTER 1

INTRODUCTION

1.1 ENDODONTIC FILLING MATERIALS

1.1.1 EARLY DEVELOPMENT

An enormous number of materials have been used for root canal filling procedures throughout the ages, including such unlikely candidates as bamboo, pitch, thistles and feathers (Grossman, 1981).

Pierre Fauchard in 1728 provided accurate descriptions of pulp cavities and root canals of various teeth and he described the filling of the pulp chambers with lead (Cruse and Bellizi, 1980a). Bourdet, the dentist of King Louis XV of France described a process of extraction, filling root canals with gold or lead, and replanting the tooth (Costich et al, 1963). However, the first recorded description of an endodontic procedure in the United States was in 1766, and has been attributed to Robert Woofendale. He cauterized pulp and packed cotton into root canal orifices (Anthony and Grossman, 1945). The credit for being the first person to place fillings into the root canals though, was given to Edward Hudson, who packed gold foil into the root canals using instruments of his own design (Anthony and Grossman, 1945). In 1839, Baker wrote in the American Journal of Dental Science that his treatment for an exposed nerve was to remove the nerve, clean the canal, and fill the canal with gold foil. This was the first published account of pulp extirpation, canal cleaning, and root canal filling (Curson, 1965).

1.1.2 GUTTA PERCHA

Edwin Truman, in 1847, introduced gutta percha - a latex derived from Malayasian trees of the family Sapotacae - which was used as a filling and denture base material (Ingle and Beveridge, 1976).

During the 1850s, wood soaked in creosote was used to fill root canals with a mixture of Hills Stopping (gutta percha, quicklime, powdered glass, feldspar and metal filings) and chloroform or eucalyptol as a sealer. By 1865, E.L. Clark, another American, was filling the roots of teeth with hot masses of baseplate gutta percha. The technique was to heat the material as hot and fluid as possible without burning it, then churn it into the canals with a hot instrument (Noyes, 1922). The principle behind this technique bears a close resemblance to a method recently devised for thermocompaction of gutta percha by McSpadden (1980).

G.A. Bowman has been credited by Ingle and Beveridge (1976) as the first to have used gutta percha as the sole material for filling the root canal. Soon after this, in 1887, S.S. White Dental Manufacturing Company began production of gutta percha points for root filling. Bowman introduced chloropercha - a solution of chloroform and gutta percha - in 1895. The chloropercha was used in conjunction with gutta percha cones to obturate root canals. This technique was also described by Rheim, in 1905 and 1912 (Anthony and Grossman, 1945).

1.1.3 PASTES AND CEMENTS

Gysi introduced his 'Triopaste' formulation in 1899. This was a cresol/formaldehyde mixture which was popular in Europe into the 1960s (Cruse and Bellizi, 1980b). Callahan presented a resin-chloroform technique for filling root canals in 1911, advocating the method as a means to seal the dentinal tubules and provide a better hermetic boundary (Cruse and Bellizi, 1980c).

The use of a cementing medium in conjunction with a gutta percha point was proposed by U.G. Rickert in 1925 (Coollidge, 1960), though chloropercha had been previously advocated by Bowman (Anthony and Grossman, 1945). Rickert's technique was later improved and became known as the lateral condensation method, which allows for the insertion of additional cones. Rickert's sealer was primarily a zinc oxide and eugenol cement.

A resorbable root canal filling paste was created by Walkoff in 1928. This paste was a mixture of chloramphenicol and iodoform, and was reported to be non-irritant to periapical tissues (Juge, 1959).

1.1.4 COPPER AND SILVER POINTS

Copper points were used as root canal fillings towards the end of the 19th Century - some dentists gold plated them to prevent oxidation and subsequent discolouration problems (Anthony and Grossman, 1945). Jasper introduced silver points for use with a special cement, neobalsam, in 1933 (Cohen and Burns, 1980). He was convinced that

standardized silver points used to obturate canals prepared with standardized instruments would result in better adapted root fillings.

1.1.5 MORE RECENT DEVELOPMENTS (eg. HYDRON)

During the past 25 years, a number of root canal sealers have been used, usually in combination with gutta percha or silver points. Sargenti and Richter brought N2 onto the market in 1959, and since then it has been the centre of considerable controversy (Sargenti, 1978). This sealer is a zinc oxide and eugenol cement, and also contains paraformaldehyde, and a corticosteroid to suppress inflammation. The vast majority of sealers are zinc oxide and eugenol types. Polycarboxylate cements and epoxy resins are also available, and solvent/gutta percha techniques utilizing chloropercha and euchapercha are used. Studies with various potential root filling materials such as silicones, cyanoacrylates and polymers have taken place and results have been encouraging with some of these. Two relatively new materials - HYDRON®*, a hydrophilic resin, and Endofill®*, a silicone/siloxane material - have both been tested for their sealing ability (Spradling and Senia, 1982). HYDRON allowed significantly less dye penetration than the control of gutta percha (laterally condensed) with a zinc oxide and eugenol sealer. Endofill® was found to allow a similar level of dye penetration to the controls.

HYDRON has been extensively tested for its biocompatibility in the medical field, but results from biological testing of the material as used for root canal filling have been equivocal (see Section 1.4).

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* HYDRON: NPD Dental Systems Inc., New Brunswick, New Jersey, U.S.A.
\+ Endofill: Lee Parmaceuticals, South El Monte, California, U.S.A.

1.1.6 DESIRABLE PROPERTIES OF ENDODONTIC FILLING MATERIALS

The variety of root canal filling materials advocated for use throughout the years has been mentioned, however many materials have been rejected by the profession as irrational, biologically unacceptable or impractical for clinical use. Suggested requirements for ideal endodontic filling materials generally, have been delineated by Grossman (1981) and were listed as follows:

1. It should be easily introduced into the root canal.
2. It should seal the canal laterally as well as apically.
3. It should not shrink after being inserted.
4. It should be imperveous to moisture.
5. It should be bacteriostatic or at least not support bacterial growth.
6. It should be radiopaque.
7. It should not stain tooth structure.
8. It should not irritate periapical tissue.
9. It should be sterile or easily and quickly sterilized immediately before insertion.
10. It should be easily removed from the root canal, if necessary.

In addition, Grossman (1981) proposed that in particular an ideal root canal sealer or cement should possess some additional qualities:

1. It should provide good adhesion to the canal wall.
2. It should have fine powder particles to allow it to mix easily.
3. It should set slowly.
4. It should be insoluble in tissue fluids.

The desirable properties of root canal filling materials as outlined by Grossman (1981) therefore concern themselves with two major areas:

(1) the sealing ability of the materials, and

(2) the biocompatibility of the materials.

1.2 HYDRON

HYDRON, a poly-2-Hydroxyethylmethacrylate (p-HEMA), is a cross-linked hydrophilic polymeric hydrogel. The conceptualization and initial development of the polymer designed for biomedical application has been attributed to Wichterle and Lim (1960), who described the use of p-HEMA for a variety of medical applications.

1.2.1 BIOMEDICAL APPLICATIONS OF p-HEMA

p-HEMA has been used in medicine for coating of surgical sutures for internal use (Singh and Melrose, 1976), as a breast augmentation material (Kliment et al, 1968a), as a burn dressing (Nathan et al, 1976), and numerous soft tissue replacement and drug delivery devices for implantation (Kocvara et al, 1967; Drobnik et al, 1974; Ratner and Miller, 1973; Luttinger and Cooper, 1967). The material has also been used experimentally as contraceptive agent - injected into the vas Deferens of male rats (Singh et al, 1982). Dental applications have included its use as a denture lining material (Kliment et al, 1968b) and a root canal filling by Benkel et al (1976).

1.2.2 SUITABILITY OF p-HEMA FOR MEDICAL PURPOSES

Ratner (1982) ascribes the following properties to hydrogels:

1. They are comprised of polymeric chains.
2. They are insoluble in water at physiologic temperature, pH and ionic strength.
3. They will swell to an equilibrium value of 10 to 98% water at physiologic temperatures, pH and ionic strength. The water content (% H_2O) is defined as:

$$\% H_2O = 100 \times \frac{(\text{weight swollen polymer} - \text{weight dry polymer})}{\text{weight swollen polymer}}$$

Several factors influence the swelling of hydrogels including the osmotic potential, cross linking density of the polymer (related to chain flexibility) and the chemical interactions between structures on the polymer and the water (such as hydrogen bonding). If dehydrated polymer is placed in water, an osmotic force will tend to force water into the water-free regions of the polymer. Strong polymer-water interactions, e.g. hydrogen bonding, will further increase the swelling. The higher the cross-link density, the greater will be the inhibition of the chains on any polymeric expansion. The equilibrium swelling is achieved when the osmotic pressure forcing water into the system is balanced by the resistive forces exerted by the polymer in limiting expansion (Ratner, 1982).

The interest in hydrogels as biomedical materials has resulted from several putative advantages of these polymers as listed by Ratner (1982):

1. The expanded nature of the hydrogels allows extraneous materials incorporated into the polymers during preparation to be washed out to leave the pure insoluble gel network.
2. Mechanical and frictional irritation to surrounding tissues is minimized due to the soft, rubbery nature of the hydrogels.
3. As these materials have a low interfacial tension with surrounding biological fluids and tissues, the forces for protein adsorption and cell adhesion are minimized.*
4. Hydrogels allow the permeation and diffusion of low molecular weight metabolities and salts, as does living tissue.+

The importance of most of the above factors for biocompatibility has not been clearly shown, though Homsy (1970) has demonstrated that leaching of toxic substances from implanted biomaterials contributed to an inflammatory response, and Lyman et al (1977) using differing diameter vascular grafts, showed that matching the mechanical modulus of such grafts to that of surrounding tissue could be important for biocompatibility.

1.2.3 COMPOSITION AND SETTING REACTION

p-HEMA is formed by the polymerization of 2-hydroxyethyl-methacrylate, in the presence of small amounts of cross linking agents such as ethylene glycol dimethacrylate. Polymerization is by conventional free radical addition and the catalyst used in most commonly benzoyl peroxide (Patent Specification No. 1446709, Patent Office, London). The structure of p-HEMA is shown in Figure 1.1.

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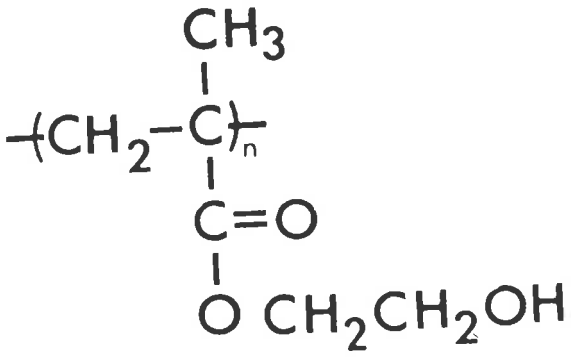
* This proposal is, however, in conflict with evidence of other authors - Horbett and Weathersby (1981).

\+ Evidence suggests that a passive hydrogel is not an appropriate analogue for dynamic connective tissue.

Figure 1.1 : Chemical Structure of p-HEMA



HYDRON was first marketed as a root canal filling material by the National Patent Development Corporation in 1978. It was sold as a paste/powder system - these were to be mixed and the resultant paste introduced into a prepared root canal where polymerization was completed. The Patent Specification for the material (Patent Specification No. 1446709, Patent Office, London) lists several materials which may be incorporated into a two component system:

Component A (liquid/semi-liquid)	Component B (solid, liquid or paste)
- hydrophilic monomers(s) e.g. 2-HEMA	- polymerization catalyst e.g. dibenzoyl peroxide
- cross-linking monomer e.g. ethylene glycol dimethacrylate	- radiopaquing agent
- radiopaquing agent e.g. barium sulphate, bismuth subcarbonate	- solvent e.g. glycerol diacetate
- reducing agent (accelerator) e.g. N, N-dihydroxyethyl 1-p-toluidine	- filler/thickening agent e.g. silica, cellulose derivatives
- antioxidant e.g. 2,6-di-t-butyl-p-cresol	
- filler/thickening agent	
- medicament or drugs	

In addition to the polymers formed by the material set out above, the patent specifies that composition may be prepared from hydrophilic co-polymers of several types, e.g. co-polymers of hydroxyalkylacrylates or methacrylates, with alkoxy alkyl acrylates and methacrylates. The preferred ranges for each of the components are also shown below (Table 1.1), however, the range may vary by considerably more as shown in the adjacent column.

TABLE 1.1 : HYDRON root canal filling material - ranges of component proportions.

Component A	Preferred Range	Range
HEMA	40-80%	25-99.95%
Diester	0.1-1%	0.05-5.0%
Accelerator	0.1-1.5%	0-1.5%
Barium Sulphate (or other) radiopaque material)	10-60%	0-0.75%
Finely Divided Silica (or equiv.)	1-60%	0-0.10%
Drug	0.1-10% (if employed)	0-10%
Component B		
Diacetin (or other diluent)	50-94%	25-94%
Benzoyl Peroxide (or equiv.)	0.5-2.5%	0.5-2.5%
Finely Divided Silica (or (equiv.)	0-10%	0-10%
Barium Sulphate (or equiv.)	10-60%	0-75%

1.2.4 BIOLOGICAL TESTING OF p-HEMA

(a) Sterilization, Surface Charge, Protein Adsorption

A considerable number of investigations of tissue responses to implants of p-HEMA have been published, since the material has been proposed for several biomedical applications. Where the material has been used for tissue replacement, e.g. breast augmentation and joint surface covering, sterilization of the polymerized material has invariably preceded its implantation. This has been carried out either by boiling or autoclaving (Kliment et al, 1968; Imai and Masuhara, 1982) or washing with alcohol (Murray and Dow, 1975). It is essential for soft contact lenses in particular to be properly washed prior to use in order to remove previously adsorbed irritants. Such sterilization procedures might well influence tissue responses to the implants. Apart from the prevention of infection at an implant site, the sterilization procedures, particularly boiling, could be expected to leach from the polymerized mass some substances which could affect cell response. Simple experiments with extracts of HYDRON (infravide) appear to have demonstrated this conclusively.

In addition, the physical form and surface charge of polymers both may strongly influence cell response to implanted materials (Smith and Williams, 1982). The adsorption of proteins onto the surface of implanted polymers is a well recognized phenomenon (Bagnall, 1977; Horbett, 1981; Horbett and Weathersby, 1981) and these surface proteins affect cellular interactions with foreign surfaces, thereby influencing the biocompatibility of implants and the responses of cells to materials in vitro (Horbett and Weathersby, 1981).

The state of polymerization or setting of a material has been clearly shown to influence its effects on tissues in vivo and on cells in culture. Spangberg, in a series of papers (1969b,c,e,f,g) described marked increased toxicity of freshly mixed root canal filling materials when compared with materials which had completed their setting process prior to testing, and more severe inflammatory responses around implanted unpolymerized samples than completely set specimens (Spanberg, 1969h).

(b) Implantation Studies

Barvic (1962) implanted set p-HEMA subcutaneously, intramuscularly and intraperitoneally into rabbits and rats for periods up to three years. He reported a mild response to the material, with connective tissue encapsulation occurring by the third day after implantation. When polymerized p-HEMA sheets were implanted subcutaneously into rats and guinea pigs (Imai and Masuhara, 1982) fibrous tissue capsules had surrounded and walled off the implants and were present after two years. No inflammatory reactions were evident at that stage, and earlier reactions were not described. These workers suggested - with apparently little evidence - that p-HEMA had a high tumorigenic potential, and they considered the capsule formation to be a pre-neoplastic change. Scanning electron microscopic (SEM) and X-ray energy dispersive analysis (EDAX) of the surface of retrieved implants clearly demonstrated the presence of calcific deposits on the implant surface. The p-HEMA specimens used by Imai and Masuhara (1982) were boiled for 30 minutes before implantation.

Intraosseous implantation of p-HEMA into the femurs of rabbits (Murray and Dow, 1975) for 24 weeks showed ossification up to the

polymeric/implant junction, with no evidence of inflammation or bone resorption in surrounding tissues. The specimens were polymerized cylinders of p-HEMA which were washed with alcohol prior to their use. Mineralization of the implant/tissue junction was evident in specimens examined by electron microscopy six months after implantation. These findings were similar to those of Sprincl et al (1973) who described calcifications along the margins of implanted macroporous gels.

Kliment et al (1968a) reported the use of p-HEMA as a breast augmentation material. They discussed a series of 55 cases of surgery and implantation procedures performed over three years. Their only criteria for evaluation were clinical, however they stated that the implants caused no reaction in the surrounding tissues, even after an unspecified "longer interval of time". No alterations in size, shape or hardness of the implants occurred. Published follow-up of the patients has not been evident, but 100% success was claimed.

(c) Fertility Control Agent

Interestingly, p-HEMA when co-polymerized with methacrylic acid (MAC), has been used experimentally for fertility control - injected (whilst polymerizing) into the vas deferens of male rats (Singh et al, 1982). The pH in the vicinity of the injected material was sufficiently low for killing the spermatozoa passing through. Singh et al (1982) also claim similar results on human spermatozoa in vitro.

(d) Burn Dressings

James and Watson (1975) claimed that 'Opsite' - a wound dressing based on p-HEMA and a polyurethane - reduced pain and increased the rate of healing. Nathan et al (1976) developed a p-HEMA-polyethylene glycol

burn bandage marketed now as HYDRON Burn Bandage*. The polyethylene glycol liquid is first dispersed over the wound, and an excess of powdered, uncross-linked p-HEMA is then dusted over the wound surface to cover the liquid. Polymerization occurs over 30 minutes and a clear, flexible, adhesive film forms. Antibiotics may be applied directly through the dressing. Healing reportedly occurs normally and fluid accumulation is minimal (Nathan et al, 1976).

(e) In Vitro Studies

In an in vitro investigation by Folkman and Moscona (1978), different dilutions of p-HEMA were pipetted onto the bottom of plastic cell culture dishes. Cell suspensions were then poured onto the tops of the p-HEMA layers. It was noted that as the dilution of the p-HEMA increased (and correspondingly the thickness of the material decreased), so too did the adhesivity of cells to the floors of the culture dishes. This phenomenon was used to investigate the role of cell shape on cell division. A number of experiments which were concurrently performed showed no sublethal cytotoxic effects of p-HEMA on the cells, even though cell adhesivity and mitotic rate was less when p-HEMA was present. Folkman and Moscona concluded that the decreased rate of cell division was merely a reflection of the rounded cell shape (due to lack of attachment to the substrate) and not due to any cytotoxic effects of the p-HEMA.

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* Hydron Burn Bandage - Abbott Laboratories.

1.2.5 ROOT CANAL FILLING MATERIAL

(a) General Background

Prior to the marketing of HYDRON root canal filling material in 1978, the manufacturers made claims for its biocompatibility and efficacy as a root canal filling material (Rising et al, 1975; Benkel et al, 1976; Goldman et al, 1975). Results of biological testing of the hydrophilic root canal filling material have, however, been equivocal. The initial investigations consisted of usage tests (Rising et al, 1975) and a clinical trial (Goldman et al, 1978). Presumably the large number of medical applications for p-HEMA was deemed by the manufacturers to provide adequate evidence for biocompatibility of the root canal filling material, without the need for any further screening tests.

(b) Biological Testing

Rising et al (1975) used HYDRON to root fill vital and pulpless monkey incisors following endodontic preparation. Following root canal filling with HYDRON, large amounts of excess material were observed in periapical bone and the periodontal ligament. Although little inflammation was associated with the material after 38 weeks, areas peripheral to the extruded HYDRON in bone and periodontal ligament contained foreign body cells and macrophages laden with particulate material. This material was assumed by the authors to be barium sulphate which had leached from the HYDRON. A further study (Kronman et al, 1977) using EDAX and SEM, revealed that the particles within the foreign body cells and macrophages were similar in appearance to barium sulphate powder particles, and high in barium content. Benkel et al (1976) also

noted an absence of inflammation in all but one case following filling and overfilling 48 monkey incisor root canals with HYDRON.

In the paper by Rising et al (1975) reference was made to an *in vitro* study published by Dreifus, Holechova and Wichterle (1962), which Rising et al (1975) claimed supported their contention that HYDRON was biocompatible. However, the polymer tested by Dreifus et al (1962) was not p-HEMA. It was in fact polyglycolmonomethacrylate, and, moreover, was boiled after setting and before testing; no quantitation of the tissue culture assay was made since it was not possible in the system used.

A clinical trial was conducted by Goldman et al (1978) using HYDRON, though no controls were quoted and the trial was only over a 12 month period. No failures were recorded during this time period in any of the 28 patients treated - based on a lack of clinical symptoms (pain, swelling), and on radiographic evidence of healing (or at least no increase in the size of periapical lesions present pre-operatively).

Several more recent studies relating to HYDRON root canal filling material have produced quite contrary results to those of Goldman's research group, even when parallel methodologies have been followed. Tanzilli et al (1981) described servere periapical inflammatory responses six months following overfilling of 10 monkey incisor root canals with HYDRON. Unfortunately these teeth were also intentionally over-instrumented, which may have contributed to the inflammatory response periapically.

A later study by Tanzilli et al (1983) in which freshly mixed HYDRON in polyethylene tubes was implanted subcutaneously in rats for 3 weeks to 6 months, described macrophage engulfment of HYDRON present in the surrounding connective tissues, and calcification in the HYDRON in the polyethylene tubes. When compared with specimens of Tubliseal root canal sealer in conjunction wth gutta percha, the HYDRON samples were surrounded by thicker fibrous capsules. The authors suggested this was a result of a more pronounced macrophage inflammatory cell response.

Langeland et al (1981) performed subcutaneous and intraosseous implantation experiments in rats and guinea pigs, and endodontic usage tests in monkeys, using freshly mixed HYDRON root canal filling material. Observation periods of up to 480 days were used for the subcutaneous implants, and up to 180 days for the intraosseous implants. Tissue in contact with the subcutaneous specimens was reportedly moderately to severely inflamed, and macrophages and foreign body cells containing HYDRON were present in surrounding tissue. After 480 days the material was still present in the tissue - associated with chronic inflammatory cells, and some neutrophilic leucocytes. The intraosseous implants showed similar results, however neutrophils were more prevalent. Results of the endodontic usage tests showed HYDRON present in periapical blood vessels and in bone marrow spaces. Langeland and his colleagues concluded that HYDRON was neither inert nor biocompatible, rather it exhibited varying degrees of toxicity; healing was interfered with in all tissue that came in contact with HYDRON.

A series of <u>in vivo</u> implantation studies using HYDRON root canal filling material was carried out by Reid (1983). He implanted polymerized HYDRON (root canal filling material) contained in open ended

teflon tubes, intraosseously and intramuscularly into guinea pigs. Following an observation time of several months, implants together with surrounding tissues were removed for histological examination. HYDRON was found to be very well tolerated by both bony and muscular tissues. Bone was found in direct apposition with the HYDRON implants, with little evidence of inflammation in surrounding tissues. HYDRON appeared to be tolerated at least as well as teflon or AH26 root canal sealer, with which it was compared. The methods used, however, did not allow assessment of early tissue responses, and since only polymerized HYDRON was tested, reactions to unbound components such as would be available during root canal filling procedures, could not be observed.

(c) Other Studies

Since biocompatibility is only one of many desirable properties of an endodontic filling material, other aspects of HYDRON have also been investigated - in particular its sealing ability and antimicrobial activity.

(i) Leakage studies

Goldman et al (1980) evaluated the resistance of HYDRON root filled teeth to bacterial invasion by exposing them to suspensions of Proteus mirabilis and Streptococcus salivarius, in vitro. Due to the hydrophilicity of the material, dye penetration methods were not used. No mention was made of the state of the HYDRON (polymerized in the presence or absence of moisture) when it was exposed to the suspensions. This factor could have been important, since when the material polymerizes in the presence of water in excess of its equilibrium content a spongy

structure will result and no further water will be absorbed; if the HYDRON polymerizes in the absence of moisture and then is exposed to it, absorption of the moisture will occur until the material's equilibrium level is reached (Migliaresi et al, 1981). The results of Goldman et al (1980) however found in favour of HYDRON. Their test system necessitated bacterial transfer along the entire length of the material/canal wall interface to achieve a positive recording for leakage. Clinically, leakage would not necessarily have to occur to such an extent to invite failure.

Rhome and co-workers (1981), in a further in vitro study using ^{14}C labelled albumin, found that root canals obturated with HYDRON leaked significantly more than those which were filled with vertically or laterally condensed gutta percha in combination with Grossman's sealer. All materials were allowed to set at room temperature and humidity, and the authors acknowledged that the 'leakage' may have been attributable in part to the hydrophilic nature of the material.

Using the same experimental model, but allowing polymerization to occur in a moist environment (the teeth were wrapped in saline soaked gauze), Director et al (1982) also found that HYDRON allowed significantly more leakage than the teeth obturated with gutta percha combined with Grossman's sealer.

Spradling and Senia (1982) used methylene blue dye penetration along root canal walls as a leakage indicator, and found that HYDRON showed significantly better sealing ability than laterally condensed gutta percha combined with a zinc oxide-eugenol sealer. The specimens

were all placed into conditions of 100% humidity at 37°C for one week prior to testing.

Kos et al (1982) carried out a comparative study of leakage of retrofilling materials. They used a modified model of Goldman et al (1980) and tested HYDRON, zinc-free amalgam and gutta percha. They found that HYDRON provided a microbially imperveous seal, while both gutta percha (without sealer) and amalgam provided poor apical sealing. The model favoured an injectable, flowing material such as HYDRON, since the control of the depth of fill was by a stainless steel wire introduced via the crown of the tooth. The results of the gutta percha filled teeth would not have been unexpected, since no sealer was used, and are certainly invalid for useful comparative purposes.

(ii) Antibacterial activity

The reported antibacterial activity of HYDRON (Kronman et al, 1979; Orstavik, 1981) may be in part responsible for the results of leakage studies which use the passage or non-passage of bacteria between HYDRON and root canal walls as the criterion for leakage. Kronman et al (1979) endeavoured to evaluate the ability of p-HEMA to support bacterial growth. Their experimental design involved placement of p-HEMA sheets into non-nutrient agar followed by innoculation with a test organism and incubation for up to 168 hours. The p-HEMA was then removed, placed in 0.1% peptone water, plated and incubated. Colonies of bacteria were observed and counted. Additionally, the powder and paste phases were separately assessed for their sterility. The results indicated that HYDRON did not tend to support bacterial growth, and that the components were sterile when packaged.

HYDRON was shown to be bactericidal against three strains of bacteria by Ørstavik (1981), but in the presence of human serum, antibacterial activity was markedly reduced or absent. The hydrophilic nature of HYDRON, together with its protein adsorptive capability, make this finding particularly significant, as the root filling material would often come into direct contact with connective tissue, thus probably reducing any antibacterial effects of HYDRON.

(iii) Paraesthesia of inferior dental nerve - case report

A case of paraesthesia of the inferior dental nerve following overfilling of a root canal in a mandibular molar has been reported (Pyner, 1980). Occurrences of paraesthesia though, are by no means restricted to the use of HYDRON (Kaufman and Rosenberg, 1980; Tanse et al, 1982; Grossman and Tatoian, 1978) and may not be entirely related to the chemical composition of a material, but could result from the physical trauma to the involved nervous tissue.

Considerable controversy still exists over the issue of biocompatibility of HYDRON and its indication as a root canal filling material. Vehement debate has been published in the literature (Kronman and Goldman, 1981; Langeland, 1981).

There is a clear indication for further evaluation of this material.

1.3 BIOCOMPATIBILITY TESTING OF DENTAL MATERIALS

Biocompatibility testing provides for the determination of the biological response to materials, and generally relies on the degree of incompatibility as a measure.

1.3.1 RATIONALE FOR BIOLOGICAL TESTING OF ENDODONTIC MATERIALS

Hensten-Pettersen (1981) stated that biocompatibility and toxicity testing is regarded as a recent concept in the evaluation of dental materials, and a large number of materials available today have come onto the market for general use with no testing having been carried out.

Endodontic filling materials invariably come in contact with vital tissue - residual vital pulp, periodontal ligament, periapical bone or periapical inflammatory tissue. Additionally, extrusion of the filling material into surrrounding tissues may occur, thus involving other tissues, e.g. the maxillary sinus or the inferior alveolar canal. Although localized tissue responses probably always occur, any life-threatening reactions are unlikely (Griffiths and Langeland, 1981). However, foreign materials in the root canal have the potential to produce systemic effects following leakage into the circulation from the site of implantation (Tucker, 1981). Thus the biocompatibility of endodontic filling materials would be desirable. The present project concerns itself with one aspect of biocompatibility studies - cytotoxicity testing in vitro - of some endodontic filling materials, particularly HYDRON.

1.3.2 RECOMMENDED STANDARD PRACTICES FOR BIOLOGICAL EVALUATION OF DENTAL MATERIALS

An array of test protocols is available for appraisal of the biological effects of dental materials (American Dental Association [ADA] and Federation Dentaire Internationale [FDI] - Recommended Standard Practices for Biological Evaluation of Dental Materials, 1979).

The methods outlined in the ADA and FDI "Standard Practices" documents have been the basis for the majority of most biological testing of dental materials since their publication. Nevertheless, with the exception of some screening tests for carcinogenicity, no statistical probability ranking for the predictive accuracy of any series of these tests has been devised. It has been suggested (Hensten-Pettersen, 1981) that evidence should be sought in the form of epidemiological studies which might assist in clarification of the long term biological hazards or risk factors associated with dental materials which have been used to date. No such evidence has been forthcoming thus far. Consequently, it is necessary to rely on "predictive" models such as the preclinical screening tests and usage tests outlined in the "Standard Practices" documents, for a guide to the probable clinical behaviour of a material, and of more importance to help in development of biologically compatible materials.

(a) Legal Requirements

Mjor (1981) implied that testing probably occurs as a formality after the production of materials, in order to comply with governmental regulations. The satisfactory performance of new dental materials in a number of biological tests is now required by law in the United States prior to their release for clinical use. No such requirements exist at present in Australia, where any biological evaluation of dental materials is the responsibility of the Pharmaceutical Control Board. The Australian Dental Standards Laboratory has so far concerned itself largely with evaluation of physical properties of dental materials, rather than their biological effects.

Researchers at the Scandinavian Institute of Dental Materials have systematically subjected several commonly used dental and endodontic materials to the tests described in the FDI "Standard Practices" (Wennberg and Hasselgren, 1981; Wennberg and Hensten-Pettersen, 1981; Tronstad and Wennberg, 1980; Ørstavik and Hongslo, 1983). This should prove to be of considerable value, since previous testing has tended to be limited to one or two types of tests, often usage tests before screening tests. This certainly was the case with the initial evaluation of HYDRON root canal filling material (Rising et al, 1975; Goldman et al, 1978).

(b) Tests for Endodontic Filling Materials

They are categorized into Initial tests, Secondary tests, and Usage tests. Initial and Secondary tests are also referred to as screening tests. Table 1.2 lists the recommended tests for evaluation of endodontic filling materials.

(c) <u>In Vivo</u> Tests

Spangberg (1983) discussed the value of <u>in vivo</u> screening tests in the evaluation of tissue responses to endodontic filling materials, and the tests themselves are adequately described in the "Standard Practices".

TABLE 1.2 : Recommended standard tests for evaluation of Endodontic Filling Materials.

SCREENING TESTS		USAGE TESTS
Initial	Secondary	
*Cytotoxicity	+Mucous Membrane Irritation Test	+Endodontic Usage Test
*Haemolysis		
*Styles test	+Subcutaneous Implantation	
+Dominant Lethal Test	+Intraosseous Implantation	
+Oral L.D.$_{50}$	+Sensitization	
+Intraperitoneal L.D.$_{50}$		

* In Vitro tests

\+ In Vivo tests

The Endodontic Usage test is the histological evaluation of tissue responses to a material following endodontic therapy in an animal - primates being recommended. Since the variables of tooth anatomy, operator skill and instrumentation may be inherent in this test it is usually carried out following the screening tests, and its findings interpreted in the light of results from all of the tests.

(d) In Vitro Tests

(i) Screening tests for carcinogenicity

Traditionally, whole animal testing has been used for the detection of carcinogenic substances (Rees, 1980). However, considering the enormous number of man-made materials in use, and the proposal that at least 80% of cancers have an 'environmental' aetiology (Rees, 1980), whole animal testing cannot adequately provide a first screen for carcinogenicity. Less cumbersome, more rapid techniques have been developed.

(ii) Ames test

The Ames test (1971) is a reasonable short-term predictive test of carcinogenicity, subject to the proviso that carcinogenicity pre-supposes mutagenicity. In the Ames test, a mutant of Salmonella typhimurium which does not require histidine in its growth medium, is exposed to a preparation of the test substance. If the mutant is exposed to a mutagenic material, it will revert to the original wild strain. A liver microsomal preparation is included in the medium to allow activation of any carcinogen. In experiments carried out by Bridges

(1976), 90% of known carcinogenic substances proved to be positive for the Ames test. Ørstavik and Hongslo (1983) tested four commonly used endodontic sealers (AH26, Endomethasone, Kloropercha NO, and Procosol), for mutagenicity in Ames test. They found that the extracts from mixed and set AH26, and the solution of its major component, expoxy-bisphenol resin, were significantly mutagenic towards one of two tester strains of Salmonella. No other material demonstrated mutagenic activity towards either tester strain.

(iii) Styles cell transformation test

By subjecting cells to viruses, transformations can be induced. Transformations can be recognized by growth characteristics such as the ability to grow on soft agar. Styles (1978) suggested that by using this growth characteristic, it was possible to screen for carcinogenicity. He found a very good correlation between the ability of cells to grow on soft agar, after exposure to a test substance, and the known carcinogenicity of a compound.

Whenever a material is positive in either of the Ames or Styles tests, the "Standard Practices" recommend referral for further evaluation in a specialized oncology laboratory.

(iv) Haemolysis

Haemolytic activity has been shown to be of significance in relation to apparent toxicity (Wennberg and Hensten-Pettersen, 1981) and haemolysis tests based on rabbit blood can be made to correlate with inhibition of cell growth (Dillingham et al, 1975).

The haemolysis test described in the "Standard Practices" seems to provide a rapid, inexpensive means of detection of water soluble toxic material, though it is not as sensitive as cell culture methods (Dillingham et al, 1975). In the haemolysis test, diluted fresh whole oxalated rabbit blood is added to a mixture of normal saline and test material, and incubated for one hour. The tube containing the mixture is then centrifuged and the supernatant is transferred to a spectrophotometric cell and absorbance of the supernatants of the test substances and controls are recorded. The per cent haemolysis is calculated as follows:

$$\% \text{ Haemolysis} = 100 \times \frac{(\text{O.D. test sample}) - (\text{O.D. negative control})}{(\text{O.E. positive control}) - (\text{O.D. negative control})}$$

If the haemolysis value is 5% or less, the material is considered non-haemolytic under the experimental conditions indicated.

The cytotoxic effects of insoluble components could not be expected to be fully expressed in an haemolysis test, which seems better designed for the evaluation of soluble substances. Spangberg (1969e) clearly demonstrated the cytotoxic potential of "insoluble" components of several root canal filling materials.

Using extracts of three endodontic materials (Kloropercha NO, Procosol and Endomethasone) Wennberg and Hensten-Pettersen (1981) carried out haemolysis tests to assess the sensitivity of rabbit, monkey, human, and guinea pig red blood cells. Monkey erythrocytes were

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* O.D. = Optical Density

found to be most sensitive at two different extract concentrations. Despite its relative simplicity and economy, haemolysis may be superfluous in a regime which includes assessment of toxicity in a cell culture system, which enables a ranked scale of toxicities.

(v) Cytotoxicity tests

Three different tests for cytotoxicity are recommended in the "Standard Practices".

The A.D.A. document suggests the Chromium Release Method described by Spangberg (1973).

Chromium Release Method: This test is based on the measurements of radioactive chromium [^{51}Cr] released from pre-labelled epithelial cells following exposure to a toxic influence. Depending on the amount of radiolabel released from the cells, the material causing the cell response is given a rating of cytotoxicity (Non-Toxic, Slightly toxic, Toxic or Severely toxic) after comparison with controls.

The Chromium Release Method has been extensively used in the study of dental material biocompatibility (Antrim, 1976; Spangberg and Langeland, 1973; Spangberg et al, 1974a,b,c; Koskinen et al, 1981), and also in immunological fields (Martz, 1976). Results are readily quantitated and hence easily subjected to statistical analysis.

However, several criticisms of this method could be made. It has been demonstrated that [^{51}Cr] chromium release does not occur until an

irreversible stage of cell wall damage is reached (Martz, 1976). Measurement of cell recovery is therefore out of the question. Some conflict exists as to the mechanism of [^{51}Cr] chromium binding to cellular constituents; Spangberg (1973) stated that it was "non-convalently bound to proteins and other cellular constituents" while Ronai (1969) maintained it was strongly covalently bound to proteins. Martz (1976) inferring a similar situation, found [^{51}Cr] chromium was released bound to cellular constituents with molecular weights of between 2,000 to 4,000.

The extent to which binding takes place and the position in which it occurs is important if correct inferences are to be made from results using such a technique.

The sensitivity of the [^{51}Cr] chromium release method has been questioned (Roper and Drewinko, 1976; Hensten-Pettersen and Helgeland, 1977). Koskinen et al (1981) found the [^{51}Cr] chromium release method satisfactory, but remarked that its use as a sole means of toxicological determination was unwise. They assessed the toxicity of root canal irrigating solutions with pre-labelled lymphoblasts and fibroblasts.

The F.D.I. recommend two tests for cytotoxicity evaluation - the Agar Overlay Method described by Guess et al (1965) and the Millipore Filter Method developed by Wennberg et al (1979).

Agar Overlay Method: A layer of vital cells is grown to confluence on the bottom of a petri dish, and covered with a thin layer of nutrient agar medium. The cells are then stained with neutral red, and standard size test specimens are placed on top of the agar. Incubation at 37^{o}C

for 24 hours follows. Vital cells are distinguished by a pink colour, whereas a toxic effect is characterized by a clear, colourless zone around the sample (non-vital cells do not accept the stain).

Hensten-Pettersen and Helgeland (1977) compared four cell culture toxicity measurement methods and found that the agar overlay method was more sensitive in its ability to detect cytotoxic effects, than the [^{51}Cr] chromium release method and colony formation-measurement of the proportion of cells able to give rise to cell colonies.

The major criticism to be made of the agar overlay method is that the material-cell contact is established by diffusion through an agar layer which, as mentioned by Guess et al (1965) may absorb toxic components released by the materials to be tested. Nevertheless the method is rapid, straight-forward and permits an objective quantitation of cell necrosis.

Millipore Filter Method: More recently, Wennberg et al (1979) developed an assay using L-cells cultured on millipore filters. Their method measured the succinate dehydrogenase activity of the cells after exposure to various dental materials.

Cells were seeded onto millipore filters placed on the bottom of tissue culture dishes. After a 24 hour incubation period the filters were placed on an agar bed, cell side down. Test materials loaded in glass rings were placed on top of the filters for 2 hours. Filters with adhering cell monolayers were stained and incubated for evaluation of succinate dehydrogenase (S.D.H.) activity, by macroscopic examination of changes in staining intensity of the cell monolayers. According to the

diameter of unstained zones and the staining density, scores from 0 to 3 were given to grade the intensity of cell response.

Tronstad and Wennberg (1980) assessed the toxicity of dental amalgams, resins, cements and 'therapeutic' pastes using the Millipore Filter Method. They concluded that the Filter Method gave a valuable indication as to which materials should be discarded and which should be subjected to further testing.

Wennberg et al, (1983) compared the agar overlay test, chromium release method and millipore filter method by an evaluation of two composite resins and a zinc oxide-eugenol cement. Although the results indicated that the tests were not interchangeable, generally it was found that better correlation of results from the in vitro tests were possible with those from implantation studies if the in vitro test was based on diffusion (millipore filter and agar overlay methods) rather than when a direct material-cell contact was established (e.g. chromium release method).

Growing of cells on millipore filters as described by Wennberg et al (1979) provided a close approximation of cells and materials without the complications of direct material-cell contact. The cell monolayer was on a confined area, which was easy to manipulate for staining.

1.3.3 OTHER IN VITRO MODELS FOR CYTOTOXICITY ASSAYS

In addition to the recommended tests in the "Standard Practices", several other methods of cytotoxicity evaluation have been described.

The ability to change a suitable number of variables of experimental design was seen by Ulreich and Chvapil (1981) as a decided advantage in their studies of the effects of various types of implantation sponges on extracellular matrix synthesis, cell division and cellular protein synthesis. The culture medium, cell type, physical state of the test material, method of establishing material-cell contact and the criteria used to assess cytotoxic effects may all be modified to suit an individual experiment. However, in the interpretation of results from *in vitro* toxicity assays, consideration must be given to all variables if relevant inferences are to be made. Moreover, some consistency of method is desirable to enable comparisons of results from one group with those of others - hence the "Standard Practices".

(a) Measurement of Cytotoxic Effects

Cell responses to toxic influences can be evaluated by several different techniques (see Table 1.3).

The diversity of chemical components to be found in various dental materials (and particularly in endodontic materials where "medicated" substances such as paraformaldehyde may be additives) tends to suggest that the pathogenesis of cytotoxicity would differ from one material to another (Browne and Tyas, 1979). An *in vitro* test which could assess the mode of cytotoxic action of a material could be an advantage, but design of such a test system would be complex and perhaps unnecessary as a screening test. The ability to assess cell recovery after exposure to materials would, however, be valuable (Wennberg, 1978; Browne and Tyas, 1979) as this is arguably more important than the measurement of initial cell damage. In previous studies, cytotoxicity has been measured by

TABLE 1.3: Criteria for the assessment of cytotoxicity *in vitro*

METHOD	REFERENCE
Cell Counting - Haemocytometer; Coulter Counter, Microscope Fields	Spangberg (1969a-f) Kawahara et al (1979) Mital and Cohen (1968) Kerestezi and Kellner (1964)
Mitosis Counting - Microscope Fields	Spangberg (1969a-f)
Vital Staining	Guess et al (1965) Imai et al (1982) Hensten-Pettersen and Helgeland (1977)
Non-Vital Staining (Dye exclusion)	Roper and Drewinko (1976)
Explant Outgrowth	Sisca et al (1968) Kawahara et al (1968)
Cell Morphology	Wilsnack (1976) Kawahara et al (1979) Das (1981) Sisca et al (1968)

TABLE 1.3 (continued)

Cell Lysis - [^{51}Cr] release	Spangberg (1973) Roper and Drewinko (1976) Koskinen et al (1981)
- [^{3}H] thymidine release	Klein and Perlman (1963)
- [^{3}H] uridine release	Lindqvist and Otteskog (1981)
Protein Synthesis [^{3}H] Proline Uptake	Helgeland (1977)
DNA Synthesis [^{3}H] Thymidine Uptake	Ulreich and Chvapil (1981) Wennberg (1976)
GAG Synthesis [$^{35}SO_4$] Sulphate Uptake	Ulreich and Chvapil (1981)
Lysosomal and Mitochondrial Enzyme Activity - Succinate dehydrogenase activity (mitochondrial)	Wennberg et al (1979) Tyas (1977) Meryon and Browne (1983)
- Acid phosphatase (Lysosome)	Tyas (1977)
Cellular Respiration	Spangberg (1969g)
Nerve Action Potential Conductance	Brodin et al (1982)

either comparing the number of cells still living (or the number killed) after exposure to an experimental influence, with control cultures, or by measurement of various metabolic activities of cells exposed to toxic materials in contrast to controls.

(i) Cell counting methods

In Spangberg's early studies of dental toxicity (1969b-g), he counted the number of cells attached to a glass culture chamber surface (presumably viable cells) and the frequency of mitotic figures per unit area as indices of cytotoxicity. While there is no doubt that these methods can provide results which are open to little misinterpretation - the cells and mitoses are actually visualized - the method suffers from being laborious and time consuming. Additionally, mitotic frequency has been questioned as a reliable index of cytotoxicity (Roper and Drewinko, 1976).

Other investigators have used haemocytometers for cell counting (Kawahara et al, 1979). Electronic cell counters, e.g. the Coulter counter, are also available. Both of these methods require that the cells be dispersed in a unicellular suspension, since clumps or aggregates may complicate counting and lead to inaccuracies (Paul, 1972). Neither method differentiates between viable and non-viable cells.

Mital and Cohen (1968) described an assay method using cell counts in the lag phase. They reasoned that as cell counts during the lag phase measured the number of cells which adhered to the surface of a culture vessel, a decrease in the cell count before replication might reflect a

functional disability less severe than necrosis, yet unrelated to growth phenomena.

(ii) Staining

Cell staining techniques for determination of cell viability have been used. Guess et al (1965) and Hensten-Pettersen and Helgeland (1977) used neutral red stain to observe vital cells which take up this stain in the agar overlay methods (see Section 1.3.2). Trypan blue was used by Roper and Drewinko (1976) to stain dead cells, (this dye is excluded by the surface membrane of living cells) which gives an indication of the number of vital cells remaining. Imai et al (1982) used crystal violet stain for vital cells and measured the absorbance of extracted dye to determine the proportions of living cells in cultures exposed to dental cements.

Staining methods, whilst providing for adequate gross examination of cultures, do not allow accurate quantitation without the further manipulation required for electronic or haemocytometer counting. Neither do they allow measurements of reversibility of cytotoxic effects, i.e. cell recovery. Dye exclusion and vital staining techniques, if successfully applied can only give a reliable answer in relation to complete cell necrosis, and cannot detect the mode of cytotoxic action of drugs or materials, or cellular changes which might occur other than death. Roper and Drewinko (1976) even concluded that dye exclusion tests lacked the sensitivity to be used as a criterion of cell survival.

(iii) Explant outgrowth

Inhibition of cell outgrowth from tissue explants was used to gauge the cytotoxic effects of dental materials in early experiments by

Kawahara et al (1968). This manner of assessment has been largely discontinued, possibly due to difficulties in quantitation, and the large number of variables which the method introduces - poor material-cell contact, difficulty of cell counting and growth determination, and non-uniform solubility of different materials. Kawahara's research group has used cell culture methods in more recent studies (1979).

(iv) Morphological characteristics

Wilsnack (1976), Spangberg (1969a), Kawahara et al (1979), Sisca et al (1968) and Das (1981), observed the different morphological characteristics of cells exposed to toxic and non-toxic materials, and used these as an indication of material cytotoxicity.

The criteria used - cell rounding and cytoplasmic shrinkage - tend to be subjective, and quantitation of results, although possible, may provide difficulties. Additionally, no information is given to allow the researcher to determine the mechanism of cytotoxic action of the material. As an aid, morphological criteria may be useful in assessment of toxicity, but alone, changes which occurred could be regarded as inconclusive.

(v) Colony formation

Colony formation or plating efficiency is that proportion of cells able to give rise to cell colonies. It has been suggested that this property is the most accurate estimate of the number of viable cells in a population (Paul, 1972; Roper and Drewinko, 1976). Any harmful environmental factor can be expected to lower the plating efficiency

(Paul, 1972), so the method should provide a sensitive criterion for toxicity testing. However, the operator's skill and quality of medium must be at a premium in order to obtain reproducible results, so "the plating efficiency must be regarded as relatively empirical, and not necessarily an absolute measure of the health state or otherwise of a cell population" (Paul, 1972). Hensten-Pettersen and Helgeland (1977) exposed human epithelial cells to set dental materials (zinc-eugenol, silicate cement and composite resin) and found that colony formation was not as sensitive an indicator of toxicity as cell growth measurements or vital staining with neutral red dye (agar overlay method).

(vi) Radioactive marker release

Release of [^{3}H] thymidine and [^{3}H] uridine from pre-labelled cells have also been applied as indicators of toxicity (Klein and Perlman, 1963; Lindqvist and Otteskog, 1981), but due to location of these substances within cells (DNA and RNA), total cell lysis would probably be necessary prior to elution of labelled markers from the cells. Hence the sensitivity of the method would probably be no better than the [^{51}Cr] chromium release method, but the binding sites are definitive, and spontaneous release might be expected to be less. However, no substantiating evidence is available to support these assumptions.

(vii) Metabolic parameters for indiction of cytotoxic effects

Several investigations have measured interference with cellular metabolic activity after exposure to dental materials, and in general the methods have provided a sensitive means of assessing cellular damage. Some understanding of the mechanism of material cytotoxicity

may also be obtained. However, if a material interfered with many metabolic pathways, only the pathways investigated would be detected. Nevertheless, it should be possible, by using different radiolabels - say [^{35}S] sulphate and [^{3}H] thymidine - to measure effects on at least two metabolic pathways simultaneously. In this case proteoglycan (PG) synthesis, and DNA synthesis would be measured. The use of radiolabelling methods should also be an advantage in obtaining quantitation of results to allow statistical analysis.

Wennberg (1976) used [^{3}H] thymidine uptake to measure the effects of water soluble substances on L929 cells, and indeed thus far the thymidine incorporation studies seem to have been restricted to investigating water soluble substances or extracts of solids as carried out by Ulreich and Chvapil (1981), and Masillamoni et al (1981).

Helgeland (1977) studied the effects of fluoride and zinc on in vitro collagen and protein biosynthesis in the rabbit pulp. She measured the differences in uptake of [^{14}C] proline into trichloracetic acid (TCA) soluble and insoluble fractions (i.e. total protein and hydroxyproline) in the presence of the materials in preparations of different pH. Similar experiments have been carried out using [^{3}H] uridine uptake as the criteria for cytotoxicity (Ceccarini and Eagle, 1971).

By observing inhibition of [^{35}S] sulphate incorporation into cells, Wiebkin and Muir (1973, 1975) assessed the effect of various macromolecular substances on cellular matrix biosynthesis. This principle has been recently utilized in toxicity studies by Ulreich and Chvapil (1981), who used inhibition of [^{35}S] sulphate incorporation into

PG as a measure of cytotoxicity. Their paper also described methods for [^{3}H] thymidine and [^{3}H] tryptophan incorporation, to assess a material's effects on DNA synthesis and synthesis of non-collagenous protein.

Cellular respiration has also been measured while materials are present in and absent from a cell culture chamber. Spangberg (1969g) measured the oxygen uptake of He La cells by means of a Warburg apparatus, and Hume (1982) has measured cellular carbon dioxide output to detect cell responses to material extracts. Spangberg's results from testing the toxicity of several endodontic filling materials with this method, correlated well with his previous studies (1969b-f) where he used cell and mitosis counting as indicators of toxicity.

The use of staining methods for detection of lysosomal and mitochondrial enzyme activity has been suggested by Tyas (1977) and also used by Wennberg et al (1979), whose in vitro assay system has been adopted as a standard screening test by the FDI. Both methods provided simple, rapid means of assessment, but the scoring system used by Wennberg et al gave only relatively gross comparisons. Tyas (1977) used a microdensitometer to detect any differences in staining and this may have provided a better measure than gross examination only. More recently Meryon and Browne (1983) have used a similar method - interspersing dentine between cells and the test material.

It must be recognised that any model which makes use of a radiolabelled precursor technique relies on the inference that the precursor is taken up and binds to a presumed site. Autoradiographic studies such as those of Heddle et al (1967) indicate that this is

indeed what occurs, so one should be able to readily identify any effect on the particular labelled pathway being observed.

(viii) Measurement of action potential conductance

Brodin et al (1982) described a method for measurement of neurotoxicity by assessment of action potential conductance of rat phrenic nerves following exposure to endodontic filling materials. They concluded that their in vitro model was of significance in relation to the clinical observation of paraesthesia following some cases of endodontic treatment.

Many assay methods have certain qualities which make them desirable: counting of mitoses in a culture chamber gives an observable indication which requires no inferences to be drawn; enzyme histochemical techniques seem relatively simple and fast; radiolabel release methods may be useful in the testing of very toxic materials; and tests using incorporation of radiolabelled precursors can be adapted to investigate effects on many metabolic processes. An ideal method would be simple, reproducible, rapid, allow for quantitation of results, and also provide the opportunity for microscopic examination of cells at appropriate stages of the experiments.

(b) Some Other Experimental Variables

(i) Cells

The most likely types to be affected by dental restorative materials are gingival epithelial and connective tissue cells, and pulp fibroblasts and odontoblasts. In the case of endodontic filling

materials in contact with periapical tissues, the fibroblasts and osteoblasts of the periodontal ligaments will inevitably be the cells affected. Occasionally, residual pulp may be present apically, in which case pulp fibroblasts would again come in contact with the filling materials. It would seem logical to utilize similar cell types for in vitro assessment of dental materials.

The majority of in vitro studies of material toxicity have used either epithelial cells (Spangberg, 1969b-g), or fibroblasts (Wennberg, 1978; Tronstad and Wennberg, 1980; Ulreich and Chvapil, 1981), or both (Spangberg, 1969f). Some of these experiments were carried out with cells from primary cultures, and some with cells from established cell lines.

Hensten-Pettersen and Helgeland (1981) carried out parallel experiments with cells from an established cell line (NCTC 2544 - human epithelial cells) and epithelial and fibroblast cells from human gingival explants. They used four different assays with each of these cell types to determine their sensitivities to toxic influence. No cell type was consistently more sensitive than the others to the effects of resin based dental materials, and the results varied with the assay method and the type of medium used.

Koskinen et al (1981) assessed the cytotoxicity of several root canal irrigating solutions - Betadine (10% polyvidon iodide), Hibitane (20% chlorhexidine gluconate), Largal Ultra (15% ethylene diamine tetracetic acid) and 5% sodium hypochlorite - by means of the chromium release method described by Spangberg (1973). They used lymphoblasts from an established cell line, and primary human skin fibroblasts. Their

results indicated that lymphoblasts may have been more readily damaged by the materials tested, though the reliability of their assay method was questioned as results were not consistently reproducible.

In development of their "Millipore Filter Method", Wennberg, Hasselgren and Tronstad (1979) used L929 cells - an established cell line of fibroblasts from mouse connective tissue. Their assay method measured the succinate dehydrogenase activity of cells grown on a millipore filter after exposure to dental materials such as silicate and zinc phosphate cements. Their reasons for the choice of L929 cells were that established cell lines are more amenable to culture, and have advantages over cells from primary cultures in maintenance and handling. This group also expressed that cells from established lines have the potential to maintain constancy of cell type throughout a series of experiments. This would eliminate the variable of any age-associated decline in metabolic activity which can occur in cells from a primary culture (Rees, 1980). However, diploid cells from primary cultures have been advocated for use in the study of material toxicity (Rice et al, 1978), since cells from established cell lines do not always demonstrate all of the specialized properties of their progenitor cells (Browne and Tyas, 1979).

A high degree of sensitivity to toxic change and "applicability" to in vivo conditions are both desirable qualities of an in vitro toxicity assay system. Although some investigators have shown apparent variation in sensitivity of different cell types to dental materials (Hensten-Pettersen and Helgeland, 1977; Koskinen et al, 1981), at the present time there is insufficient evidence to conclude that the type of cells chosen would affect the outcome of a test to a significant extent. Until

such time as more data are reported indicating superiority of sensitivity of one cell type over another, it would seem preferable to carry out parallel tests with an established cell line and cells from a primary culture.

(ii) Material-cell contact

The state in which materials are introduced into an *in vitro* system (i.e. set or unset) and the manner in which these materials are allowed to contact the cells both may have an influence on the results (Browne and Tyas, 1979).

Most dental restorative materials, including endodontic filling materials, are mixed in the surgery immediately prior to insertion into the tooth in an unset state. Several reports of both *in vivo* and *in vitro* experiments have attested to the high initial irritation caused by dental materials followed by regression of toxic effects soon after setting (Spangberg, 1969a-h; Wennberg, 1976; Wennberg and Hasselgren, 1981). In order to elucidate the irritant potential of a material *in vitro*, it would be desirable to introduce the material into a test system in an unset state and allow it to set under conditions which parallel clinical use, whilst ensuring satisfactory material-cell contact. Few investigations have achieved this in the past.

Obtaining adequate material-cell contact is not a problem with soluble materials, such as root canal irrigating solutions, as these may be directly added to the culture medium in appropriate dilutions (Masillimoni et al, 1981). A more complex situation arises when insoluble substances are to be introduced into an *in vitro* test system.

Good material-cell contact can be obtained by placing a specimen of test substance directly onto a cell monolayer (Rosenbluth et al, 1965), or by seeding cells directly onto the surface of a material (Spangberg, 1973; Leirskar and Helgeland, 1972). However, the physical properties of a material (size, shape, weight, surface roughness, surface tension) have been shown to affect cell response. Using a novel method to assess the effects of compressive forces applied to cells in vitro, Nakamura and Thonard (1972, 1973) showed that the presence of material directly on top of a cell monolayer may affect the supply of nutrients to the cells, which would complicate evaluation of cell response to a material's toxic effects. Results of experiments by Grinnell et al (1972) showed that cellular adhesion to different solid surfaces varied with the physical characteristics of the solid surfaces such as surface tension.

Unless the effects of such factors can be readily measured, evaluation of results will be unnecessarily complicated. Folkman and Moscona (1978) clearly demonstrated that cell shape is influenced by surface texture and this in turn could significantly affect cell metabolism; as assessed by uptake of [^{3}H] thymidine.

Murphy et al (1976) described a method for in vitro toxicity testing of root canal filling materials. Freshly mixed material was placed into a small diameter glass tube, which was placed horizontally in a culture dish. A cell suspension was then added into the dish. This allowed diffusion of toxic products from the ends of the tube, and apparently normal setting of the material. Although a completely normal setting process would have been unlikely, this method was a more appropriate means to simulate usage conditions than previous attempts.

Whether or not this should be the aim of *in vitro* assay for cytotoxicity is questionable, but Browne and Tyas (1979) have recommended that *in vitro* assays should simulate *in vivo* conditions.

Three other groups of workers have also described innovative systems for *in vitro* biomaterial toxicity testing. Guess et al (1965) described the agar overlay method, which has been widely used in the evaluation of dental materials (Mjor et al, 1977; Hensten-Petersen and Helgeland, 1977, 1981). This method relied on diffusion of toxic products through an agar gel to produce an effect on cells beneath the agar (see Section 1.3.2).

Tyas (1977) placed a Millipore filter in a well in the lid of a culture dish; the filter separated the medium and cells (which were a monolayer on the bottom of the culture dish) from the setting material on top of the filter. Tyas (1979) later altered this system by decreasing the distance between the cells and the filter, to ensure more complete diffusion of toxic products to the cells.

Wennberg et al (1979) overcame the problem of agar's absorption of toxic products using another Millipore filter method (see Section 1.3.2).

Browne and Tyas (1979) stated that the use of extract solutions or suspensions is an advantage, as serial dilutions can be made which allow determination of the concentration at which a material is no longer toxic. However, this may be of general value for the "soluble" components of the test material alone, since only the "dissolvable" components will vary from one dilution to another. Thus endeavours to

rank the dilutions of the test materials themselves from least toxic to most toxic (by effective titres) could be misleading, because the nominally "insoluble" components which have also been shown to be toxic (Spangberg, 1969e) will not be quantitatively accounted for.

Spangberg (1969c,f) used distilled water to extract toxic agents from set endodontic filling materials before mixing them with He La cell and skin fibroblast suspensions. He assessed the cell response by counting the number of cells undergoing mitosis after exposure to the extracts. Wilsnack (1976) and Ulreich and Chvapil (1981) extracted test substances in culture medium, and carried out assays to detect the effects of rubber, silicone rubbers, PVC plastic, nlyon, and various sponges on tissue culture cells. Wilsnack (1976) used direct cell observation methods to determine cellular morphological changes after exposure to material extracts, together with serial dilution tests of extracts to assign a cytotoxicity level to each material. Rubber materials were found to have the highest cytotoxicity and produce the most marked morphological changes. Ulreich and Chvapil (1981) used medium labelled with [^{3}H] thymidine and [^{35}S] sulphate, to assess cellular DNA and PG synthesis after exposure to different extract dilutions.

In the preparation of extracts of dental materials for use in toxicity studies, the presence of serum in the extractant may cause a decrease in toxicity of the extract (Hensten-Pettersen and Helgeland, 1977). Often extraction procedures are designed to strengthen evidence of toxic agents rather than to simulate the <u>in vivo</u> extraction which occurs over protracted periods, and this may be achieved by use of extractants such as distilled water or phosphate buffered saline (Hume,

University of California Los Angeles - personal communication) e.g. relative levels of ionic dissociation etc.

Different methods of providing material-cell contact have understandably produced variable cytotoxicity "ratings" for many materials. Hensten-Pettersen and Helgeland (1977) used different cell culture techniques to assess the toxicity of three dental restorative materials - silicate cement, zinc oxide-eugenol cement and composite resin. They used direct seeding of cells onto set specimens, addition of extract-medium onto a cell monolayer and the agar overlay method. In each test the rank order of toxicity was found to be the same, but the absolute toxicity varied with the method used, indicating differences in sensitivity in the model systems.

Methods which provide adequate material-cell contact without the complicating effects of agar, large medium volume, or direct physical material-cell surface interactions should allow a more realistic appraisal of the biological properties of a material. The "filter method" described by Wennberg et al (1979) appears to satisfy these needs, though an agar gel base may not provide ideal nutritional support for cell growth for more than 24 hours (Guess et al, 1965).

1.4 IN VIVO VS IN VITRO TESTING

While *in vivo* tests, and especially usage tests may be desirable prior to clinical testing of biomaterials, the results of usage experiments for endodontic materials may vary markedly with several factors. Tooth anatomy, completeness of root canal debridement, the presence of infection, operator skill and the animal model chosen can

all influence results significantly (Spangberg, 1969a; Rowe, 1980). In acknowledgement of these more uncontrollable influences, some doubt could be raised as to the validity of using an endodontic usage test in the assessment of tissue irritation of root canal filling materials. Initial surgical trauma may also complicate the evaluation of *in vivo* screening tests (Wennberg, 1976).

Several investigators have alluded to the apparent non-correlation of *in vivo* and *in vitro* toxicity studies (Hensten-Pettersen, 1981; Mjor, 1980; Rees, 1980). However, careful interpretation of observed *in vivo* effects and full consideration of *in vitro* experimental design can provide an accurate assessment of possible toxic effects. It has been assumed that a toxic *in vitro* effect of a certain level is an indication for non use of a material. This is not necessarily so. Consideration must be given to the intended action of a material. Calcium hydroxide - a material used for the induction of hard tissue "bridge" formation at the apices of incompletely developed teeth - invariably is very toxic in cell culture systems (Tronstad, 1974). This is often cited to demonstrate the inability of *in vitro* systems to predict performances of material *in vivo*. The results of *in vitro* experiments though, appear to give a good indication of the nature of action of this material. Calcium hydroxide has a pH of 12 to 12.5 (Tronstad and Wennberg, 1980; Heithersay, 1975) and it acts on vital tissue by causing an initial superficial necrosis 1 to 1.5 mm in depth, including a thin layer of coagulation necrosis which is in close contact with the vital tissue beneath (Schroder and Granath, 1971). The remaining pulp is relatively unaffected (Mejare et al, 1976). Hard tissue formation apparently occurs as a result of underlying tissues producing collagen in response to the coagulation necrosis; the collagen is later mineralized and the

coagulated tissue is calcilfied (Schroder, 1973). Thus although the use of this material may result in hard tissue formation, i.e. healing, its initial action is that of a locally, superficially toxic material. Rather than showing the invalidity of *in vitro* tests, this situation perhaps points out the usefulness of carrying out both screening tests and usage tests before clinical trials, and the ability of *in vitro* tests to help ascertain the mechanism of action of a material.

1.4.1 *IN VITRO* TESTS - WHAT IS REQUIRED?

Biological evaluation of new medical and dental materials is necessary to prevent potentially harmful effects occurring in humans through the use of unsuitable compounds. *In vitro* toxicity testing regimes can supply useful information in the screening phases of evaluation.

An established line of fibroblast cells, say L-cells, would be suitable as the "standard" line for experimenters if fibrous connective tissue is the most likely affected tissue.

Culture media should be of a common standard formulation and pH for all tests undertaken. Methods of obtaining material-cell contact and measurement of cytotoxic effects should also preferably be standardized. Under some circumstances, standardization of all these factors may not be possible or desirable, but any variations should be clearly noted in reports. The "Standard Practices" documents produced by the ADA and FDI have achieved a great deal in the area of standardization of test procedures, however a continuing re-evaluation must take place to ensure that methods used are the most useful available.

The use of a range of biocompatibility tests is desirable to enable a more complete evaluation of proposed biomaterials. At present *in vivo* and *in vitro* screening tests should both be used, though it may be that in time *in vivo* screening tests become supplanted by elegant *in vitro* methods. This does not infer that usage tests and clinical trials will not be required, rather that initial decisions as to whether or not a material reaches the stage of usage might be made on the basis of an *in vitro* test.

Several advantages would ensue if this becomes possible: more control over experimental conditions; reduced cost, reduced usage of animals for experimentation, and relatively easier quantitative analysis. Langeland and Langeland (1981) have pointed out the great difficulties in accurate subjective interpretation of results in histological specimens.

The "Filter Method" described by Wennberg et al (1979) seems to be a most promising *in vitro* system for the evaluation of "insoluble" or set and setting materials.

At the present time it would be unacceptable to rely entirely on *in vitro* tests for screening purposes. Endeavours should be made to improve present systems and to devise new *in vitro* models which may provide a greater understanding of the manner in which a material will behave when used clinically, as well as allowing evaluation of general toxicity.

CHAPTER 2

MATERIALS AND METHODS

2.1 CELLS AND CELL CULTURE MAINTENANCE

Cells from a fibroblast cell line - NCTC Clone L929 (L-cells) (Commonwealth Serum Laboratories, Melbourne, Australia) were used for all experiments. L-cells were supplied in 25 ml tubes in Medium 199 supplemented with 10% foetal calf serum. Cells were seeded into 25 cm^2 flasks (Lux Scientific Corporation, California, U.S.A.) following washing twice with 5 ml of Hanks Balanced Salt Solution (HBSS), and trypsinization with 0.1% trypsin + 0.02% ethylenediaminetetracetic acid (EDTA).

All cultures were maintained in Medium 199 (Flow Laboratories, Virginia, U.S.A.) and supplemented with 15% foetal calf serum (Flow Laboratories) and 50 µg/ml gentamycin (Flow Laboratories). Prior to use all medium was filtered through sterile 0.45 µ Millipore filters (Millipore Filter Corporation, Massechusetts, U.S.A.) and pH was adjusted to 6.8 with 5.8% sterile sodium bicarbonate (Flow Laboratories). Cell cultures in the enclosed flasks were incubated at 37°C and medium was changed as required until confluent cell monolyers developed in the flasks.

2.2 TEST MATERIALS AND CONTROLS

The materials tested were:

(i) Unpolymerized HYDRON* (freshly mixed HYDRON),

(ii) Pre-polymerized HYDRON* (HYDRON allowed to polymerize in an humidified atmosphere at 37°C for 24 hours),

(iii) Freshly mixed AH26** root canal sealer,

(iv) Freshly mixed Tubliseal+ root canal sealer.

The basic constituents of (iii) and (iv) are listed in Table 2.1.

Table 2.1: Root Canal Filling Material Constituents

1.	AH26 (silver-free)		
	Epoxybisphenol Resin	-	Resin
	Bismuth oxide	80%	Powder
	Hexamethylene tetramine	20%	
2.	TUBLISEAL		
	Zinc oxide		Base
	Barium sulphate		
	Oleo resins		
	Thymol iodide		Accelerator
	Oils		
	Modifiers		
	Eugenol		

(v) Controls used were filters with viable and ethanol-killed cell monolayers, onto which inverted glass cups filled with glass beads were placed. This was to represent the weight of the test specimens (0.65 ± 0.05 g).

* HYDRON Root Canal Filling Material - NPD Dental Systems, Lot No. 106

** AH26 - De Trey, Zurich, Switzerland - Expiry date 11/87.

\+ Tubliseal - Kerr, Michigan, U.S.A. - Batch No. 116.

2.3 TESTING PROCEDURES

Several different experiments were performed to ascertain various aspects of the effects of HYDRON on L-cells.

(1) 24 h incorporation of [^{3}H] thymidine and [^{35}S] sulphate by cells exposed to HYDRON and other materials.

(2) The ability of cells to recover following 24 hour exposure to HYDRON and other materials.

(3) The effects on radiolabel incorporation of controlling fall in pH caused by HYDRON.

(4) The effects of liquid extracts (HYDRON extracted in whole medium for up to 14 days) on cellular activities.

2.3.1 BIOSYNTHETIC INCORPORTION OF [^{3}H] THYMIDINE AND [^{35}S] SULPHATE

Sterile millipore filters (13 mm in diameter and with a pore size of 0.45 µm) were pre-soaked in culture medium for 1 h, then placed into 24-well Linbro plates (Flow Laboratories) - one filter per well. At this time the cell monolyers previously established in the flasks were washed twice with HBSS, harvested with trypsin-EDTA, and resuspended in fresh medium to a concentration of 10^{5} cells/ml (haemocytometer counted). Each filter was covered with one millilitre of the cell suspension. For each experiment cells used were from the same subculture.

To establish a sufficient cell monolayer on the filters, the multi-well plates with filters and cells were incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2 in air.

(a) Histology

Following incubation, two filters from each multi-well plate were chosen at random and microscopically examined to assess the extent of cell growth. Filters were fixed in 100% ethanol then subjected to hydration through a series of hydrated ethanols (90% - 70% - 50% ethanol) and stained with Erlich's haematoxylin. Standard dehydration steps to xylene and subsequent mounting in Xam followed.

(b) Scanning Electron Microscopy

Two filters from each of three experiments were prepared for Scanning Electron Microscopy (SEM), primarily to see if any cell processes extended through the filters at this stage.

Filters with cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.6 for 2 h at room temperature. They were then transferred to 0.1 M cacodylate buffer containing 6% sucrose, which was changed twice before post-fixing in 2% osmium tetroxide in cacodylate buffer. Another wash in cacodylate sucrose buffer followed. Fixed specimens were dehydrated through a graded series of alcohols from 35 to 100%.

The filters were critically point dried using liquid carbon dioxide as the transitional fluid, mounted on copper stubs with silver dag adhesive then carbon and gold coated prior to examination in the scanning electron microscope.

(c) Radiolabel Incorporation Assay

Filters with cell monolayers not stained for light microscopy or prepared for SEM were washed twice with 5 ml HBSS then placed cell side down onto mesh organ culture grids in Falcon 3010 organ tissue culture dishes (Falcon Plastics, Los Angeles California, U.S.A.) (Figure 2.1.) 1.2 ml of culture medium 199 containing radiolabelled [^{35}S] sulphate 2 µCi/ml) and [^{3}H] thymidine (2 µCi/ml) was added to the inner wells of the culture dishes (Figure 2.2). This was sufficient to ensure contact of the medium with the cells throughout the experiment.

Test specimens in standardized glass cups were then placed onto the cell-free upper surface of the filters and left in place for 24 hours - incubated in an humidified atmosphere of 5% CO_2 air. The humidity was ensured by saturation of the cardboard wicks in the outer wells of the culture dishes with sterile deionized water (Figure 2.3).

To end the experiment, specimens were removed from the cultured dishes, and all filters were washed twice with 5 ml HBSS then fixed in 100% ethanol for 15 min. Following ethanol fixation, the filters were placed cell side up onto a stainless steel grid 12 cm x 12 cm, and washed for 16 h in 1 l of a continuously stirred solution of HBSS containing non-radioactive 0.1M thymidine and 0.1M sodium sulphate (to exchange free radiolabel), and 0.1M cetylpyridinium chloride (CPC). Both ethanol and CPC will precipitate proteoglycan on the filter (Roden et al, 1972). The ionic concentration was below the critical electrolyte concentration for CPC precipitation of these polyanions.

Each filter was then washed twice in fresh salt solution before being placed into 5 ml scintillation fluid (see Appendix). Radioactivity was measured in a Packard Tricarb scintillation spectrometer, using machine set windows for sulphur (=[^{14}C]) and tritium. Figure 2.4 outlines the testing procedure.

Since results for experimental and controls have been reported as ratios, machine efficiency and quench curves were not taken into account.

Four samples of each material were tested. Radioactivity measurements were repeated five times for each specimen. The entire experiment was repeated five times with fresh samples.

2.3.2 BIOSYNTHETIC INCORPORATION OF [^{3}H] THYMIDINE AND [^{35}S] SULPHATE FOLLOWING REMOVAL OF HYDRON

In addition to the 24 h incorporation studies (Section 2.3.1), experiments were performed to ascertain whether the L-cells could recover to any extent after removal of the test materials.

Following the usual 24 h incorporation regime, specimens were removed and filters were washed twice with 5 ml HBSS. Half of the filters with cells were fixed and counted as before; the remainder were placed into fresh [^{3}H] and [^{35}S] labelled media and incubated for a further 24 h prior to fixation and radioactivity monitoring.

The incorporation levels of [^{3}H] thymidine and [^{35}S] sulphate were compared for cells exposed to HYDRON then fixed (24 h), and cells exposed to HYDRON then given an opportunity to recover for 24 h in fresh

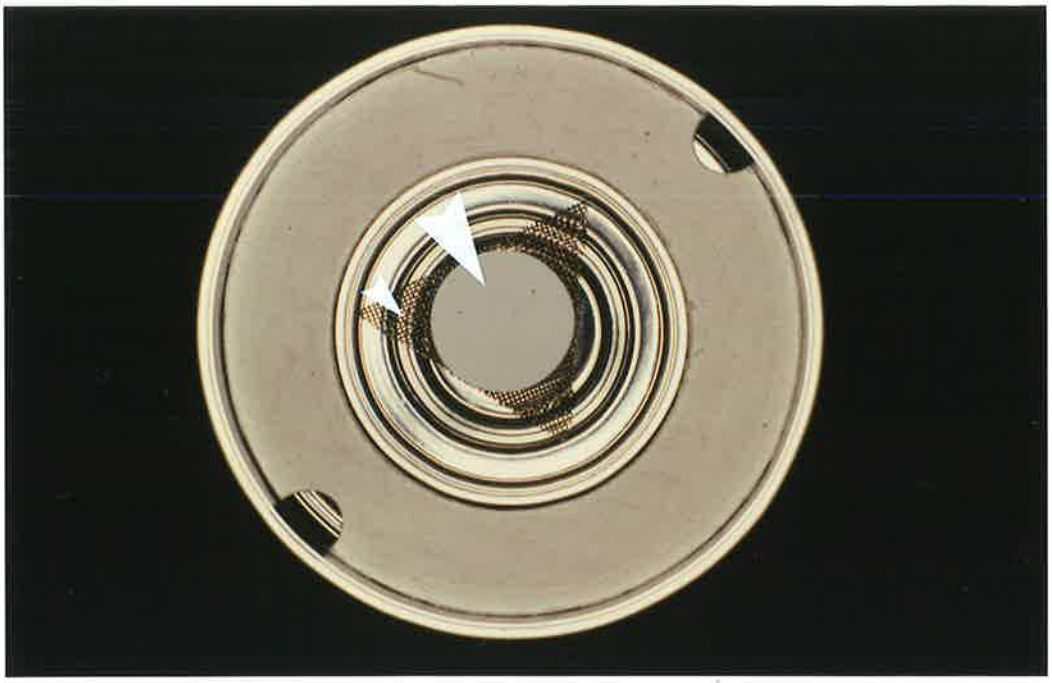


Figure 2.1 - **Testing assembly I**
Filter with cells attached (large arrow)
Organ culture grid (small arrow)

Figure 2.2 - **Testing assembly II**
Inner well with labelled medium (large arrow)
contacting cells on filter (small arrow)

Figure 2.3 - Testing assembly III
Outer well with H_2O soaked cardboard wick (large arrow)
Test specimen in glass cup (small arrow) upturned onto cell-free side of filter
Underlying radiolabelled medium (double small arrow)

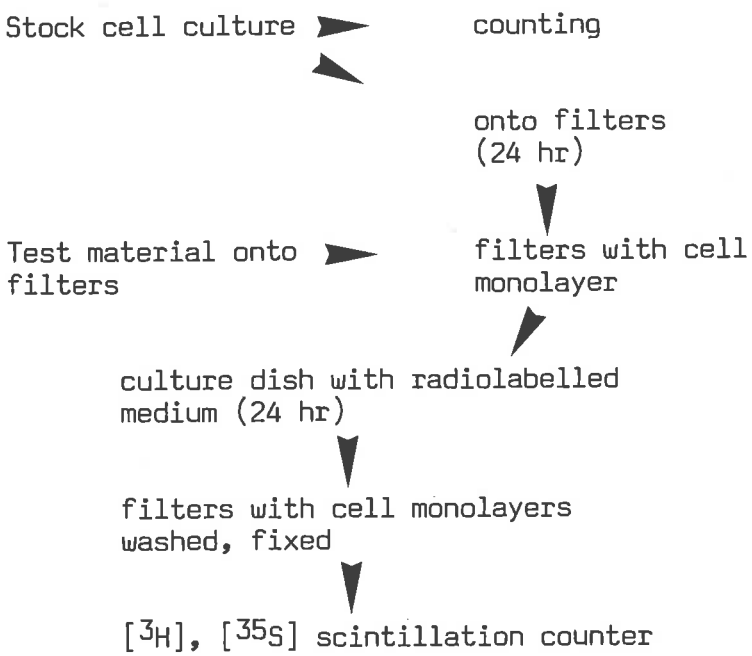


Figure 2.4 - Testing procedure

media (48 h total). Four specimens each of unpolymerized HYDRON and prepolymerized HYDRON were used for the usual counting and recovery groups. Controls with live cells were incubated for 24 h in labelled media then placed into fresh labelled media for a further 24 h. The experiment was repeated five times with fresh samples.

2.3.3 THE EFFECTS OF MEDIA pH CHANGES ON INCORPORATION LEVELS

An observation relating to HYDRON tested incubations was that despite the buffering capacity of Medium 199, a decrease in the pH of the medium began to occur immediately following application of HYDRON onto the filters (pH 6.8 to 4.5). Parallel experiments were undertaken to examine the extent to which pH might influence radiolabel incorporation levels.

Controls used were as in 2.3.1. Four other groups were tested: unpolymerized and prepolymerized HYDRON tested as in 2.3.1, and unpolymerized and prepolymerized HYDRON with 0.1 ml 5.8% sodium bicarbonate added to the medium but otherwise as tested in 2.3.1. This amount of sodium bicarbonate was determined to be sufficient to prevent the decrease in pH over the incorporation period. The experiment was repeated five times with fresh samples.

2.3.4 THE EFFECTS OF HYDRON EXTRACTS ON INCORPORATION LEVELS

Ten unpolymerized HYDRON specimens in the standard glass cups were each placed into 2.5 ml of Medium 199 (supplemented with 15% FCS and gentamycin 50 µg/ml) for 24 h. The same specimens were then removed and replaced into fresh media for a further 24 h. This procedure was

repeated for 14 days. Each daily batch of medium extract was collected and pooled, so 20 to 25 ml of extract from each day was available for testing. The pH was adjusted to 6.8 with 5.8% sodium bicarbonate, and the extracts were filtered through 0.45 µm millipore filters. Radiolabelled [^{3}H] thymidine and [^{35}S] sulphate was added to test batches until the concentration for each isotope was 2 µCi/ml. Extracts from days 1, 3, 7, and 14 were chosen for testing.

Following establishment of cell monolayers on the filters as previously described (Section 2.3.1) the filters were washed twice with 5 ml HBSS and placed cell side up into fresh multi-well plates. Aliquots of 1.2 ml of the radiolabelled media extracts from days 1, 3, 7, and 14 were added to each of four wells to cover the filters. Controls were cell covered filters in [^{3}H] and [^{35}S] supplemented fresh whole media.

Control and test specimens were incubated for a 24 h incorporation period at 37°C in an humidified atmosphere of 5% CO_2 air. Fixation, washing and radioactivity counting was then caried out as previous described (Section 2.3.1). Incorporation levels of [^{3}H] and [^{35}S] for the extracts and controls were compared. Each experiment contained four samples of each test extract. The experiment was repeated four times with fresh samples.

2.4 DATA COMPILATION AND STATISTICAL ANALYSIS

The nature of the data generated i.e. real numbers, not ranked values, allowed ready statistical analysis of results.

Data from repeated experiments were pooled in their respective groups. Student's T test was applied to test for differences between groups of importance.

Data and statistical analysis are in Appendix II.

CHAPTER 3

RESULTS

3.1 CELL CULTURE AND HISTOLOGY

Culture and maintainence of L-cells proceeded uneventfully under the conditions previously described (see Figure 3.1). Histological and SEM examination of seeded filters 24 h after seeding showed that the cell suspension concentration and filter condition were appropriate (Figures 3.2, 3.3). No cell processes were visible on the reverse (cell-free) side of the filters (Figure 3.4).

3.2 RADIOLABEL INCORPORATION EXPERIMENTS

3.2.1 TWENTY-FOUR HOUR INCORPORATION OF [^{3}H] THYMIDINE AND [^{35}S] SULPHATE BY L-CELLS - Effects of endodontic filling materials.

(a) HYDRON

Unpolymerized HYDRON caused a marked decrease in incorporation of [^{3}H] thymidine compared with the control group of filters with viable cell monolayers ($P < 0.001$). A significant suppression of inorganic [^{35}S] sulphate incorporation was also effected by this material ($P < 0.001$).

Prepolymerized HYDRON also caused decreased incorporation of both radiolabelled precursors, but to a significantly lesser extent than the unpolymerized material ($P < 0.01$).

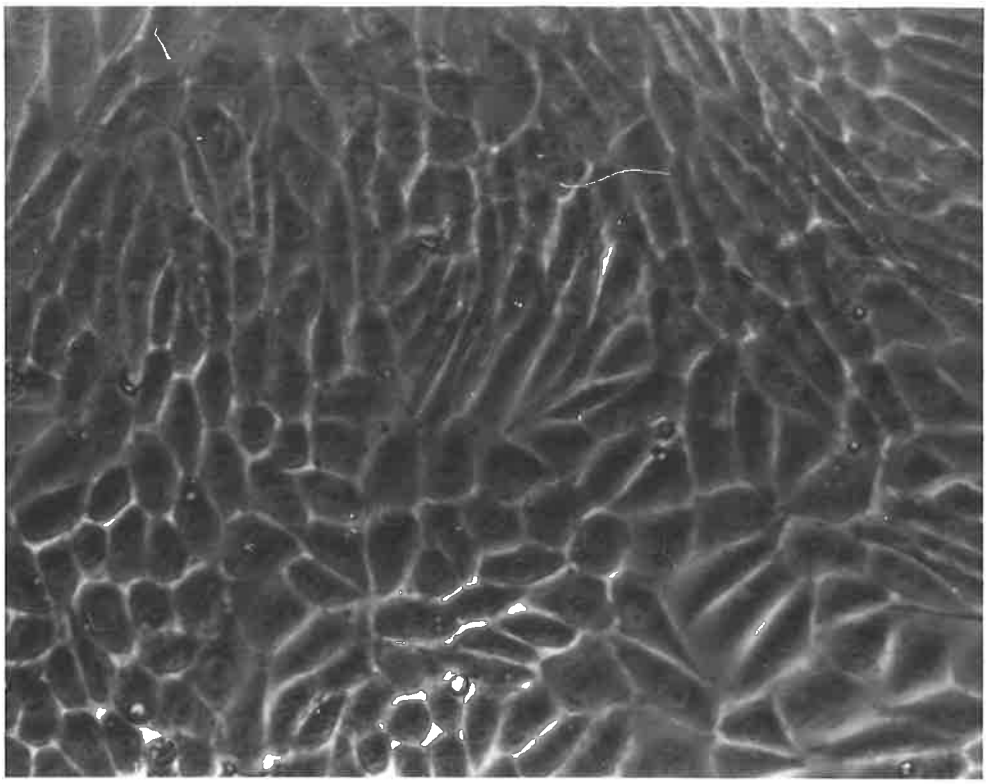


Figure 3.1 - L-cells in culture x 250

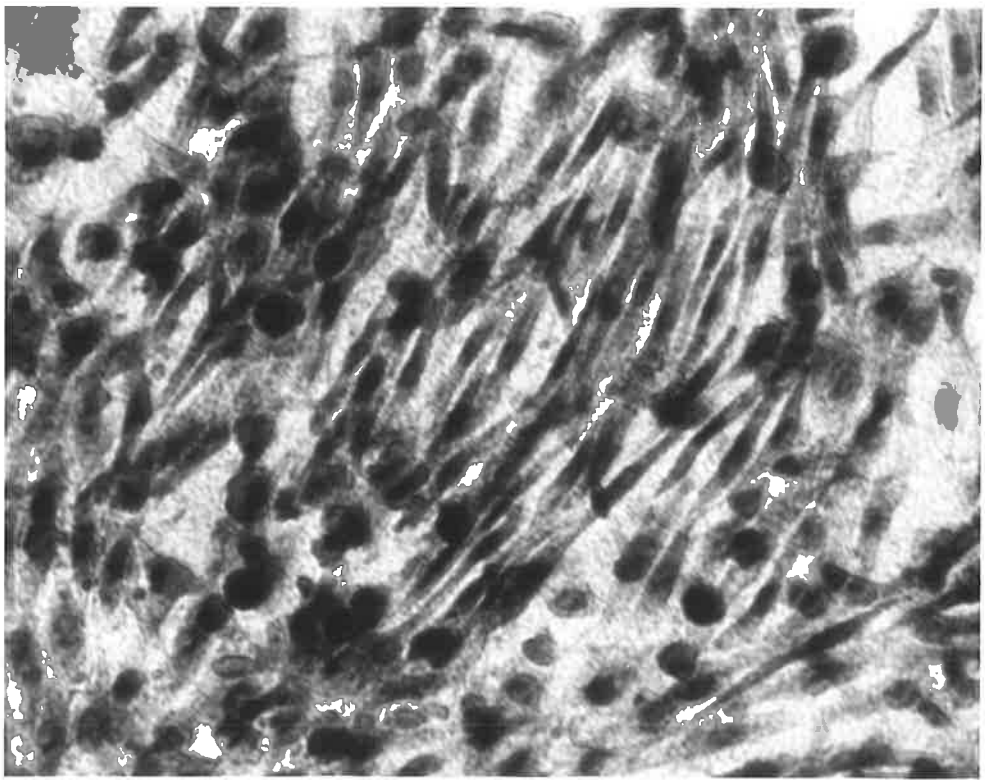


Figure 3.2 - L cells cultured on Millipore filter 24 hours after seeding x 250

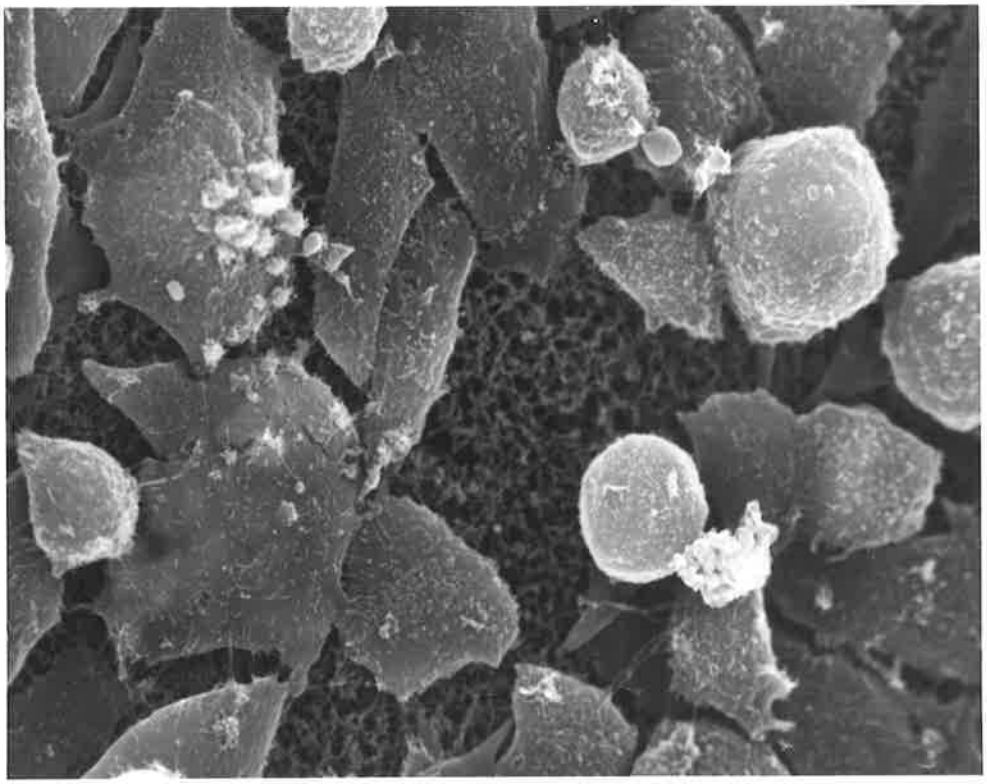


Figure 3.3 - L-cells on Millipore filter - 24 hours after seeding SEM x 1000

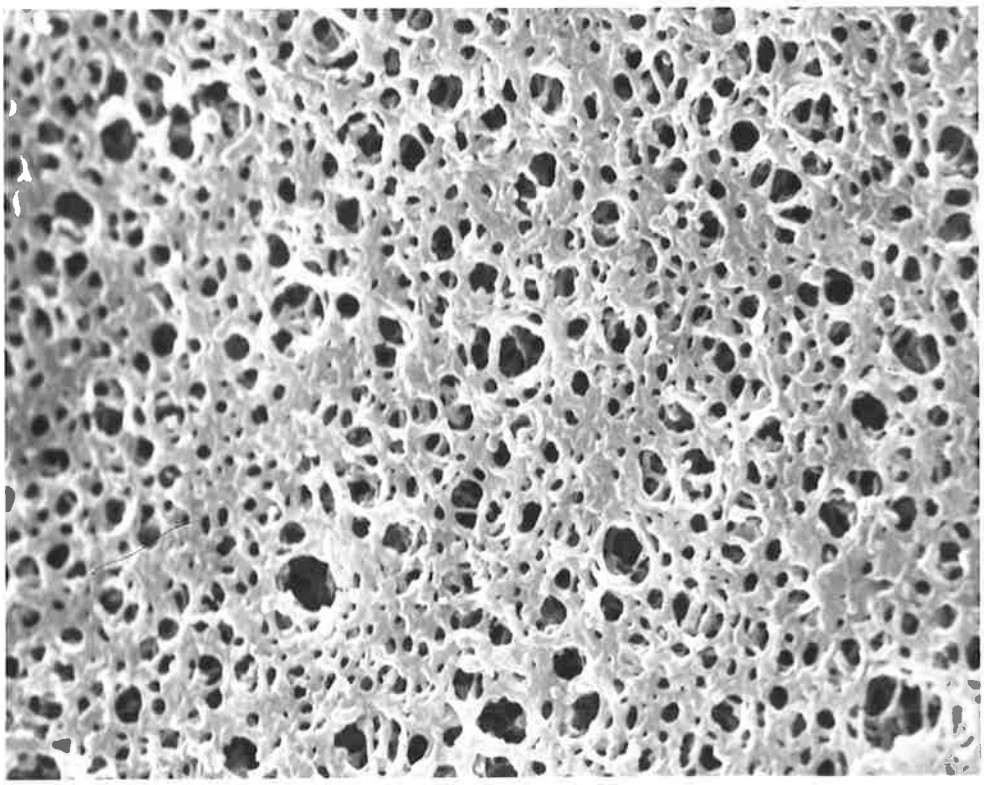


Figure 3.4 - Cell-free side of seeded Millipore filter - 24 hours after seeding SEM x 5000

(b) Other materials

Freshly mixed AH26 caused a significant decrease in incorporation of both radiolabels compared to controls, while TUBLISEAL showed even greater ability to inhibit incorporation of [^{3}H] thymidine and [^{35}S] sulphate than either AH26, or HYDRON in its prepolymerized or unpolymerized states.

Figures 3.5(a) and (b) illustrate relative radioactivity incorporation levels for controls and cells exposed to test materials.

3.2.2 BIOSYNTHETIC INCORPORATION OF [^{3}H] THYMIDINE AND [^{35}S] SULPHATE FOLLOWING REMOVAL OF HYDRON

Cells exposed to unpolymerized HYDRON, then replaced into fresh labelled medium did <u>not</u> show significantly increased levels of incorporation of either [^{3}H] thymidine or [^{35}S] sulphate over those cells not given the opportunity to recover. However a small increase was evident for both radiolabel measurements.

Significant recovery of thymidine incorporation did occur for the cells exposed to prepolymerized HYDRON ($P < 0.01$). The ability of the cells in this group to synthesize extracellular matrix material ([^{35}S] incorporation) was also increased, but not significantly. Figures 3.6(a) and (b) show the radiolabel incorporation levels for cells exposed to HYDRON for the 24 h incorporation period then fixed, and those cells which had the materials removed after the 24 hour period, and were then replaced into fresh labelled medium for a further 24 h prior to fixation and counting.

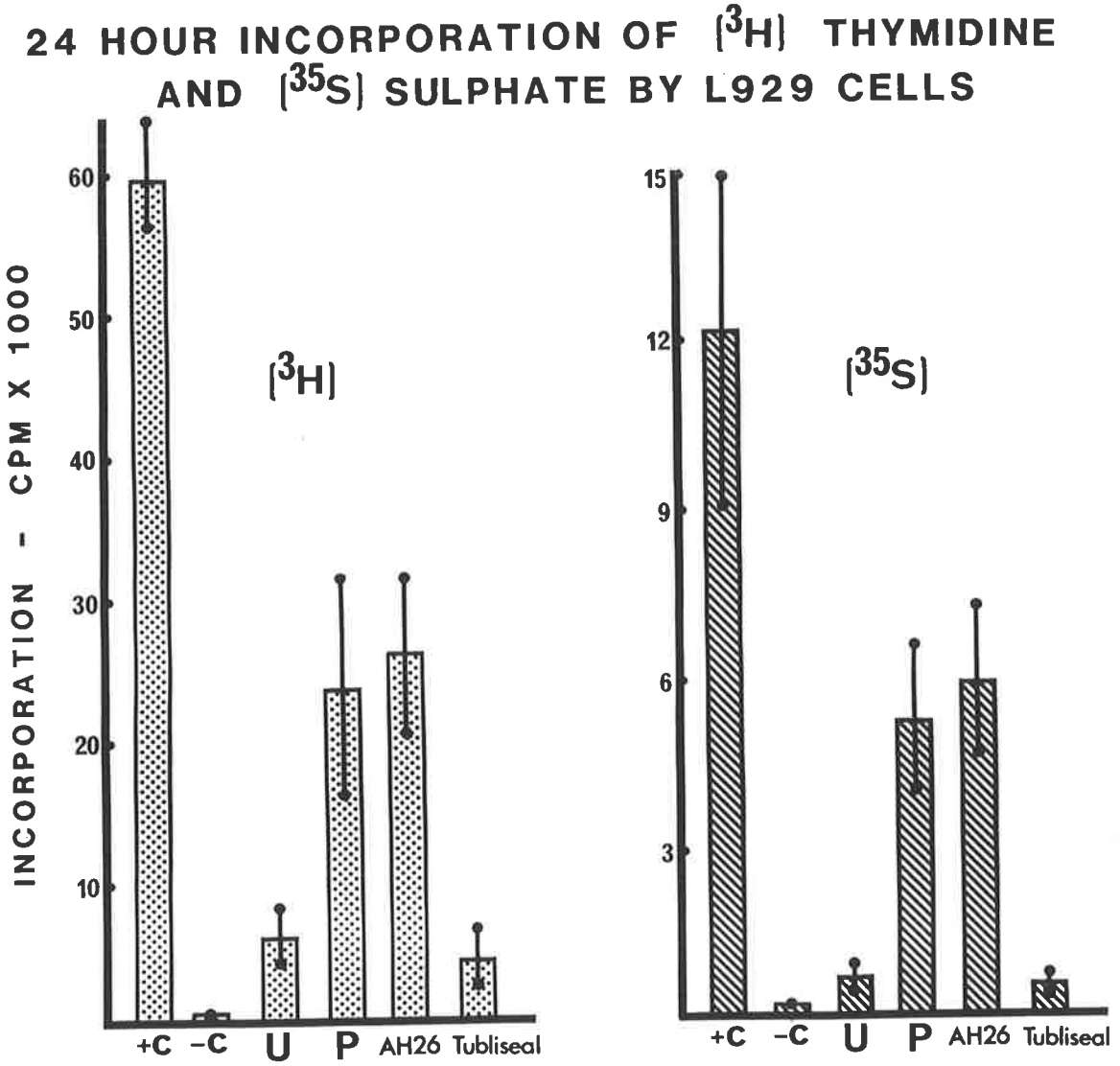


Figure 3.5(a) **Figure 3.5 (b)**

Key:
- +C control - live cells
- -C control - killed cells
- U unpolymerized HYDRON group
- P prepolymerized HYDRON group

Both unpolymerized HYDRON and prepolymerized HYDRON caused significantly decreased incorporation of [^{3}H] thymidine and [^{35}S] sulpahte ($P < 0.001$). Prepolymerized HYDRON was significantly less toxic than unpolymerized HYDRON ($P < 0.001$). Freshly mixed Tubliseal caused the greatest decrease in incorporation of both radiolabelled precursors.

INCORPORATION OF [^{3}H] THYMIDINE AND [^{35}S] SULPHATE FOLLOWING REMOVAL OF HYDRON

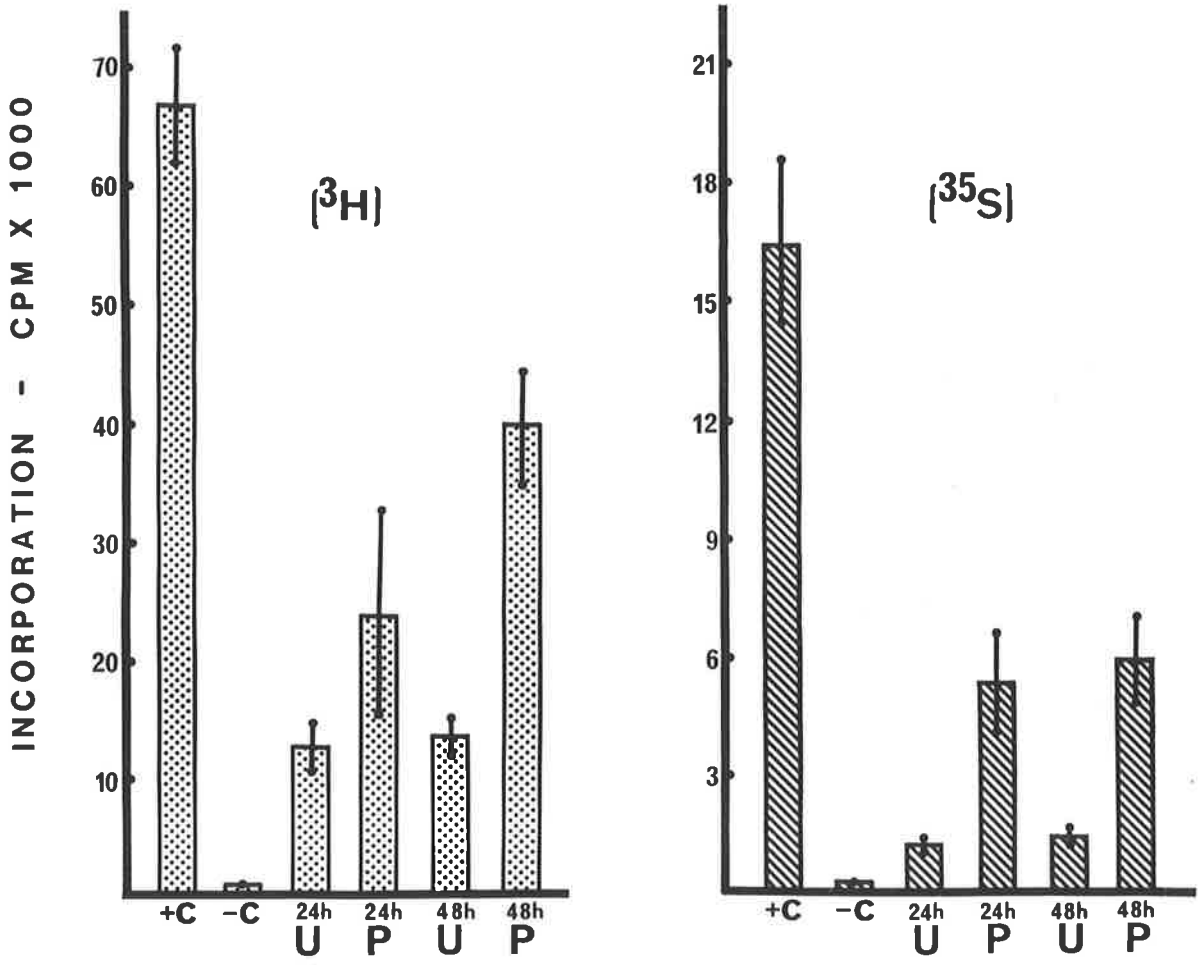


Figure 3.6(a) **Figure 3.6(b)**

Key:
- +C control - live cells
- -C control - killed cells
- U24h 24 hour incorporation - unpolymerized HYDRON group
- P24h 24 hour incorporation - prepolymerized HYDRON group
- U48h 24 hour incorporation with unpolymerized HYDRON on cells plus 24 hours incorporation after removal of HYDRON and replacement of media.
- P48h 24 hour incorporation with prepolymerized HYDRON on cells plus 24 hours incorporation after removal of HYDRON and replacement of media.

[^{3}H] thymidine incorporation was significantly increased in cultures after removal of polymerized HYDRON ($P < 0.05$). [^{35}S] sulphate incorporation was not markedly affectd ($P > 0.05$).

Removal of unpolymerized HYDRON specimens did not allow significant recovery of cell metabolism.

3.2.3 THE EFFECTS OF MEDIA pH CHANGES ON RADIOLABEL INCORPORATION

Cells in both unpolymerized and pre-polymerized HYDRON groups showed significantly greater incorporation of [^{3}H] thymidine when the medium pH was maintained at 6.8, than when no attempt was made to prevent the pH fall which occurred in other groups for media with HYDRON specimens ($P < 0.001$), see Figure 3.7(a).

Radioactive sulphate incorporation appeared to be little affected, regardless of the pH changes which occurred (Figure 3.7(b) though significantly increased incorporation was evident for the unpolymerized HYDRON group.

3.2.4 THE EFFECTS OF HYDRON EXTRACTS ON RADIOLABEL INCORPORATION

Whole media extracts from HYDRON specimens up to 14 days old all exhibited significant ability to depress [^{3}H] thymidine uptake by L-cells when compared with controls. This decreased incorporation was significantly less with day 14 extracts than with day 1 extracts ($P < 0.001$). A consistent increase in the incorporation was evident as the specimen age increased. (Figure 3.8(a)).

A parallel pattern occurred for [^{35}S] sulphate incorporation levels. (Figure 3.9(b)). In particular, synthesis of extracellular matrix material was greatly reduced in cells incubated in day 1 and day 3 extracts. Increased incorporation levels also occurred with increasing specimen age.

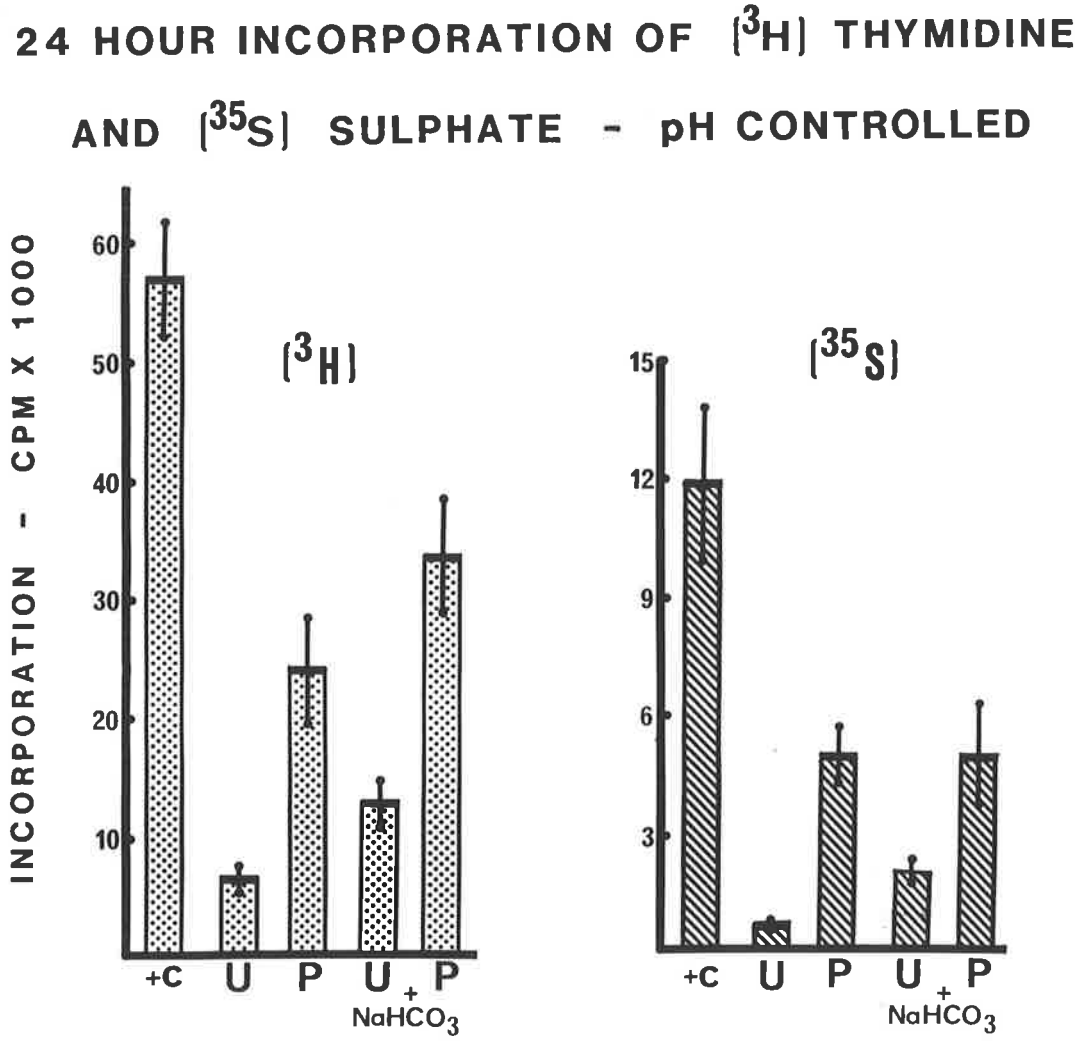
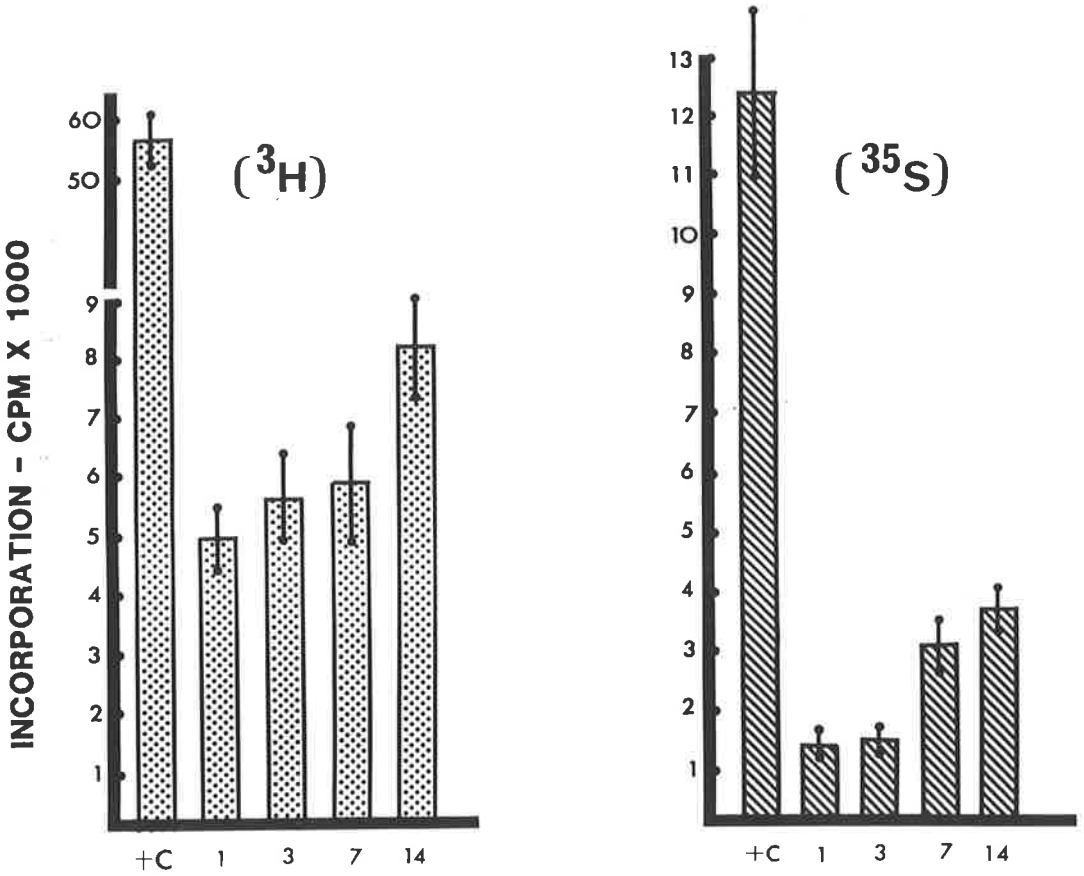


Figure 3.7(a) **Figure 3.7(b)**

Key:
- +C control - live cells
- U unpolymerized HYDRON group - pH 4.5
- P prepolymerized HYDRON group - pH 4.5
- U + $NaHCO_3$ unpolymerized HYDRON group - pH 6.8
- P + $NaHCO_3$ unpolymerized HYDRON group - pH 6.8

Cultures in media at pH 4.5 showed significantly decreased rates of [^{3}H] thymidine incorporation compared with cultures in media at pH 6.8. ($P < 0.001$). [^{35}S] Sulphate incorporation was not affected to the same extent, though the cultures exposed to unpolymerized HYDRON showed significant increase ($P < 0.001$).

24 HOUR INCORPORATION OF [^{3}H] THYMIDINE AND [^{35}S] SULPHATE BY L CELLS EXPOSED TO HYDRON EXTRACTS



Specimens age in days - i.e. days previously spent in media

Figure 3.8(a) **Figure 3.8(b)**

Key:
- +C control - live cells - fresh media
- 1
- 3 Specimen age in days: number of days previously by specimen
- 7 in serially changed media
- 14

Significant depression of incorporation levels of [^{3}H] thymidine and [^{35}S] sulphate occurred even with the 14 day extracts ($P < 0.001$).

However there was a tendency towards increased incorporation for [^{3}H] thymidine and [^{35}S] sulphate as the specimen age increased.

CHAPTER 4

DISCUSSION

In the absence of work by others, and further work by the author himself directly in this area of research, this discussion will focus on the two main aspects of this research project:-

(1) the in vitro testing method developed, and

(2) its application for the testing of HYDRON and evaluation of some of the biological effects of HYDRON measured by this method.

4.1 TESTING METHODS

Useful in vitro toxicity testing methods utilizing Millipore filters as (a) cell growth substrates and (b) material-cell contact interfaces have previously been utilized for the testing of dental materials (Wennberg et al 1979, Tyas 1977).

The method adopted in this project can be validated biochemically - evidenced by the sensitivity of cell cultures to both soluble and "insoluble" substrates. Cell responses to unpolymerized and prepolymerized HYDRON, AH26 and Tubliseal, were measured by comparison of incorporation of radioactive precursors for the processes of cell division (DNA-synthesis) and synthesis of extracellular matrix material. While all materials did affect the rates of both biosynthetic pathways, significant differences in effects were recorded for HYDRON, AH26 and Tubliseal (see Results - Chapter 3).

Evidence for the suitability of Millipore filters as a growth surface has been obtained from histology and electron microscopic observations (Figures 3.2, 3.3, 3.4). The filters also provided an appropriate controlled interface for material-cell contact, where "insoluble" specimens were tested. Furthermore, cultures grown on filters could also be used for testing the effects of soluble materials on metabolism.

The L-cell line was chosen for its capacity to be easily maintained in culture. This cell line is also specified for use in in vitro assays outlined in the 'Standard Practices', probably on account of its ready availability.

In vitro toxicity testing of endodontic materials is an important aspect of material evaluation, and is a good starting point for evaluation of materials in vivo. Nevertheless, cell culture methods may be unrealistically sensitive for the prediction of the true tissue toxicity.

The incorporation of [^{3}H] thymidine into DNA is a recognized measure for cell viability in culture (as opposed to individual cell susceptibility), and its calculation is thus an appropriate process for evaluation of cell responses in the present project.

In respect of matrix secretion as a measure of maintainence of culture integrity, incorporation of radioactive amino acids into general protein synthesis does not give definite information about the functional molecules synthesized. Thus the radioactive marker [^{35}S] sulphate has been used. The rationale embodies the following: the

carbohydrate attachment to the protein cores of matrix protein-polysaccharides (proteoglycans) is post-translational, and the sulphation is partly dependent upon correct molecular configuration. Therefore, the measure of [^{35}S] sulphate incorporation into these completed intercellular molecules represents more closely some of the actual biosynthetic events which occur in the maintainence of tissue integrity.

The testing method employed in this project has been adopted, since it embodies a number of appropriate features, namely:-

1. measurement of effects on metabolic processes rather than cell survival.
2. controlled material-cell contact.
3. adaptability for testing soluble and "insoluble" substances.
4. production of data suitable for quantitative analysis.

4.2 HYDRON

4.2.1 State of Polymerization

In root canal filling procedures HYDRON is introduced into the root canal in an unpolymerized state. The polymerization of the material may of course be just as critical to tissue responses as the polymerized material *per se*. Therefore, some attempt has been made to determine the effects on cell cultures of both unpolymerized and polymerized HYDRON. Indeed, significant differences in cell responses were noted, indicating the production of by-products or conditions involved in polymerization which did depress the rates of cell division and extracellular matrix synthesis.

4.2.2 Effects of pH

Cultures exposed to both unpolymerized and prepolymerized HYDRON, in which the pH was allowed to fall following application of the specimens, exhibited significantly greater decreases in the rates of cell division than cultures which were maintained at pH 6.8 throughout the experiment. This effect has also been noted by others (Helgeland, 1977).

Inorganic [^{35}S] sulphate incorporation however, was not influenced to a significant extent. Cell division and matrix synthesis have been reported to be mutually antagonistic (Abbott and Holtzer, 1966). Thus the production of extracellular matrix material by cells in these cultures may be pre-eminent.

Whether the noted effects of pH would be as dramatic *in vivo* is arguable, since the buffering capacity of vital tissue would probably be effective in reducing such changes in pH.

4.2.3 Reversibility of Cell Responses

Although initial responses of tissues to applied materials is undoubtedly important, the ability of injured cells to recover following such an insult also deserves consideration. Hence, an effort was made to determine the reversibility of cell responses to HYDRON.

Cultures exposed to unpolymerized HYDRON showed some evidence of recovery (not statistically significant) following removal of the HYDRON. Those cultures onto which prepolymerized specimens were placed

showed significant signs of recovery (marked increase in [^{3}H] thymidine incorporation) after removal of HYDRON.

It may be reasonable to assume that cultures more adversely affected initially (eg by unpolymerized HYDRON) would take more time to completely recover. The results herein would support this view.

4.2.4 HYDRON Extracts

The possibility that continued elution of toxic materials from HYDRON might occur over longer time periods was also tested.

Any putative toxic components were serially eluted from specimens of prepolymerized HYDRON with daily changes of fresh culture media up to 14 days. Samples of these daily media changes were then toxicity tested after being adjusted to pH 6.8 and the addition of radiolabelled precursors.

The significant depression of metabolism even with day 14 media indicated continued availability of toxic products for this time period *in vitro*. The extent to which this might occur *in vivo* is unknown. However, it could reasonably be expected to be less damaging to vital tissues due to their better overall recuperative capacity.

Results from experiments with HYDRON extracts were consistent with the concept of an ongoing but slowly decreasing availability of toxic materials from polymerized HYDRON in contact with tissue fluids.

CHAPTER 5

CONCLUSION

5.1 METHODOLOGY

The use of a cellulose acetate filter as a growth surface for the cells provided conditions which were suitable for testing of "insoluble" materials such as HYDRON. The method was also successfully adapted for evaluation of the toxicity of liquid extracts from set HYDRON specimens.

Radiolabel uptake methods used have enabled assessment of effects on two biosynthetic pathways, and these events would occur prior to cell death. The procedure should therefore, be more sensitive than techniques such as the chromium release method, or dye exclusion and viable cell staining methods. These assays rely on complete cell lysis or irreversible cell membrane damage to occur before results are available, while the method developed for the presently described experiments would enable earlier detection of toxic effects by showing alterations in the rate of metabolism. Thus the radioactivity incorporation technique adopted has been used to establish the degree of reversibility of cell responses to HYDRON.

Since no cell processes were evident on the cell-free sides of the filters at the time of testing, the test material-cell contact could be controlled - so reducing the effects of different material surfaces on cell function. Test specimens could be readily tested in their set state or during polymerization.

The cell activities measured could be expressed quantitatively, which is an advantage for data analysis and comparison.

5.2 HYDRON

HYDRON caused a substantial decrease in the rate of cell division and a decrease in the biosynthesis of extracellular proteoglycan matrix. Freshly mixed HYDRON caused a significantly greater fall in incorporation of both [^{3}H] thymidine and [^{35}S] sulphate than the prepolymerized material. This result could help to explain some differences between results of previous *in vivo* studies relating to HYDRON, where the material has been used either prepolymerized or freshly mixed.

Exposure of cultures to prepolymerized HYDRON resulted in a significant increase in [^{3}H] thymidine uptake following the removal of the HYDRON. However, cells exposed to unpolymerized HYDRON did not recover significantly during the experimental period, indicating a more pronounced initial effect on metabolism. This initial effect however, was no more severe than that produced by another frequently (clinically) used root canal sealing agent ie TUBLISEAL.

The fall in pH of media underlying the HYDRON specimens and the decrease of radiolabel uptake which this effected indicated that this may be one mechanism by which HYDRON could affect local cellular metabolism. It also indicates that the material is not "insoluble" as claimed by its manufacturers, since some components must leach out to cause the change.

Although the fall in medium pH caused by HYDRON adversely affected metabolism, other factors also contributed. This was evidenced by the ability of HYDRON extracts (where medium pH was adjusted to 6.8) to cause depression of the rates of [^{3}H] thymidine uptake and [^{35}S] sulphate incorporation. The decreasing influence of extracts on metabolism with the HYDRON specimen age (ie days of prior extraction) indicates an improved cell tolerance of the material with time.

In relation to other endodontic filling materials tested in the foregoing manner, HYDRON appeared to be more or less toxic, dependent upon its state of polymerization.

5.3 DIRECTIONS FOR FURTHER RESEARCH

Although one of the aims of this particular project was to develop an *in vitro* toxicity testing model, it would be appropriate for HYDRON to be exposed to the full range of *in vitro* tests outlined in the 'Standard Practices'. This would be useful to gain greater knowledge of the material's effects in a broader range of tests, and also to compare the results of the standard tests with the results presented for this project.

Further evaluation of the individual components of of HYDRON would be desirable to ascertain which component(s) or aggregates of components were most responsible for toxic effects; thus establishing a basis for possible modification or rationalization of the components or their proportions, consistent with desirable physical properties.

A considerable amount of *in vivo* testing of HYDRON has been carried out (see Chapter 1), however, little of this has been done according to the 'Standard Practices' methods. This aspect of testing should be further pursued for HYDRON, and if possible some comparison made to ascertain the predictability or otherwise of the *in vitro* model described in this report, for tissue reaction *in vivo*.

Although interpretation of the results of *in vitro* toxicity testing of endodontic materials (and dental materials in general) may not readily predetermine clinical success, the development of predictive *in vitro* and *in vivo* tests for particular materials and devices remain a major need, and an area on which further materials research should focus.

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LIST OF APPENDICES

APPENDIX I

Scintillation Fluid Composition

80 g Napthalene - AJAX Chemicals, Sydney, Australia

4 g BBOT (2,5-bis[5'-tert-BUTYL-BENZ-OXAZOLYL-(2')]THIOPHENE) - SIGMA Chemical Company, St. Louis, U.S.A.

520 ml Toluene - AJAX Chemicals, Sydney, Australia

Made up to one litre with 2-methoxyethanol - AJAX Chemicals, Sydney, Australia

APPENDIX II

Data and Statistics

1(a) 24 hour incorporation of [^{3}H] thymidine by L-cells.

1	2	3	4	5	6
Control Live Cells	Control Dead Cells	Unpoly-merized HYDRON	Prepoly-merized HYDRON	AH26	Tubliseal
53894	258	14106	48726	32663	3123
62350	297	5884	35431	23177	3495
63721	254	10818	32870	20257	2672
58627	276	13217	36952	26269	2991
48373	222	12520	7662	19545	9466
55603	210	5208	14720	19960	13882
58000	209	8843	20145	22137	12836
54967	231	6751	14875	20249	11641
57118	403	1342	16000	25894	2633
61776	257	2402	15580	27376	1874
64203	323	2937	16971	25512	2102
56489	402	2744	17647	31269	2231
55816	173	7328	27101	29937	4271
65902	209	5173	33601	32642	3965
72027	328	5127	26819	38723	3772
53011	127	5573	31142	37273	4101
59920	291	5379	22317	26921	3639
59860	223	4361	24222	28883	4711
67123	479	8887	23193	34550	2793
62286	198	4296	19764	31695	2942
			Mean Values		
59553	269	6645	24287	27747	4957
			Standard Deviations		
5533	85	3676	9853	5820	3741

T tests

Groups	T value	P value
1 and 3	35.617	<0.001
3 and 4	-7.501	<0.001

1(b) 24 hour incorporation of [^{35}S]sulphate by L-cells.

1	2	3	4	5	6
Control Live Cells	Control Dead Cells	Unpoly-merized HYDRON	Prepoly-merized HYDRON	AH26	Tubliseal
17220	102	916	6154	6123	463
15404	77	666	6996	5024	299
14424	77	885	7666	5435	427
16991	82	1010	7123	5465	590
10576	64	297	3904	5102	412
12797	48	266	3791	5239	295
12749	36	187	4032	4864	410
13120	71	193	4411	4553	396
14965	83	1403	5377	6243	1398
14728	89	1125	5624	7760	1105
15719	137	625	6408	7229	780
14410	62	850	5560	8093	666
8795	26	700	3469	6160	620
6837	18	710	5281	4445	485
6991	24	501	5366	6010	553
7724	37	483	4190	4288	491
12240	65	890	7785	6666	390
9176	81	925	6971	7983	787
10268	49	763	2671	8642	653
11397	19	681	6188	8104	886
Mean Values					
12327	62	704	5448	6171	605
Standard Deviations					
3240	30	318	1475	1375	278

T tests

Groups	T value	P value
1 and 3	15.962	<0.001
3 and 4	-14.054	<0.001

2(a) Incorporation of [^{3}H] thymidine after removal of HYDRON.

1	2	3	4	5	6
Control Live Cells	Control Dead Cells	Unpoly-merized HYDRON 48 hr.	Prepoly-merized HYDRON 48 hr.	Unpoly-merized HYDRON 24 hr.	Prepoly-merized HYDRON 24 hr.
72379	420	16261	46285	14010	28652
64821	290	17493	49173	16431	27421
66768	327	17012	43294	15998	29365
70094	483	21286	41286	16205	28743
64398	320	15276	39670	13246	23464
67291	363	13471	41291	12738	22906
70933	237	9508	43242	14024	21877
69172	381	12292	38279	12971	24654
58671	287	16170	28382	12179	23589
64990	321	9233	33764	10231	25795
67262	228	11286	37421	8783	24608
61190	263	14175	32645	12223	22704
57670	319	11267	35450	9671	31028
62929	204	12408	37683	12485	30293
74260	321	10986	38291	12923	29224
59821	286	9872	42625	13062	28642
68920	401	16223	45283	14213	26694
71293	385	15021	39702	16192	27543
74869	702	16872	41286	13031	27201
65487	325	13286	38660	13295	28228
		Mean Values			
66660	343	13970	39686	13195	26618
		Standard Deviations			
4971	108	3164	4874	2081	2740

T tests

Groups	T value	P value
1 and 3	0.914	>0.05
3 and 4	2.619	<0.05

2(b) Incorporation of [^{35}S] sulphate after removal of HYDRON.

1	2	3	4	5	6
Control Live Cells	Control Dead Cells	Unpoly-merized HYDRON 48 hr.	Prepoly-merized HYDRON 48 hr.	Unpoly-merized HYDRON 24 hr.	Prepoly-merized HYDRON 24 hr.
15267	120	967	7249	891	5268
14983	79	1283	6837	1073	5491
16171	89	1491	6971	1791	5704
15888	108	873	7031	1210	5329
14720	65	1228	5793	1193	5604
18699	77	1402	5565	1209	5576
16371	84	1093	6631	939	5823
12042	109	1181	5450	1428	5921
17628	87	1529	4872	1428	6438
21083	94	1024	7041	1639	6202
15267	128	1479	6389	1010	6113
13891	63	1951	4437	875	5987
14391	64	1311	4951	1014	5886
18202	56	1781	4236	1168	6792
16863	33	1636	7933	1437	6238
16227	68	1430	6985	1397	6149
20316	96	1951	4660	1392	6031
18729	59	1404	7159	1406	5297
16238	169	1287	3725	1129	5488
17092	75	1365	6431	1187	5701
		Mean Values			
16503	86	1383	6017	1240	5852
		Standard Deviations			
2180	30	294	1204	245	401

T tests

Groups	T value	P value
1 and 3	1.66	>0.05
3 and 4	0.04	>0.05

3(a) Incorporation of [^{3}H] thymidine at different pH levels.

1	2	3	4	5	6
Control Live Cells		Unpoly-merized HYDRON pH 4.5	Prepoly-merized HYDRON pH 4.5	Unpoly-merized HYDRON pH 6.8	Prepoly-merized HYDRON pH 6.8
	56720	8739	31228	14244	38292
	63459	7042	29283	15081	38726
	61376	7629	22309	1613	34330
	48622	5043	21240	14221	36267
	57228	6261	29463	12091	41015
	51243	4037	20086	13165	29624
	56628	5836	19278	12067	28302
	52437	6991	18625	11873	31283
	57861	7849	22387	11428	30021
	60094	4368	25262	10662	29072
	56292	4616	30624	12649	28694
	61043	5124	28443	12943	34302
	49298	6093	22467	13121	36946
	57864	4044	17891	15267	36863
	58869	6241	22634	16421	38249
	59132	7760	21021	12224	39021
	58321	1924	20473	11029	31962
	62401	6029	29283	10867	32084
	55504	7218	26242	12136	37293
	51627	4467	27161	11242	35064
Mean Values					
	56801	5866	24270	12217	34371
Standard Deviations					
	4075	1669	4347.5	2952	3972

T tests

Groups	T value	P value
2 and 4	-8.376	<0.001
3 and 5	-7.67	<0.001

3(b) Incorporation of [^{35}S] sulphate at different pH levels.

1	2	3	4	5	6
Control Live Cells		Unpoly-merized HYDRON pH 4.5	Prepoly-merized HYDRON pH 4.5	Unpoly-merized HYDRON pH 6.8	Prepoly-merized HYDRON pH 6.8
	12092	1129	4998	928	7023
	14629	867	4372	607	4217
	15037	724	5091	704	3022
	14221	683	4867	863	4438
	12616	483	4409	291	5098
	10862	729	4321	1103	4623
	9123	504	5862	691	2869
	8794	697	5264	866	7931
	9938	891	6094	904	6226
	10244	704	5867	895	4093
	12624	699	5823	1920	7124
	13024	880	4211	1695	7653
	15221	496	6283	1498	6828
	14062	991	5258	2257	6720
	12290	1284	6025	2057	6437
	10862	875	5868	3211	6661
	9198	938	5722	2021	5214
	12042	764	6214	1876	4083
	11221	883	7428	1924	6946
	10937	985	6309	2138	5827
Mean Values					
	11952	810	5514	2022	5651
Standard Deviations					
	1946	205	831	384	1523

T tests

Groups	T value	P value
2 and 4	12.4656	<0.001
3 and 5	-0.354	>0.05

4(a) Incorporation of [^{3}H] thymidine by L-cells in HYDRON extract.

1	2	3	4	5	6
Control Live Cells	Control Dead Cells	1 Day Extract	3 Day Extract	7 Day Extract	14 Day Extract
51927	299	4387	4302	4954	7101
52005	243	4647	4691	5840	8802
64459	429	4729	5744	3606	8812
48724	207	5348	5527	7933	7168
50247	128	4591	4782	5686	8821
58218	209	4622	5219	4893	9250
59043	227	4893	6848	5491	9858
57224	324	4124	6242	6821	8247
49876	162	5020	7051	6942	7485
53206	192	6219	5228	6386	7783
61422	243	5431	5898	5706	7280
62301	171	5387	6285	4932	7910
53267	202	5292	6392	6921	8246
54298	286	4668	6111	6028	8751
58231	326	4385	5805	5873	7286
53279	301	5280	5721	6627	7593
		Mean Values			
55489	247	4939	5765	5881	8150
		Standard Deviations			
4755	77	529	752	1006	830

T tests

Groups	T value	P value
1 and 3	42.2596	<0.001
1 and 6	39.2266	<0.001
3 and 6	-13.047	<0.001

4(b) Incorporation of [^{35}S] sulphate by L-cells in HYDRON extract.

1	2	3	4	5	6
Control Live Cells	Control Dead Cells	1 Day Extract	3 Day Extract	7 Day Extract	14 Day Extract
10976	94	1105	1685	2983	2864
11803	68	1396	1247	3409	3902
13210	102	1187	986	2469	3941
12245	74	1288	1984	2917	3785
11641	69	1274	2023	3821	3662
14028	83	1083	1423	3222	3704
13279	78	1409	1608	2679	3528
12041	86	1273	1240	2406	4244
15624	91	1684	1894	2808	3878
12865	129	1312	1088	2776	4021
8791	78	1267	1113	2682	3976
9046	74	1089	1249	2998	2984
13292	39	291	1624	3205	3274
12103	45	1469	1583	3671	3387
13058	67	1572	1292	3865	3665
14294	83	1866	1365	2752	3824
Mean Values					
12394	79	1285	1463	3041	3665
Standard Deviations					
1770	21	342	322	454	377

T tests

Groups	T value	P value
1 and 3	24.6516	<0.001
1 and 6	19.2956	<0.001
3 and 6	-18.707	<0.001