A HISTOLOGICAL EVALUATION OF THE BIOCOMPATIBILITY OF HYDRON IMPLANTED IN GUINEA PIGS

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DEDICATION

This volume is dedicated to my wife Christine
and my children John, Penelope and Jill.
Without their love, understanding and support
completion of this volume would not
have been possible.

ERRATUM

Page 3	line 9:	hydrofloric should be hydrofluoric
Page 59	line 12:	extipated should be extirpated
Page 65	last sentence:	found in should be founded on
Page 112	line 4:	enought should be enough
Page 116	line 9:	were should be was
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Page 124	line 16:	quantitated should be quantified
Page 127	line 9: line 13:	gradicule should be graticule Student t-tests should be Student's t-tests
Page 155	line 15:	a word 'revealed' or 'showed' seems to have been omitted
Page 160	line 3:	entirity should be entirety
Page 171	legend Fig. 4.34:	demarkated should be demarcated
Page 180	lines 20 & 21:	$P = 0.05$ should be $P \leqslant 0.05$
Page 226	line 4:	'the' (third word) has been omitted
Page 229	footnote:	May and Barker should be May and Baker

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PRÉCIS

Hydron®, a hydrophilic long chain hydrocarbon polymer (poly-hydroxyethyl methacrylate) was introduced as a purportedly biocompatible root filling material in 1978. Prior to its introduction as an endodontic material, Hydron had been used in a variety of medical and surgical applications such as breast augmentation. In these contexts Hydron was introduced to tissues as an implant which was pre-polymerized, sterilized and leached to remove residual impurities. When employed as an endodontic root filling material Hydron is introduced to the root canal unset and polymerization takes place *in vivo*. Consequently any impurities present in the material, including residual monomer, remain *in situ*.

A review of the literature on the biocompatibility of Hydron revealed that much of the experimental work on this material involved the use of pre-polymerized and purified Hydron. Since differences exist between Hydron in this form and Hydron as used in an endodontic context a study was designed with the broad objective of examining aspects of the biocompatibility of Hydron when used as a root filling material.

A variety of *in vitro* and *in vivo* model systems can be employed to examine various aspects of the "biocompatibility" of any material which is to be used as an implant or of a material which remains in contact with living tissues *in vivo* for long periods of time. Endodontic materials fall within the latter category. Ideally a biological compatibility test should simulate the actual in use situation and conditions appropriate for the

material being tested. The present study was designed to allow investigation of certain tissue responses following *in vivo* implantation of Hydron root filling material in guinea pigs.

The model system devised involved the implantation of short lengths of 0.8 mm inner diameter Teflon tubing into the mandibles and muscle of guinea pigs. The Teflon tubing acted as a carrier for the Hydron and control materials used - namely AH26 and solid Teflon. The original research protocol used in the present investigation required the implantation of unset Hydron within the carrier tubes. Assessment of histological material, in pilot studies using this protocol, revealed however that spillage of Hydron into the tissues at the ends of the Teflon tubing occurred. This made standardized, post-implantation histological assessment of tissue reactions impossible.

Subsequently, the research protocol for the main study employed the use of Hydron which was polymerized within the Teflon tubing before implantation. Although polymerized before implantation the Hydron was not otherwise altered or treated to remove impurities.

A total of 216 experimental and control implants were carried out in this study, 108 intrabony implants and 108 muscle implants. At intervals ranging from 2 days to 26 weeks post-implantation animals were sacrificed and implant sites dissected out and processed for histological examination by light microscopy. In the case of the intramuscular implants, the following parameters were assessed microscopically:

(a) the nature and extent of any inflammatory response tissues adjacent to the implant face;

(b) the nature and extent of any "capsular zone" adjacent to the implant material;

(c) foreign body giant cell response;

(d) macrophage response and distribution;

(e) the presence and distribution of fat cells;

(f) the nature of any mineralization.

Where possible, statistical analysis of results was carried out. However, where this was not feasible, the results were described and similarities and differences between the various reactions noted. In the case of the intraosseous implants the nature of any bone repair occurring adjacent to the implant materials was examined in detail.

Analysis and study of the results obtained from both intraosseous and intramuscular implant specimens revealed that Hydron elicited only a minimal inflammatory response in tissues following implantation. In general, the assessed tissue reactions and responses to Hydron, AH26 and Teflon implanted in muscle were similar and were consistent with those reactions expected of relatively biocompatible materials. Phagocytosis of Hydron by macrophages was observed. A finding of interest was the occurrence of mineralization of Hydron along the implant-tissue interface in muscle specimens. Analysis of bone implant specimens demonstrated that bone regeneration occurred in contact with implanted Hydron.

DECLARATION

This thesis is submitted in part fulfilment of the requirements for the Degree of Master of Dental Surgery, of the University of Adelaide. Candidature for the degree was satisfied by a Qualifying Examination in 1980.

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

RALPH JOHN REID

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CHAPTER I.

HYDRON - A LITERATURE REVIEW.

1.1 INTRODUCTION

Hydron is the trade name given by the National Patent Development Corporation (N.P.D.) to products consisting of poly hydroxyethyl methacrylate (poly-HEMA) and certain additives. The product is marketed by Hydron Limited, in the United States of America and by various branches of this company in other countries. Hydron Limited is a company jointly owned by N.P.D. and Smith and Nephew Associated Companies Limited.

Poly-HEMA was first produced by Otto Wichterle and Drahoslan Lím of the Institute for Macromolecular Chemistry, Czechoslovak Academy of Science, Prague. The material was first reported in 1960 (Wichterle and Lím, 1960). The HEMA monomers were prepared by the alcoholic re-esterification of methyl methacrylate with glycol, the di- and mono-esters being selectively extracted from the reaction mixture. It was stated that polymerization of the monomer could take place with the monomer containing a variable amount of water. Depending on the amount of water, either sponges or gels were formed. They claimed (without supporting evidence) that the living organism (tissues) was not irritated by the material.

Two patents exist which outline the methods of preparing articles made of hydrophilic polymers (Wichterle and Lím, 1961; Wichterle and Lím, 1965). Another patent outlines the composition of the material when used as a tooth root canal filling material (National Patent Development Corporation, 1976).

1.2 CHEMICAL NATURE

Poly hydroxyethyl methacrylate is formed by the polymerization of hydroxyethyl methacrylate, usually in the presence of small amounts of cross-linking agents, such as ethylene glycol dimethacrylate. Polymerization is by conventional free radical addition, the usual catalyst employed being benzyl peroxide.

The structure of poly hydroxyethyl methacrylate is shown in Figure 1.1.

Cross-linked poly-HEMA is insoluble in strong acids such as hydrofloric and all common organic solvents. In the absence of water the material is hard, clear and brittle, resembling poly-methyl methacrylate. Depolymerization will only take place if it is heated above 200°C. When polymerized in the absence of cross-linking agents, a linear polymer is formed, which is much less stable, for example it is soluble in alcohol (Simpson, 1969).

If dehydrated cross-linked poly-HEMA is hydrated, it gradually swells and becomes soft. The final water content is 37%. The monomer and water are completely miscible. However, if the solubility of water in the polymer is exceeded during polymerization, then a sponge is produced. The mean size of the diameter of the channels in the sponge varies, depending mainly on the water content. Other conditions of polymerization also affect the final product but to a lesser extent. These sponges can be washed in water to completely remove any impurities. The poly-HEMA can withstand boiling and autoclaving (Barvic et al, 1967).

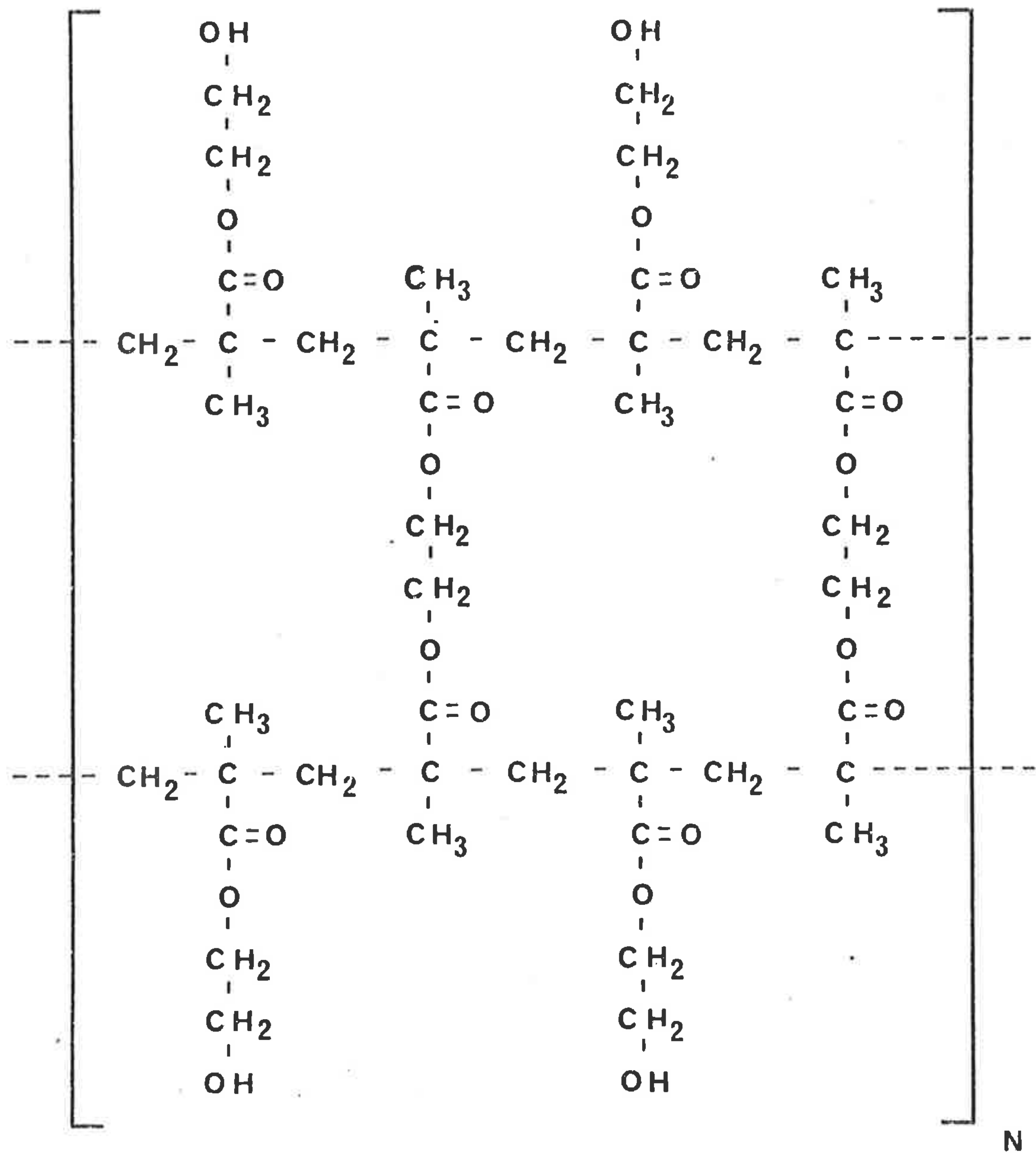


FIGURE 1.1

The material is used as either a sponge or a gel in medicine. After polymerization in a mould of the required shape, the poly-HEMA is washed to remove any impurities and sterilized by autoclaving (or boiling for asepsis).

When poly-HEMA is employed as a root canal filling material, a paste and powder are combined and introduced into the previously debrided and sterilized root canal space (Goldman et al, 1979). Polymerization takes place *in situ*. There is only one reference to the composition of this material, (Kronman et al, 1977) which claims that the powder consists of 99.5% barium sulphate and 0.5% benzyl peroxide. The patent pertaining to Hydron as a root canal filling material, (National Patent Development Corporation, 1976) lists a number of materials that may be incorporated in a two material system:

PART A LIQUID/SEMI-LIQUID	PART B SOLID/LIQUID/PASTE
may contain:	may contain:
monomer (HEMA)	polymerization catalyst
cross-linking monomer	radiopaque material
active filler	active filler
reducing agent and antioxidant	thickening agent
radiopaque material	

It then goes on to list a number of mixtures that could be used. However, the amounts in each of these mixtures vary over a wide range.

Commercially available monomer (HEMA) varies from 50% to 85% purity (Sothman, 1980). However, the material can be further purified by vacuum distillation.

1.3 BIOLOGICAL TESTING OF POLY-HEMA

1.3.1 TISSUE CULTURES

Dreifus et al (1962) grew explanate cells from fragments of new born rat kidneys, in an animal-extract medium. The pre-polymerized and pre-sterilized material was placed close to, but not in contact with, the growing cells. Incubation was for two days at 37°C.

Fibroblast-type cells, wandering cells, and epithelial cells grew out from the tissue and established contact with the test material. Some even grew over this material. These results compared favourably with the positive control (partially polymerized methyl methacrylate). Subsequent staining showed the cells which contacted the poly-HEMA to be morphologically normal.

Because size and surface morphology of the test samples varied and since both could affect the outcome of the experiment, results from one sample could not be compared with the others. It appeared that different cell types grew out from different kidney samples, another variable outside the experimenter's control. Some indication of gross toxicity (or lack of it) was perhaps the only conclusion that could be drawn from such a methodology. However, this was the only reference in the literature of testing using tissue culture techniques.

1.3.2 IMPLANTATION

(i) Gel

Little experimentation has been done with Hydron in the form of a gel. However, when Barvic (1962) implanted purified discs subcutaneously and intraperitoneally in rats, a minimal response was observed.

(ii) Sponges

Following the implantation of sponges cutaneously in rats (Barvic et al, 1969) there was little response other than thin capsule formation. There was a serous and cellular exudate into the sponge. Organization of this exudate took place near the implant-tissue interface and eventually connective tissue filled the spaces of the sponge. Unfortunately, 4 weeks was the longest period post-implantation studied.

Calan (1970) observed that the pore size of the sponge seemed the critical factor affecting the ingrowth of tissue. A large pore size encouraged a tissue ingrowth, while a small pore size inhibited such ingrowth. The remainder of the sponge was filled with a fluid which was compared, both with normal interstitial fluid, and with blood. Interstitial fluid contained more chloride and less potassium than blood. The fluid from within the poly-HEMA sponge contained more chloride than did blood but less than interstitial fluid. Thus it differed from both blood and interstitial fluid. The fluid was not static. Calan demonstrated an increase and then a decline in the concentration of an antibiotic, following an oral course of the drug.

This researcher and others have found excellent tissue tolerance. However all these studies have been for relatively short time periods (two months or less).

When the poly-HEMA sponge was implanted subcutaneously for longer periods (Winter and Simpson, 1969; Winter, 1970; Smahel et al, 1971) the initial response was excellent acceptance by tissues. However, after a period of four weeks, a local round cell infiltration in isolated areas of the tissue was observed. Local discreet areas of the connective tissue in the sponge also began to stain basophilically. These areas when stained according to the method of Von Kossa indicated the presence of calcium salts.

Concurrently, there was an increase in the cellularity of the connective tissue stroma which then took on the appearance of granulation tissue. Ossification of the previously mentioned Von Kossa positive areas then occurred by intramembranous ossification. This ossification continued until the whole pore space was filled with woven bone. Thereafter, there was evidence of remodelling of the woven bone. In the 1969 paper, Simpson reported that calcification of poly-HEMA sponge occurred following subcutaneous implantation in rats for six months.

What effects chemical modifications and changes in the physical structure of poly-HEMA might have on calcification were investigated by Šprinel et al (1973). They could detect no change in the process of calcification following chemical changes to the backbone of the polymer by the introduction of iogenic groups. However, changes in the physical structure of the poly-HEMA greatly influenced the degree and distribution of the resulting calcification. Homogeneous and macroporous gels underwent calcification only rarely. When calcification did occur in these materials it was confined to the margin of the implant. Macroporous gels (sponges) exhibited massive calcification at the margin of the implanted poly-HEMA. Very high porosity sponges exhibited massive calcification in the centre of the implant. Šprinel explained this phenomenon "by the degree to which the implant is penetrated by the newly

formed tissue, as well as by the difference in accessibility of nutrition to the penetrating cells depending on the porosity of the implant".

1.3.3 COMPATIBILITY WITH BLOOD

Singh (1969) implanted a piece of Hydron, about the size of a postage stamp, in the wall of the right atrium (dog) for two months. The implant was so positioned, that one end was sutured into the atrial wall and the other end was free in the cavity of the atrium. The Hydron had previously been decontaminated and autoclaved.

There was no thrombus formation on the free end of the implant. A small amount of thrombus was adhering to the Hydron-heart wall interface. Macroscopically, this thrombus could be easily separated from the Hydron, but not so from the atrial wall. Microscopically, suture material could be seen at the base of the thrombus and Singh concluded that probably these sutures had initiated the thrombus. In some cases there was calcium deposition in the thrombus adjacent to the Hydron.

The poly-HEMA appeared compatible with blood. Where any implant meets the endothelium there is likely to be thrombus formation because of disturbances in flow. This case was no exception. Although the sutures could be the causative agent the Hydron could not be entirely excluded from being part of the causation of such a thrombus. This experiment would have been

more meaningful if the implant had remained in place for a longer time period and the procedure had been repeated so that the sutures were buried.

1.3.4 CARCINOGENICITY

The only reference to possible carcinogenicity is made by Barvic (1962). He claimed that implanted discs of poly-HEMA gel did not increase the incidence of spontaneous sarcoma development in rats. No mention is made of the length of time the implants were in place.

1.3.5 ANTIMICROBIAL ACTIVITY

A number of micro-organisms (gram-positive cocci, gram-negative rods and a gram-positive yeast) were incubated in conjunction with strips of poly-HEMA (Kronman et al, 1979). They claimed the results of these experiments showed that the poly-HEMA not only did not support microbial growth but inhibited it. They also claimed that the powder and liquid were self-sterilizing.

The first count of the number of microbes present was taken 24 hours after their introduction into the culture vessel. It is possible that the organisms could have been "trapped" within the sponge network and therefore not be evident. It would have been more meaningful to thoroughly disintegrate the material, and carry out counts at 0 (to establish a baseline), 24, 48, and 72 hours. Glass slides were

a poor control because of the dissimilarity between their surface and that of spongy poly-HEMA. Testing the powder and the liquid as the experiment did, probably only indicated that the components were sterile when packaged.

1.4 APPLIED MEDICAL RESEARCH

In all cases, the poly-HEMA was pre-polymerized and then any residual materials (such as unreacted monomer, catalyst, etc.) were thoroughly removed either by washing, boiling, or autoclaving.

1.4.1 SOFT CONTACT LENSES

All research concerned with soft contact lenses seemed to take for granted that the material was biocompatible. The research, for example Takahaski et al (1966), Buxton and Locke (1971), was mainly concerned with such things as: oxygen transfer across the lens; fitting the lens; easy sterilization of the lens by the patient; ways to stop the lens drying out and the suitability of case selection for these lenses.

1.4.2 HOLLOW TUBE REPLACEMENT

Reinforced poly-HEMA tubes have been used experimentally in various animals to replace such organs as the common bile duct (Levowitz et al, 1969), the ureter (Kocvara et al, 1967; Warren et al, 1967) and major blood vessels (Warren et al, 1967). The majority of the prostheses mentioned functioned satisfactorily.

The ends of the tubes united well with the tissues they were attached to, and the lumina remained patent. In the case of the major blood vessels, thrombus formation on the wall of the prosthesis does not seem to have taken place. However, there seems to be no follow-up of these experiments.

1.4.3 SUTURES

Singh and Melrose (1971) inserted two rows of continuous sutures in the right atrial wall of an experimental animal. The sutures of one row consisted of Hydron-coated terylene while the sutures of the other row consisted of terylene (the sutures most commonly employed during cardiac surgery). The control sutures caused a thick fibrin deposition on the affected heart surface and thrombus formation. Numerous giant cells indicated a marked foreign body reaction to the terylene. The Hydron-coated sutures were covered with a minimal layer of fibrin and there was no foreign body reaction to the material or thrombus formation. Calcium deposits were not seen around any of the sutures.

The Hydron was well tolerated by the heart muscle tissues during the short time of this experiment. However, the two week period that the sutures were in place, would seem too brief a time to draw any conclusions concerning biocompatibility.

1.4.4 JOINT PROSTHESES

In a series of research publications, Taylor et al (1971), and Murray and Dow (1975) altered the formulation of the Hydron to give it the consistency of articular cartilage. The articular surface of the distal femur was exposed, and a defect cut through the cartilage in the direction of the long axis of the femur. Into this defect was inserted a rod of Hydron. The end of the rod was so shaped that the articular surface of the distal femur was covered by a layer of Hydron. The purpose of this particular implant was to try to achieve a self-locking implant which would cover any deficiency in the articular cartilage.

As well as investigating the replacement of articular cartilage with poly-HEMA, this group also investigated the bone-implant interface. Histologically, the implant was well accepted by the bone. Healing resulted in bone either directly in apposition with the implant or separated from it by a very thin fibrous capsule. Electron micrographs were interpreted to indicate that the layer of collagen adjacent to the implant was ossifying. Murray and Dow (1975) concluded from this that the capsule would eventually ossify. This last conclusion would appear to be supposition and no longer term studies have been reported, to either prove or disprove it. This was a difficult area in which to assess the histological response of bone to an implant (see Section 2.4.3 where this technique was reviewed for investigating biocompatibility in bone).

In another experiment, Murray et al (1978) investigated the abrasion of Hydron caused by the opposing articular cartilage. They concluded that in the form in which they used Hydron, it was not sufficiently tough for this application. Kon and de Visser (1981) investigated a number of poly-HEMA sponges as articular cartilage replacement materials. They concluded that sponges with pore sizes from 50-100 μm were too weak to serve as a weight bearing material. However, sponges with a pore size up to 50 μm, when replacing articular cartilage, were covered with either fibrous tissue, fibro-cartilage or with hyaline cartilage. The last occurrence was rarely observed. They concluded that Hydron with a pore size of up to 50 μm showed promise as a replacement for articular cartilage and should be further investigated.

1.4.5 PLASTIC SURGERY

Hydron sponge with a pore size of 40-80 μ has been used for breast augmentation (Kliment et al, 1975). The implant was reinforced at the base and lower edges by a polyester mesh to facilitate suturing. Kliment reported 55 cases over five years with excellent results: no reaction in the surrounding tissues even after long time intervals, and no fibrous scar formation. However, Winter (1970) reported radiopaque shadows in some cases eighteen months to four years post-operatively. Possibly calcification may take place in such implants, but as yet biopsy material has not become available. Calcifications would tend to make this material unsuitable for this use.

1.4.6 BURN DRESSINGS

Following research with experimental animals (Nathan et al, 1974), Hydron has been used in a clinical trial by Warren and Snelling (1980) as a burn dressing. After debridement of the wound, a layer of polyethylene glycol (poly-HEMA solvent) was sprayed onto the burn. Then poly-HEMA powder was sprayed onto the wound. When the polyethylene glycol evaporated, one complete layer of Hydron resulted. Three to eight complete layers were applied over the wound. The complete dressing needed to be removed and re-applied every three days until healing was completed.

Warren and Snelling considered the procedure time-consuming and tedious. The dressing integrity was frequently broken by cracking, peeling or ineffectual drying. Although wound colonization did occur beneath the Hydron dressing, this phenomenon did not occur more frequently than beneath other commonly used burn dressings. In its present form, Warren and Snelling considered that Hydron did not produce results superior to other regimes for hospitalized burn patients.

Brown (1981), when treating burns, applied Hydron already dissolved in polyethylene glycol. He thought Hydron proved a valuable asset in treating limited burns, because of its ease of application, its transparency (enabling visualization of the wound), its flexibility and the ease with which it could be removed.

1.4.7 OTHERS

A number of uses have been proposed, for which it was thought Hydron could be suitable. These included:

filling voids created by lung lobotomies;

cosmetic repair in loss-type injuries, especially those involving the face and other external parts;

as sponges for soaking up blood during surgery;

dressing for haemostasis (especially of parenchymatous organs during surgery) (Barvic et al, 1967);

as a membrane for cell chromatography (Pristovpil et al, 1972);

prostheses for the stapes in tympanoplastic operations (Hubacek and Tesarik, 1964).

Levowitz et al (1968) suggested a number of the above uses with antibiotic incorporated in the polymer. They also suggested using the material as the vehicle for such drugs.

1.5 DENTAL RESEARCH AND APPLICATIONS

1.5.1 RESEARCH BY GOLDMAN

All of the early research concerned with Hydron as a root canal filling material, has emanated from Tufts University, Boston. The research team was headed by Melvin Goldman.

In the first published results of experiments using Hydron as a root canal filling material (Rising et al, 1975), the incisors of monkeys were root filled with either Silastic 382, Silastic Medical Adhesive Type A, or Hydron. Control canals were sealed with gutta-percha and Kerr Pulp Canal Sealer. Both vital and infected non-vital teeth with periapical radio-lucencies were treated. The preparation of the canals followed normal endodontic procedures. The teeth and periapical regions were evaluated histologically, approximately six months and nine months following treatment.

Although inflammation was noted around the excess material (forced into the periapical tissues) in all cases, it was suggested that there was less inflammation associated with the Hydron. Rising claimed that:

(a) A lack of persisting periapical inflammation was an indication that healing was progressing.

(b) The Hydron was better adapted to the canal walls than the other materials.

If the material was to be considered a success, after nine months, substantial osseous repair would be expected in the case of the non-vital teeth with periapical pathology. A lack of inflammation, without associated repair, could not be construed as healing. The use of canals obturated with Gutta-percha and Kerr Pulp Canal Sealer as the control, was not a good choice. Spångberg (1969d) showed Kerr Pulp Sealer to be

one of the least biocompatible endodontic materials of the group he studied. No mention was made of the control teeth when discussing the results.

Benkel et al (1976) endodontically treated a similar group of monkeys' incisors. Both vital and non-vital teeth were included and the material remained *in situ* for four months and eleven months (approximately). Benkel claimed biocompatibility in all cases except three. These last three results were thought to be due to lack of adequate debridement of the canal. Fibroblasts were said to be invading the extruded Hydron. The material seemed to be well adapted to the walls of the canal and in some instances it was claimed that it had penetrated the dentinal tubules. The Hydron in some specimens, eleven months post-insertion, was said to be calcifying.

Small granules were noted in the cytoplasm of some histiocytes (and of some other cells) in the results of both Rising's and Benkel's research. To investigate these particles, Kronman et al (1977) re-examined some of Benkel's histological material using different techniques. The tissues examined contained extruded Hydron that had been *in situ* for eleven months. Following the location of an area of histiocytes containing the above mentioned granules, the section was examined with a scanning electron microscope. Kronman claimed that the particles were barium sulphate after comparing the S.E.M. images of Hydron powder (said to contain 99.5% barium

sulphate). X-ray spectra of the area showed the presence of calcium and barium. A high concentration of calcium occurred adjacent to histiocytes containing phagocytosed granules. While the particles observed in the histiocytes were the same general shape, their size and appearance seemed to be different. Kronman explained this difference (which he said was in size only) by postulating a possible coating action from a calcific process. However, this was not substantiated. From the elemental distributions illustrated, barium sulphate was present within the phagocytosing histiocytes. That the phagocytosed material was barium sulphate coated with poly-HEMA (which is essentially what dental Hydron consists of) was not investigated.

A clinical trial employing Hydron as a root canal filling material, was reported by Goldman et al (1978). The longest recall period was twelve months. Goldman claimed there were no failures following the treatment of thirty-two teeth with Hydron and that the material was biocompatible, non-toxic and caused no adverse reactions. He also claimed that the material was easily and quickly introduced into the canal(s), and was subsequently easily removed from the canals for post crown preparations. Goldman et al (1979) discussed the techniques for mixing and delivering Hydron root canal filling material.

1.5.2 OTHER RESEARCH

The results of investigations by other researchers would indicate that Hydron root canal filling material was less ideal than claimed by Goldman.

Langeland et al (1981) carried out an extensive investigation into Hydron root canal filling material. The physical aspects of the material were investigated as well as its biocompatibility. The biocompatibility of the material was evaluated in implantation tests (subcutaneous tissue and bone) as well as in a usage test.

The physical aspects were investigated by bench curing the material under a number of differing conditions. When curing took place in normal room conditions, considerable heat was produced by the setting reaction. This Hydron, when set, was very hard. When exposed to water during setting, the Hydron either did not cure or if it did cure, did so with "uncontrolled expansion".

Observations made following implantation in subcutaneous tissue for periods up to sixteen months post-implantation, were characterized by moderate to severe inflammation. Langeland reported the presence of "neutrophilic leukocytes" in the tissues adjacent to the Hydron for the longest implantation period and the presence of Hydron in "vessels at all observation periods".

Observations made following intraosseous implantation of the Hydron, for periods up to six months post-implantation, revealed features similar to those observed following subcutaneous implantation of the material. Inflammation was noted not only in the tissues adjacent to the implanted Hydron but also in the "marrow spaces and interstitial areas further away". Transported Hydron was noted in the same areas where inflammatory cells were present.

Following endodontic treatment of baboon teeth, microscopic observations of the teeth and adjacent periapical tissues, up to six months after treatment, revealed the presence of Hydron in "the vessels of the remaining pulp tissue at the level of the foramen, in vessels in the periapical tissue and in foreign body cells in the periapical marrow spaces". This was despite the fact that radiographs of involved teeth did not indicate the presence of Hydron in any periapical tissues.

Langeland concluded that "Hydron was transported in vessels, in macrophages and in foreign body cells from the area of placement. It caused inflammatory reactions in all tissues into which it was implanted". The type of "vessels" in which the Hydron was allegedly transported were not defined and there were considerable differences in the times for which the Hydron was implanted in the various tissues. These findings had been earlier reported in brief by Langeland and Olsson (1980).

Tanzilli et al (1981) compared monkeys' teeth root filled with Hydron, to similar teeth root filled with gutta-percha and Kerr's Tubli-seal. Maxillary and mandibular incisors were employed in this study. These teeth were either overinstrumented and overfilled or underinstrumented and underfilled with either of the above materials respectively. Following normal canal debridement, enlargement and obturation, the materials were allowed to remain *in situ* for six months post-operatively. The teeth and adjacent tissues were subsequently examined both radiographically and histologically.

A severe inflammatory response was observed in the tissues adjacent to canals that had been overinstrumented and overfilled with Hydron. The predominant cell type of this response was the macrophage although lymphocytes were also present. Phagocytosed Hydron was noted in the macrophages both within the canal and also in the adjacent periapical and periodontal tissues. Granulation tissue was observed in the apical 2-3 mm of the canal. Incomplete calcifications were present in the apical foramen region of several canals and this tissue resembled cementum. Minor resorptions were present on the lateral and apical root surfaces.

Following overinstrumentation and overfilling of the canal with gutta-percha, a severe inflammatory reaction consisting predominantly of lymphocytes resulted. There was little or no resorption of the root filling materials nor was there any

ingrowth of granulation tissue into the canal. There was no calcification of the apical region of the canal. Root resorptions were present but confined to the apical region.

When the incisors were underinstrumented and underfilled with Hydron, a mild inflammatory reaction resulted in the periapical tissues. Macrophages again were the predominant cell type. Some calcification of the apical foramen occurred in all specimens. Hydron was noted within this calcified tissue as well as in the periapical and periodontal tissues. However, the amount of phagocytosed Hydron appeared to be less than that observed following overfilling with Hydron. Some slight root resorptions were noted.

The tissues adjacent to underinstrumented and underfilled canals obturated with gutta-percha and sealer, exhibited little or no inflammation. Calcification of the apical canal occurred in all specimens and root resorption was not observed.

These authors concluded that the Hydron acted as a matrix for calcification. The phagocytosed particles observed in the tissues appeared the same as particles of Hydron in the root canal. It therefore seemed unlikely that the phagocytosed material was barium sulphate dissociated from the polymer (compare with Kronmann et al, 1977). Hydron appeared to be a less than ideal root filling material because of the difficulty

experienced manipulating the material, the resorption of the material that occurred and the inflammatory response that followed its introduction into the canal.

Rhome et al (1981) compared the sealing ability of laterally condensed gutta-percha, vertically condensed gutta-percha and Hydron. Sixty-three extracted maxillary human teeth were employed in this study. These authors measured the amount of leakage of radioactively labelled human serum albumin sealed in the middle third of the endodontically treated teeth. Those obturated with Hydron showed a 30-40% leakage of the labelled albumin from the root canal during the six months of this investigation. This compared with a 10-17% leakage from laterally condensed canals and 10-14% leakage from vertically condensed canals.

Statistically there were no significant differences between the amounts of leakage that occurred from laterally condensed or vertically condensed canals. However, there were significant differences between the amounts of leakage that occurred following both methods of gutta-percha condensation and obturation with Hydron. The authors concluded significantly greater leakage occurred following obturation with Hydron than occurred following obturation with either vertically or laterally condensed gutta-percha.

Linden et al (1980) compared AH26, Cavit, Hydron, N2, Pulpdent and Zinc Oxide-Eugenol used as root canal filling materials. These researchers employed an in use test, the root canals being overinstrumented and overfilled. The assessment was based on a histological examination of the periapical tissues in contact with the various endodontic sealers for up to two months. Hydron and N2 were found to be the least biocompatible of the materials examined.

Mandor (1981) described a case of internal resorption that he treated endodontically with Hydron root canal filling material. Radiographically, the material did not appear to fill the defect completely. However, twenty-one months after obturation, the tooth remained asymptomatic and functional.

A number of articles (Clinical Research Associates, 1979; Kessler, 1980) concerning the clinical use of Hydron as a root canal filling material, have been published. These appeared to be a resume of Goldman's research without the addition of any original material.

Goldman and Langeland have confronted each other in the Letters to the Editor section of the Journal of Endodontics (Vol. 7, no. 10, 1981). While points made by both appear to be valid, there was little clarification as to why such fundamental differences as inflammation and foreign body reactions should occur following relatively similar experimental procedures.

CHAPTER 2.

BIOCOMPATIBILITY TESTS

with special reference to endodontic materials.

2.1 INTRODUCTION

A material implanted into living tissues will have an effect upon those tissues and perhaps the entire organism. This is of special significance with dental materials since the contact is usually of long duration. In dentistry, the compatibility of an endodontic material is of special significance since it is in direct contact with vital tissue. Since the aim in much endodontic therapy is to achieve or aid "healing" of pulpal or periapical tissues it is axiomatic, therefore, that the question of "biocompatibility" of the different endodontic materials available is fully understood.

2.2 DEFINITIONS

Biomaterials have been defined as: *"Systemically, pharmacologically inert substances designed for implantation within or incorporation with living systems"* (Clemson Advisory Board for Biomaterials, 1974). Biomaterials have also been defined as: *"Implants replacing biological materials"* (Park, 1979). These definitions exclude such substances as grafts and entities such as prostheses which remain outside an intact epithelium. Biocompatibility is the study of the reactions between the host and a biomaterial.

2.3 THE OBJECTIVES OF BIOCOMPATIBILITY TESTING

Many materials have been used in medicine and dentistry in such a way that they contact living tissues and as such can be defined as biomaterials. The properties of an ideal biomaterial are poorly understood except that some hold that an ideal biomaterial should ultimately be replaced entirely by tissue identical to that it originally replaced (Park, 1979). An ideal biomaterial would also need to be broken down and excreted from the body entirely, without causing damage.

Many materials have been used as biologic implants in the past and have failed, to some degree, to fulfil the criteria of an ideal material. The properties of materials that cause undesirable sequelae have been studied and, in fact, most tests are directed towards establishing the presence or absence of these undesirable properties. Biocompatibility testing, as presently practised, is not necessarily directed towards establishing desirable properties of a material, but rather towards establishing the absence of undesirable properties. The further a material deviates from being ideal, the easier it is to assess its undesirability. Therefore, it is logical to first test whether the most undesirable properties are present or not. The final test, however, must always be how the material reacts when used clinically. This notwithstanding, the better the testing methods employed before a material is used clinically, the less likely it is that an adverse reaction will be encountered following its use.

The aim of any test of biocompatibility is to be able, as much as possible, to predict how the tissues will react when the particular test material is placed in contact with them. To achieve this a number of questions need to be asked:

(1) Is the material toxic; does it release any agents into the tissues which are injurious to them?

This first question is fundamental, and requires further sub-division into:

(a) Is the material so toxic that it will be damaging to the whole organism?

(b) What are the effects of diffusion products on local tissues?

(c) What effect does the whole material have on the tissue with which it is likely to come in contact?

(2) Is the material a sensitizing agent leading to an allergenic response?

(3) Is the material mutagenic?

(4) Is the material stable in a biological environment?

(Autian, 1975)

It is only after questions 1-3 have been adequately answered that question 4 can be investigated. This may be achieved with "usage" tests and then clinical trials.

It is not considered necessary in establishing biocompatibility, that a material be subjected to all the tests that exist (Federation Dentaire Internationale, 1979). However, a series of tests should be chosen, from which it is hoped significant answers can be obtained for the questions posed. By so testing a material, much more satisfactory information can be obtained than by using the "human guinea pig" method whereby the material is first used clinically and only tested if some untoward effects are noticed. Unfortunately, this has all too often been the method employed in the past.

2.4 TESTS FOR BIOCOMPATIBILITY

There exist a large number of tests that can be used to establish the biocompatibility of any particular material. The A.D.A. (American Dental Association, 1979) and the F.D.I. (Federation Dentaire Internationale, 1979) have recently published documents which have attempted (at least in the field of dentistry) to standardize this very difficult field. Most of the tests listed in these documents are mentioned in the following section of this review.

There are numerous ways in which the various biocompatibility tests can be grouped. For the purpose of this review I have chosen to group them as follows:

1. *Tests for Gross Toxicity*
2. *Tests for Cytotoxicity*
3. *Tests for Tissue Response*
4. *Tests for Mutagenic Response*
5. *Usage Tests in Animals*
6. *Clinical Trials*

(Table 2.1)

1. *TESTS FOR GROSS TOXICITY*	2. *TESTS FOR CYTOTOXICITY*	3. *TESTS FOR TISSUE RESPONSE*	4. *TESTS FOR MUTAGENIC RESPONSE*
(i) L.D.$_{50}$ Tests *(a) Oral route* *(b) Intraperitoneal route* (ii) Acute Inhalation Tests	(i) Tissue Culture Tests *(a) Direct counting methods* *(b) Direct seeding methods* *(c) Millipore filter methods* *(d) Cell respiration methods* (ii) Other Methods *(a) Haemolysis* *(b) Muscle cell response* *(c) Endothelial cell response*	(i) Implantation Techniques *(a) Soft tissue* *(b) Intraosseous* (ii) Allergenicity Tests *(a) Guinea pig maximization test* *(b) Other methods*	(i) Ames Mutagenicity Test (ii) Styles Cell Transformation Test (iii) Dominant Lethal Test (iv) *In Vivo* Tests (v) Miscellaneous Methods

TABLE 2.1:

In any programme for testing the biocompatibility of a particular material, a test or tests from each of the four groups indicated in Table 2.1 would be carried out initially. Only after these tests have been concluded should *usage tests in animals* or *clinical trials* be entertained. The last two tests are entirely dependent on the final use envisaged for the material. Therefore, each material would virtually need an individually designed trial to test its unique properties. Since a discussion of such tests is beyond the scope of this review, no further mention will be made of *usage tests in animals* and *clinical trials*. They have been mentioned here only for completeness.

2.4.1 TESTS FOR GROSS TOXICITY

(i) L.D.$_{50}$ Tests

These are screening tests to preclude the possibility of gross adverse reactions to the material. If possible the material should be introduced via the route by which the body is most likely to come in contact with it.

(a) Oral route

Liquid, or pulverized solid in a suspension of a suitable medium, is administered by stomach tube to the test animals. These are observed daily (for at least two weeks) for death or toxic effects. When a material is impossible to emulsify, a solid dose is introduced directly into the oesophagus when the animal is lightly anaesthetized. It is considered that if the L.D.$_{50}$ dose is 1.0 mgm/kg or greater, the material is suitable for further testing (Weil, 1952).

(b) Intraperitoneal route

The test material, in the case of liquids, is injected into the peritoneal cavity of laboratory animals. Solids are first emulsified before being injected. Following this procedure the animals are observed for seven days for death or toxic effects (Weil, 1952).

(ii) Acute Inhalation Test

This is a screening test to preclude the possibility of gross adverse reactions consequent to inhalation.

The experimental animals are subjected to an atmosphere containing the test material for six hours daily, five days per week for a selected number of weeks. Haematological analyses and urinalysis are performed weekly and the test is assessed on the basis of these results and $L.D._{50}$ calculations. If the test material is not volatile, it can be atomized and the "dusty" atmosphere introduced to the test chamber (Gage, 1953 and 1959).

These tests have been used to advantage in identifying potentially hazardous materials in the industrial environment (Gage, 1959). Tests for gross toxicity would appear to be of little value when screening dental materials for biocompatibility.

2.4.2 TESTS FOR CYTOTOXICITY

This group of tests basically measures the effect or non-effect any test material has on cells *in vitro*. The results are never absolute and must be expressed relative to known toxic and non-toxic materials (controls). Cytotoxicity tests are most helpful in developing a toxicity profile of an untried material

Cytotoxicity testing methods measure the effects of diffusable "toxic" products from a given test material. If the test material can be in intimate contact, without actual physical contact, the effect the material has on the adjacent cells is dependent only on the "toxicity" of the material (Wennberg, 1978).

One advantage of using cell cultures is that human cells are readily available and thus species specificity is eliminated.

A number of workers first applied cell culture techniques to dental materials in 1955 (Grand, 1955; Kawahara et al, 1955). A wide variety of cell types have subsequently been used in conjunction with these methodologies. Examples include:

Normal human cells (Wilsnack, 1976);

Malignant human cells (Spångberg, 1969a; Wennberg et al, 1979; Imai, 1979);

Monkey kidney cells (Metcalfe, 1971);

Mouse fibroblasts (Wennberg, 1976);

Foetal chicken cells (Cruischank et al, 1960; Kawahara et al, 1955).

The scope of this field makes it difficult to detail all the methodologies that have been used. Consequently, only a small number of representative techniques are described in this review.

For the purpose of this section of the review various cytotoxicity tests can be divided into two broad groups:

(i) those methods using tissue culturing techniques;

(ii) those methods using other than tissue culturing techniques (refer Table 2.1).

(i) <u>Methods Using Tissue Culturing Techniques</u>

A monolayer of cells is established in a suitable culture vessel containing an appropriate culture medium. A standard sized specimen (or a standard amount in suspension) of the test material is added to the culture medium, and this mixture incubated for a specific time. Diffusion of toxic materials from the test material into the medium should affect the cells. Measurement of a variety of cellular parameters forms the basis of assessment in these techniques.

Irrespective of the cell type used in these techniques, results with the same material appear to be similar (Spångberg, 1969a). However, some cells are easier to cultivate *in vitro* and some seem to be more sensitive for the purpose of such tests (Spångberg, 1969a). For these reasons, the usual cells used have been malignant human epithelial cells from the cervix (He La cells) or mouse fibroblasts (L-cells).

(a) Direct cell number count

Counting the number of cells or the number of mitotic figures in a representative microscopic field after exposure to the test material, is employed as the assessment method for this technique. Spångberg (1969b) used this technique to rank the toxicity of the following products, at that time commonly employed in endodontics: silver points, gutta percha, gutta percha germicidal, silver amalgam, chloropercha, calcium hydroxide paste, AH26, tubli-seal, diaket, N_2 and Riebler's paste. This particular methodology requires that the test material be introduced into the system as a liquid. However, when the material under investigation is a solid, toxins can be introduced after dissolution in a suitable solvent.

Counting the number of cells is a time consuming and tedious procedure. Numerous methods have been proposed to evaluate results from this type of technique without having to physically count the cells. A number of these techniques are outlined below.

Agar Overlay Method

This method was used by Guess et al (1965) to assess the toxicity of samples of 100 plastic biomaterials. After establishing a cell monolayer, an agar overlay (Eagle's medium + 1% agar + 1% calf serum) is poured over the cells and allowed to solidify. The cells are stained with neutral

red (a vital dye) and the test material then pressed into the agar overlay. Similar pieces of known toxic and non-toxic materials in the same overlay act as controls. After incubation, assessment of cytotoxicity is based on the extent of a relatively clear zone of non-vital cells around the test material, compared with that found around the controls.

Chromium Release Method

This method was employed by Spångberg (1973) to establish the relative toxicity of a number of standard phenol solutions. Sodium chromate is non-covalently bound to cell proteins and other cell constituents. It is reduced during binding. Material released following lysis of a cell, therefore, cannot be re-bound to other cells.

In this methodology a cell monolayer is established and then labelled with $Na_2^{51}CrO_4$. These labelled cells are incubated in the presence of the test material. Following incubation, some fluid is drawn from the test chamber, centrifuged and the resultant supernatant examined for radioactivity in a gamma-counter.

The amount of chromate released into the cell medium should be a good measure of the number of cells destroyed by the toxins diffusing from the test material. In fact about 70% to 95% of the radioactivity is released into solution from affected cells. However, because of the minimum handling of these cells, the method is sensitive and accurate (Wennberg, 1978).

Spångberg and Langeland (1973) used the chromium release method to evaluate the relative toxicity of a number of endodontic materials. These included a number of Gutta-percha points and Chlorapercha. The endodontic sealers included Tubli-Seal, AH26, N_2, Proco-Sol, Rickert's Pulp Canal Sealer, Rickert's Modified Pulp Canal Sealer, resin chloroform, Kerr experimental root canal cement. All materials tested were highly toxic when freshly prepared, although the Kerr experimental root canal cement was less toxic than the other experimental materials. Chlorapercha, after chloroform evaporation, had the lowest toxic effect of all set materials.

Thymidine Uptake Method

This method was employed by Wennberg (1976) to establish the relative toxicity of a number of standard phenol solutions. When cells were expected to recover following exposure to the test material, this method was particularly applicable (Wennberg, 1978).

An established monolayer is incubated in the presence of the test material. This material is then removed and the remaining cells further incubated with labelled thymidine. Unused thymidine is removed and the DNA of the remaining cells hydrolyzed. The resulting radioactivity is then measured. The effect the test material has on the cells is compared with controls.

Wennberg (1980) used this method to evaluate the relative toxicity of the following root canal antiseptics: Jodopax 0.04%, Biosept 0.1%, Hibitane 0.1%, Chloramine 5% and Sodium Hypochlorite 0.5%. No differences in the toxicity or the tissue irritating effect of all the above materials, with the exception of Chloramine 5%, were observed. There was good correlation overall between the results of this method and those obtained *in vivo*, with the same test

However, Mjör (1980) noted that the correlation between the results obtained by the thymidine uptake method and *in vivo* results was not convincing when the antiseptic solutions were investigated at concentrations similar to those used clinically.

The above methods differ only in that they use different properties of cells to measure how many cells have been affected by the test material and the control(s). In the studies described above there was an almost linear relationship between the parameter measured and the number of cells affected.

(b) Direct seeding

In this direct seeding method, the cells are seeded directly onto films of the test material and suitably incubated. This method only applies to materials capable of being preformed into suitable test films. Therefore, this method is virtually restricted to polymers. After

fixation, the number of cells attached to the particular film is determined by measuring the amount of dye (crystal violet) these cells absorb. This is effected by extracting the dye and measuring it spectrophotometrically. To confirm the accuracy of the above method, it was compared with a direct cell counting method. There was good correlation (Imai et al, 1979).

Unlike most tissue-culture methods the direct seeding method uses fixed cells. This is seen to have a number of advantages in that assessments can be made at the operator's convenience, the assessment method can be repeated as many times as needed, and should a different staining method be required, the previous stain can be removed and the cells re-stained.

According to Imai, the use of crystal violet uptake as a measure of the number of attached cells present does not take into account any morphological alterations in the cells - that is, altered cells will absorb approximately the same amount of dye as a similar number of "normal" cells. Imai et al employed either pyronin G and methyl green or azur II and eosin (Giemsas) to investigate this aspect of their results.

(c) Millipore Filter Method

A cell monolayer is established on a millipore filter, the filter inverted and then the test material placed in contact with this side of the filter. The advantage of the method is that the test material establishes an intimate relationship with the cells without actual contact. In this way, the milieu of the cells adjacent to the test material is affected only by the "toxins" from the material and not by its physical presence. Following incubation for a suitable time, the cells are stained for the presence of succinate dehydrogenase activity (an indicator of cell respiration). Measuring the area of cells around the test material and controls that show reduced (or no) metabolic activity is the basis of the assessment (Wennberg et al, 1979).

Using this method, Wennberg investigated the toxicity of cold-curing methyl methacrylate, zinc phosphate cement and silicate cement (to illustrate the methodology). Care is needed in choosing the filter, as some millipore filters have been manufactured from material which is itself cytotoxic (Weibkin, 1980).

(d) Measurement of Cell Respiration

Cell respiration is considered a particularly sensitive indicator of normal cell function. It is thought to be the first system of cells affected by a material having an adverse effect on those cells (De Roberts et al, 1975).

In tests employing this method, cells in culture are incubated in a closed vessel (such as a Warburg apparatus) and the rate of utilization of oxygen measured. The test material (liquid or solid in suspension) is added and the rate of oxygen utilization measured. Assessment consists of comparing the oxygen utilization rates before and after the addition of each particular material tested.

Spångberg (1969c), a pioneer in the field of biocompatibility testing of endodontic materials, used this technique to rank a number of commonly used endodontic materials. The materials investigated were: silver, zinc phosphate cement, Gutta percha, Gutta percha Germicidal, chloropercha (Nygaard-Østby), calcium hydroxide, AH26, Tubli-Seal, N_2, Riebler's paste and Diaket. All materials affected cell respiration. The weakest affect was produced by silver and zinc phosphate cement. Diaket caused the greatest affect, followed by chloropercha, N_2, Tubli-Seal, Riebler's paste, AH26, Gutta percha Germicidal, calcium hydroxide and Gutta percha.

A number of other methodologies have also been used to measure cell respiration. These include lactate production and glucose utilization (Helgeland and Leirskar, 1972) and the rate of production of labelled carbon dioxide production from labelled succinic acid (Vale et al, 1980). While the methodology of these experiments differs considerably, each compares the metabolic activity of a cell population before and after the introduction of the test material.

(ii) Methods Using Other Than Tissue Culturing Techniques

(a) *Haemolysis*

This technique measures the adverse effects toxins from potential biomaterials have on red blood cells,by utilizing the unique properties of erythrocytes. Haemolysis testing is considered a particularly appropriate test when a material is expected to come into contact with blood. However, it is also considered to be a good test of toxicity in general (Federation Dentaire Internationale, 1979).

In this method,the test material is incubated in contact with whole blood (usually rabbit). Should any red blood cells be lysed, the blood pigments are released into solution. After removing unaffected erythrocytes by centrifugation, the concentration of haemoglobin in the resulting solution is measured spectrophotometrically. This is compared with a solution from similar blood similarly incubated with a control (Autian, 1977).

Dillingham et al (1975) used haemolysis to evaluate the cytotoxicity of polymethyl methacrylate.

(b) *Muscle cell response*

This method allows the test material to come into contact with living tissue, without the complication of surgical trauma.

In this method, first described by Wennberg et al (1979) non-epithelized indentations are created in the thigh muscle of a rabbit. Into these indentations are placed the test material and control materials respectively. Following contact between these materials and the muscle for a specific time, the blocks of muscle containing the materials are dissected out and 10-20μ sections prepared. These are stained for the demonstration of succinate dehydrogenase activity. This method was used by Wennberg to rank the toxicity of silicate cement, zinc phosphate cement and a 4% solution of phenol. The results using these materials were compared with those obtained when the indentations were filled with physiological saline. Freshly prepared silicate cement produced the greatest effect.

The cells around the created indentations act as cells of a tissue culture. However, they remain part of the functioning animal. As well as acting as a test of cytotoxicity, this methodology allows implantation, where the effects of the surgical operation are eliminated.

(c) *Endothelial cell response*

This test measures the effect materials have on endothelial cells *in vivo*.

The experimental animal is injected intradermally with the test material (with solids, a suspension in Ringers solution is used). After a specific time, a dye (Evans blue)

is administered intravenously. The Evans blue is bound to plasma albumins which are not normally permeable to the endothelial lining of blood vessels. However, once an inflammatory reaction is initiated, the permeability of certain blood vessels (mainly post capillary venules) is increased. Plasma proteins with bound dye then leak into the tissues. The greater the amount of dye in the tissue, the greater the amount of plasma protein that leaks out and this is in proportion to the intensity of the inflammatory reaction. The amount of dye in the tissue is estimated by punching out a standard sized piece of dyed tissue, extracting the dye and quantitating it spectrophotometrically (Udaka et al, 1970).

Rutberg et al (1977) used this method to rank a number of solutions commonly used in endodontics. The solutions investigated were EDTAC (according to Nygaard-Østby), 0.1% Zephiran, 0.04% Iodopax, 1% sodium hypochlorite, 5% sodium hypochlorite, 3% hydrogen peroxide and an experimental antimicrobial agent. The 5% sodium hypochlorite caused the most extensive damage.

The method outlined is based on experiments initially performed last century by Arnold (1875) using cinnabar.

Since the introduction of tests of cytotoxicity, they have been widely used in the screening phase of test programmes recommended for the biological evaluation of dental materials (Wennberg, 1978). However, more standardization of the methodology is required, since by altering the medium volume to specimen size ratio, almost any effect from no toxicity to extreme cytotoxicity has been demonstrated (Hensten-Pettersen and Helgeland, 1977).

Care also needs to be taken when interpreting results. This was evident following a comparison of results from a number of experiments conducted by Spångberg in 1969. In these experiments ten endodontic materials were ranked according to their toxicity using a number of methods. The toxicity of calcium hydroxide, when ranked following a direct cell counting method, was more toxic than six of the other materials. However, when ranked according to toxicity as measured by the material's effect on cell respiration, calcium hydroxide was more toxic than only two other materials. Spångberg concluded that this difference was produced by the calcium hydroxide impairing the cell's ability to adhere to the glass of the culture vessel.

Deficiencies are also apparent in other methods. For example, when using the agar overlay method, the material is pressed into the overlay. The material's physical presence will alter the respiration of the cells directly beneath it. As well as this, some toxins are bound to the agar, further distorting the result (Wennberg, 1978). Calcium hydroxide containing materials bind with the labelled unreduced chromate, preventing its uptake by cells in the chromate release method. This method is therefore unsuitable for measuring the effect such materials have on cells (Wennberg, 1978).

Tests of cytotoxicity are not always indicative of clinical behaviour. According to the results of cell culture investigations, zinc oxide-eugenol is markedly cytotoxic, while silicate cement has a slight cytotoxic effect (Hensten-Pettersen and Helgeland, 1977). The reverse applies to their clinical behaviour (Seltzer and Bender, 1975).

The results obtained from cytotoxicity tests are not absolute. This is because of difficulties related to standardization of test samples and because of the inability of the operator to precisely define what is an acceptable result and what is an unacceptable result with respect to the material being tested. The best that can be achieved is a relative assessment of materials. Thus it is extremely important in cytotoxicity tests to choose a well tried

material of known toxicity with which to compare the test material. The problem of test material concentration was noted by Holland (1978) while using the chromium release method. Holland found that cadmium at concentrations of around 50 m mol/litre was non-toxic. At higher and lower concentrations, it was toxic. This surprising result was explained as being due to protein precipitation, including the ^{51}Cr fraction or some stabilization of the cell membrane at this concentration of cadmium.

Tissue respiration tests are probably the most sensitive and meaningful of the tests listed in Section 2.4. However, they require a particularly exacting technique and a great deal of very specialized equipment. Cell counting techniques are relatively easy to perform and are quick and cheap. They can be repeated easily to eliminate once only results.

Documents of biological testing such as those produced by the American Dental Association (1979) and the Federation Dentaire Internationale (1979) justify the use of many of the above mentioned tests because they are the methodologies used for testing pharmacological compounds. Pharmacologists, however, question the relevance of many such tests (Mjör, 1980).

2.4.3 TESTS OF TISSUE RESPONSE

How any particular tissue of a living animal reacts when a biomaterial comes into contact with it is of obvious importance when testing the biocompatibility of that material. Cytotoxicity tests are concerned with the toxicity of the material *in vitro*. When the material is tested *in vivo*, responses such as inflammation and immune responses may be activated as well as single cell responses such as altered respiration or degeneration-necrosis. Tests of tissue response logically follow cytotoxicity tests.

(i) Implantation Techniques

Implantation studies have utilized techniques which can be broadly divided into those where soft tissue sites have been employed and those employing intraosseous implantation sites.

(a) Soft tissue implantation

Techniques

A variety of techniques involving the implantation of test materials into animal soft tissues have been documented. In general, three main groups of soft tissue implant sites have been employed:

Subcutaneous tissue (Dixon and Rickert, 1933; Browne and Friend, 1968; Langeland et al, 1969; Olsson et al, 1981);

Muscle (Laing et al, 1967; The United States Pharmacopeia, 1975; Gourlay et al, 1978);

The peritoneal cavity (Le Veen and Barberio, 1949; Lam et al, 1974).

The methods of "implanting" the various test materials into the different tissue sites have also varied and include:

Surgical implantation (Dixon and Rickert, 1933; Laing et al, 1967; Browne and Friend, 1968; Langeland et al, 1969; Gourlay et al, 1978; Olsson et al, 1981);

Via a trochar needle (The United States Pharmacopeia, 1975);

Employing free and attached peritoneal implants (Le Veen and Barberio, 1949; Lam et al, 1974).

The form in which test materials have been used in soft tissue implantation studies have also varied. Some investigators have used pre-set shaped, polished and sterilized materials (Dixon and Rickert, 1933; Laing et al, 1967). Most endodontically related studies have employed un-set material contained in an inert tube (Browne and Friend, 1968; Langeland et al, 1969; Olsson et al, 1981). Gourlay (1978) employed finely ground test material contained in a gelatin capsule. Investigators utilizing the peritoneal cavity either attached the test material to the peritoneal wall (Lam et al, 1974) or allowed the material to remain free in the peritoneal cavity (Le Veen and Barberio, 1949).

Methods of Assessment

The aim of any soft tissue implantation study is to gauge the extent and severity of responses following contact with the test material. Numerous criteria have been used to measure the extent and severity of the tissue reactions to implanted materials.

Most assessment protocols have relied on descriptive histology, from which various conclusions have been drawn. The aspects of the histology usually stressed have been the degree and type of inflammatory reaction present and the thickness of the capsular response (Le Veen and Barberio, 1949; Browne and Friend, 1968; Langeland et al, 1969; Lam et al, 1974; Olsson et al 1981). Some investigators have chosen only one of these parameters and based their assessment of biocompatibility on this one aspect only (Laing et al, 1967). These methods appear to be relatively subjective.

The presence of fat has been used to rank the biocompatibility of a large number of commonly implanted biomaterials. The materials investigated using this method of assessment included various stainless steel alloys, ceramics, an epoxy reinforced fibreglass and a number of plastics (Kaminski et al, 1977). As pointed out by Kaminski, the "*adipose fat cell is primarily a storage cell with few protective qualities; consequently, adipose fat cells would be more susceptible to the presence of toxic materials. Therefore, from*

a functional standpoint, the appearance of adipose fat tissue in the protective pseudo-membrane indicates a decreased need for protection. We believe that the early appearance of adipose tissue indicates lesser implant toxicity and thus biocompatibility." (Kaminski, et al, 1977). According to these investigators there appeared to be a good correlation between results using this method and those using "more conventional" parameters of biocompatibility.

Several researchers have attempted to devise a meaningful system by which assessments can be expressed in a more objective manner. Guttuso (1963) arbitrarily classified tissue reaction as mild, moderate and severe using indicators of tissue response such as density and type of inflammatory cells present, the thickness of the capsular response, the degree of vascularity and the "effect" the implant had on adjacent tissues.

Havgen and Mjör (1978) used a similar approach when assessing the biocompatibility of a number of periodontal dressing materials. The materials investigated were Coe-pak, Peri-pak and Ward's Wonderpak. This method is currently recommended by the Federation Dentaire Internationale (1979) when assessing soft tissue implantation studies. The Federation Dentaire Internationale recommends that a number be substituted for mild, moderate and severe for ease of presentation.

Stereologic quantitative analysis of the volumetric densities of cells adjacent to implanted tubes containing a variety of periodontal dressing materials was used by Marion et al (1980) to assess biocompatibility. Although there would appear to be a number of technical difficulties in applying stereological methods there was, according to Marion et al (1980), broad correlation between their results using stereologic methods and more commonly used assessment methods.

The most comprehensive (and least subjective) assessment method encountered when reviewing the literature was that of Gourlay et al (1978). The method employed by Gourlay et al was based on a method initially devised by Sewell et al (1955) to grade tissue responses to various suture materials. The method detailed by Gourlay et al (1978) involved the quantitation of six parameters. These were:

(1) degree of muscle cell damage;

(2) total thickness of the reaction;

(3) number of polymorphonuclear leukocytes and erythrocytes;

(4) number of eosinophils, lymphocytes and foreign body giant cells;

(5) number of fibroblasts and mononuclear phagocytes;

Summation of (3), (4) and (5) gave the final parameter;

(6) overall cell density.

The area assessed was a standard x400 field of tissue. Each of the first two indicators was assigned a grade (from 1 to 8). Next the numbers of the particular cell types indicated in (3), (4) and (5) were counted in the designated field. From this data a grade for parameters (3) to (6) was assigned. Each indicator was then weighted according to a scale and a standard tissue score calculated according to the formula:

$$STS = \frac{\text{T.S. (mean)} - \text{Lowest Observed T.S.}}{\text{Highest T.S} - \text{Lowest T.S.}} \times 100$$

The final outcome of this method is that a single number score can be derived for an individual test material. Although this system has the advantage that it is objective and quantitative, the model described by Gourlay et al does have a deficiency in that their weighting system is not rationalized.

(b) Intraosseous implantations

Techniques

Intraosseous implants in animals have been used to assess the response of bone in close proximity to, or in contact with a biomaterial.

A number of models for the study of intraosseous implants have been proposed. These models have employed a variety of sites:

The mandible - a defect cut into the mandible into which test material is introduced (Feldmann and Nyborg, 1962; Spångberg, 1969d; Hoover et al, 1980; Olsson et al, 1981);

The cranium - a groove cut in the cranium (Havgen and Mjör, 1978);

The tibia - a window cut through the cortex to expose the intermedullary tissue (Wenger et al, 1978);

The femur - a defect cut within the cortex (Friend and Browne, 1969; Lawrence et al, 1975);

The intermedullary tissue approached through the articular cartilage (Taylor et al, 1971).

The form in which the test materials have been introduced in intraosseous implant studies include:

Un-set material contained in a variety of inert vehicles. Spångberg (1969d) and Olsson et al (1981) employed teflon cups, while Friend and Browne (1969) and Wenger et al (1978) employed polyethylene tubes.

Un-set material completely filling the defect (Lawrence et al, 1975; Hoover et al, 1980; Havgen and Mjör, 1979) pre-set and pre-shaped material (Feldmann and Nyborg, 1962; Taylor et al, 1971).

Assessment Methods

The aim of any intraosseous implantation study is to gauge the extent and severity of any response of the tissues that might follow contact with the test material. In the models reviewed, the implant virtually completely filled the defect. Cortical bone, being composed mainly of inorganic salts, is virtually incapable of exhibiting any reaction except via the relatively sparse population of osteocytes and the small amounts of stromal tissue. Consequently, the area of necrotic osteocytes

adjacent to the implant was usually the sole observable response. Thus only degenerative aspects and some resorptive changes could be investigated and no account could be taken of inflammatory and reparative aspects of the reaction. In the context of an endodontic material, repair of the bony lesion would seem to be perhaps the most important aspect. Spångberg (1969d) attempted to assess the minimal quantity of healing clot between the implant and the bone.

The area assessed needs to be carefully chosen. Havgen and Mjör (1979), utilizing a groove cut in the cranial bones of the rat, assessed an area affected not only by the material in contact with the cut bone, but also the material contacting intact and damaged periosteum as well as the damaged periosteum itself. Taylor et al (1971) assessed the bone marrow reaction to a material inserted via the knee joint. The implant traversed the epiphyseal plate and thus was in an area of great flux. This made identification of those aspects of the reaction due solely to the implant, difficult. Further, synovial fluid could be carried into, or could percolate into the wound. The effect that this alkaline fluid, composed mainly of plasma proteins (predominantly albumim), lymphocytes and synovial cells (Jaffe, 1975) might have on subsequent healing, would be unknown.

The choice of controls is very important when relating the results between different materials implanted in bone. An unfilled lesion (Hoover et al, 1980; Lawrence et al 1975) would appear to be a poor choice. A replica of the implant of the test material, made of a known inert material, appears to be the only acceptable negative control.

(ii) Allergenicity Tests

Many materials, when introduced into the body are potential immunogens. Materials may either be complete antigens or haptens. Most endodontic materials are likely to act at least as haptens (Morse, 1980). A number of tests have been proposed to investigate the possibility of a biomaterial producing an allergenic response.

(a) Guinea pig maximization test

In this method a guinea pig is injected intradermally with the following materials:

Freund's complete adjuvant alone;

test material alone;

test material emulsified in Freund's complete adjuvant.

Test materials unable to be emulsified, are applied as a powder incorporated in petroleum jelly. After seven days a patch of the test material is bandaged over the injection sites for 48 hours. Following a further 2 weeks, the animal is challenged

with the test material at a distant site. Interpretation of the results are based on the proportion of sensitized animals found in the tested population (Mugnusson and Klingman, 1969).

The use of an adjuvant (Freund's complete adjuvant) in the guinea pig maximization test preferentially induces a cell mediated response. The granulomatous lesions produced are more easily graded than lesions produced without such an adjuvant.

(b) *Other methods*

Torabinejad et al (1979) recently reported an investigative study in which they attempted to assess the ability of endodontic materials to sensitize a host.

The pulpal tissues of a number of teeth of the experimental animal are extipated and the incubated *in vitro* in the presence of the test material. The pulpless teeth are prepared for root fillings using normal endodontic procedures. At obturation, the teeth are intentionally overfilled with the test material. A number of investigations subsequently follow:

Antibody formation - by a passive haemagglutination test;

Delayed hypersensitivity -

(i) by a lymphocyte transformation test,

(ii) skin sensitization by injecting intradermally the homogenized pulp incubated in the presence of the test material. This last test is essentially the same as a guinea pig maximization test except that sensitization of the animal's tissues occurs (if it does occur) *in vitro*.

It is of interest to note that Adamkiewicz et al (1978), Feiglin and Reade (1979) and Adamkiewicz and Peković (1980) have recorded that immunologic sensitization may occur via the pulp tissues or the root canal. The use of the root canal, rather than other easier routes for introducing the material (subcutaneous or intradermal injections) would not seem justified. Instead, considering the difficulties of gaining access to the periapical tissues of experimental animals and the inability to control the dosage of the test (possibly sensitizing) material, it would seem to be a most inappropriate experimental route.

The allergic response to a material, following its implantation, may well be interpreted as a low grade chronic inflammatory reaction. It is therefore necessary to include a test of allergenicity in any comprehensive biocompatibility testing programme.

(iii) Discussion

As previously indicated, a number of tissues have been proposed as experimental implantation sites. Factors such as ease of access, interpretation of any ensuing tissue response and the anticipated usage of a biomaterial must be taken into account with each particular tissue type.

Subcutaneous tissue, as used by Dixon and Rickert (1933), Browne and Friend (1968), Langeland et al (1969) and Olsson et al (1981) is readily accessible and the non-oriented nature of the tissue makes implantation technically less difficult than in other tissues. However, the implants tend to move in this tissue, thus making it difficult to locate specimens post-implantation. This movement can also traumatize the adjacent tissue.

Muscle, as used by Laing et al (1967), the United States Pharmacopeia (1975) and Gourlay et al (1978), is surgically less accessible than subcutaneous tissue and it is more difficult to systematically orient implants in this tissue. However, once the implant is in place, it is less prone to movement than are subcutaneous implants. Because of the highly structured nature of muscle, the shape of meaningful controls must correspond exactly to that of the test material.

The peritoneal cavity, as employed by Le Veen and Barerio (1949) and Lam et al (1974) is a large, easily accessible space in which to insert implants. However, the tissues encountered

within the peritoneal cavity differ significantly from tissues which dental material might affect. Consequently, in the dental context, peritoneal tissues are probably only of value in drawing broad conclusions regarding biocompatibility in general.

Implantation methods within mineralized bone such as those described by Feldmann and Nyborg (1962), Friend and Browne (1969), Spångberg (1969d), Lawrence et al (1975), Havgen and Mjör (1978), Wenger et al (1978), Hoover et al (1980) and Olsson et al (1981) ignore in the author's assessment the effects the test material might have on osseous repair. The area adjacent to the implant is usually devoid of cells following the surgery necessary to cut the osseous defect and remains accellular for the duration of the experiment.

Both *in vitro* and *in vivo* methods of biocompatibility testing allow the rating of materials on the basis of toxicity. Relatively few studies have attempted to compare results obtained utilizing more than one method. When such comparisons have been made, there was usually poor correlation (Mjör et al, 1977; Mjör et al, 1977a and Hensten-Pettersen, 1980). This was highlighted recently by Hensten-Petersen (1980), (see Figure 2.1).

According to Mjör (1980) it would appear that better correlation exists between more toxic substances than between less toxic substances. It is likely that dental materials would be found in the area where poor correlation exists.

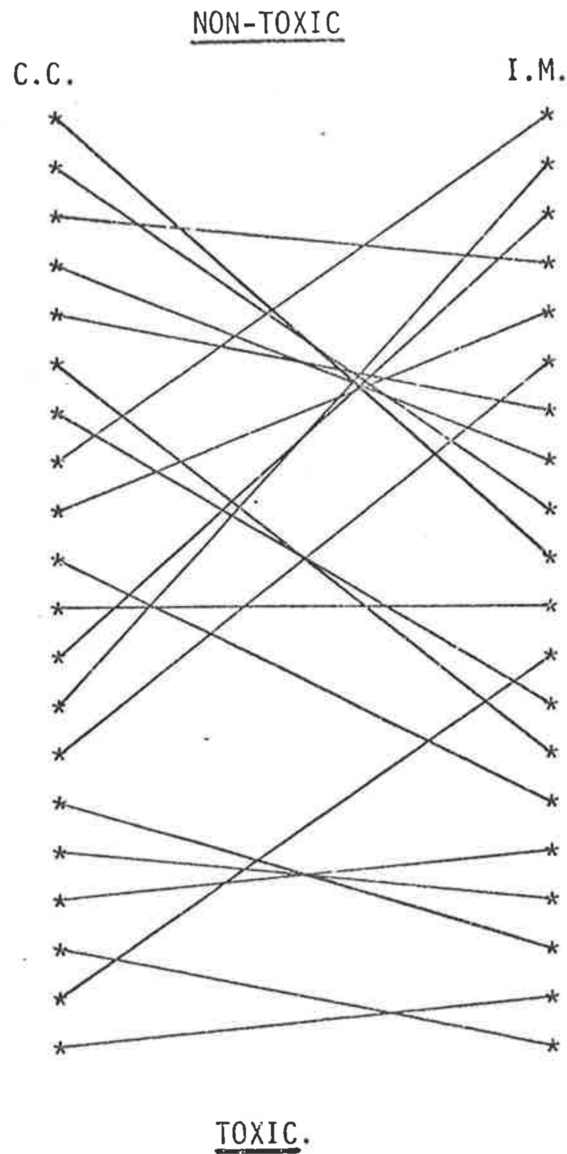


FIGURE 2.1: Table illustrating the results of a study carried out by Hensten-Pettersen (1980) in which twenty biomaterials are ranked according to the cell culture techniques (C.C.) and intramuscular implantations (I.M.). The individual materials and their respective ratings within their respective C.C. and I.M. groups are indicated by reference to the solid connecting lines.

2.4.4 TESTS OF MUTAGENIC RESPONSE

A number of so-called short term tests of (mutagenic response) carcinogenicity (Bridges, 1976) have been proposed which employ a wide range of organisms from bacteriophages and bacteria, through yeasts and insects, to mammalian cells in culture. The basis of all short term tests for carcinogenicity is that they show the capacity of an agent to cause DNA damage. However, *in vitro* tests cannot indicate whether man is susceptible or resistant to the test substance (Gardner, 1980).

A large number of systems designed to screen materials for mutagenic potential have been described in the published literature. In the context of this review, only a small number of the more "popular" tests will be outlined.

(i) Ames Mutagenicity Test

In this test a mutant, histidine dependent strain of Salmonella typhimurium is plated onto a histidine free nutrient (minimal media). Following the addition of the test material, the plated nutrient is incubated. A small amount of histidine is usually added to this minimal media to allow some growth to take place and to ensure that the test material affects multiplying bacteria. Many potential mutagens, unless first metabolized do not become active. For this purpose, the microsomal fraction of homogenized rat liver (S_9) is added to the system.

When the test material is not mutagenic, only a few colonies of S.typhimurium grow, due to random mutation back to the wild type which synthesizes histidine. Should the test material cause mutagenic change, however, conversion to non-histidine dependent Salmonella occurs more frequently and many more viable colonies grow on the minimal media. The test is performed with strains of both base-pair substitution mutan Salmonella and frameshift mutant Salmonella. It is assumed that materials that cause a significant increase in reverse mutations have a high probability of being mutagenic and carcinogenic in mammalian species (Ames et al, 1973 and Ames et al, 1975).

McCann et al (1975) tested 300 chemicals, known to be carcinogenic in mammals. They found using the Ames mutagenicity test, over 90% of these materials produced a positive result.

(ii) Styles Cell Transformation Test

The transformation of mammalian cells *in vitro* has been used to study mutagenic change. There are many cell transformation assays, each having a different end point such as change in: morphology, plating efficiency, serum requirements, nuclear size, enzyme activity, growth in semi-solid agar, cytoskeletal structure and antigenicity. The growth in semi-solid agar is usually the last characteristic to appear in transformed cells and appears to be a mutational event (Styles, 1980). The Styles cell transformation test is founded in this premise.

In the Styles test the cells of a standard line are grown according to a standardized regime. A filtered supernatant of rat liver microsome (S_9) and the test material are added to the suspended cells and the mixture incubated. Following incubation for a specific time, the cells are centrifuged and then re-suspended in an agar and nutrient suspension. A transformation frequency (shown by colony growth) two and half times greater than that of control cultures is considered a positive result (Purchase et al, 1976).

(iii) Dominant Lethal Test

This test attempts to establish the mutagenic potential of a material on gonad tissues. Other tissues of the body can be affected without influencing results.

A number of male mice are dosed with the test material (or a control material). These males are then mated with a series of females over a period of time. The uteri of the mated females are examined at a suitable interval post-coition for evidence of foetal death and malformation. Early post-implantation foetal deaths are considered the best indicator of a mutagenic response (Anderson et al, 1976a and Anderson et al, 1976b).

(iv) *In Vivo* Tests

The induction of DNA damage would appear to be the first step in a complex set of processes leading to tumor formation (Green, 1980). Tests such as the Ames mutagenicity test, or the Styles cell transformation test demonstrate DNA damage *in vitro*. Mutagenicity tests can also be conducted *in vivo*.

(a) Method of Heddle (1973)

Assessment of the extent and nature of chromosome damage caused following exposure to a test material, as expressed by the bone marrow cells,constitutes the basis of this test,as described by Heddle (1973).

In this model, the animal is dosed with the test material via the most appropriate route. A bone marrow smear is subsequently examined microscopically and all types of chromosome abberations (including chromosome gaps) noted. A number of known carcinogens have registered positive results using this method (Anderson, 1978).

(v) Miscellaneous Methods

A number of little tried methods for detecting mutagens have been proposed. In the future some of these may well be of value in predicting the mutagenicity of substances. However, at this point in time, these methods would have to be considered experimental. They include:

degranulation of rough endoplasmic reticulum of rat liver (Williams and Rabin, 1971);

loss of labelled DNA from rough endoplasmic reticulum (Purchase and Lefevre, 1975);

in vivo enhancement of biphenyl-2-hydroxylation activity in rat liver microsome preparations (McPherson et al, 1974);

estimating the amount of DNA synthesis in repair immediately following damage as measured by the uptake of ^{3}H thymidine (Han and Stick, 1975);

measuring the difference in the killing ability (both positive and negative) of a lysogenic bacteriophage, known to result from DNA damage (Green, 1980).

It is obvious that carcinomatous change produced by the introduction of a material into the body is undesirable. Mutations may well be equally deleterous to mankind. Mutations in germ cells are usually recessive and may not be apparent for several generations.

Prior to the introduction of short term screening tests, long term carcinogenicity studies were the basis of testing the carcinogenicity of a test material. A well conducted long term carcinogenicity study was so slow and so expensive that the result had to be regarded as definitive, simply because the test would never be repeated. The figures quoted for 1977 (Wenck and Collum) were that a short term test would take less than one month and cost $1,000 while a long term test would take more than two years and cost $100,000 per chemical. For this reason, short term tests can be repeated more readily. Unfortunately, when they are repeated they do not always give the same answer. This may also be true for long term tests, but since they are seldom repeated it is not a practical difficulty. Moreover, different short term tests may give different answers and the more tests that are used the more likely it is that one of them will give a positive result. It all becomes a little confusing. The confusion is likely, however, to represent a more realistic picture of carcinogenic risk than the arbitrary simplicity of results from a single test (Green, 1980).

The Ames test is an excellent primary screening test because it is sensitive and it gives a reasonably low frequency of both "false" positives and negatives (Green, 1980). When short term tests were first introduced, they were a strictly a "yes/no" assay. In recent years a debate has begun about whether

or not these tests (in particular the Ames test) can be used quantitatively, i.e. to grade the potency of a mutagen (Ashby and Styles, 1978; Ashby and Styles, 1978a; Ames and Hooper, 1978). This question remains far from resolved.

The Ames mutagenicity test is the best documented and most widely accepted screening test to date. Tests such as the Ames test measure only point mutations. However, major categories of genetic damage of concern to the human population are:

gene mutations;

chromosome re-arrangement;

abnormal numbers of chromosomes;

mitotic recombination and gene conversion;

stimulation of unscheduled DNA synthesis and inhibition of DNA repair.

The general consensus of workers in this field is that the majority of chemicals that are mutagenic, produce more than one class of genetic alteration and thus would be detected as mutagens (de Serres, 1977). The possibility must remain, however, that some mutagens will remain undetected.

All *in vitro* tests of mutagenicity use a liver supernatant fraction to allow potential mutagens to be metabolized into their active form. Probably 80% of the difficulties in bacterial tests arise from the metabolizing system; most of the remaining 20% arise from failure to understand how to measure mutation (Green, 1980). Activation by a liver supernatant fraction does not cover

all situations. Some chemicals need reduction rather than oxidation for activation, e.g. metabolism by the gut flora, while others need activation by organs other than the liver or by components other than microsomes (Anderson, 1978).

There is no special reason to believe that a mutation test, for instance, is better than any other of the categories of proposed tests. Even if the somatic theory of carcinogenesis is accepted, it does not mean that mutation in mammalian cells *in vivo* follows the same mechanism as histidine reversion in S.typhimurium. Tests must be judged on the basis of their simplicity, sensitivity, freedom from artifact and ability to differentiate mutagens from non-mutagens (Green, 1980).

Perhaps the most promising use of the short term test is quality control, not only with regard to formulated products, but also with regard to the technical grade product constituents (de Serres, 1977).

CHAPTER 3.

MATERIALS AND METHODS.

3.1 PILOT STUDIES

3.1.1 INTRODUCTION

In the formative stages of this project, many experimental models concerned with the *in vivo* biocompatibility testing of a range of materials were reviewed (Section 2.4.3). These studies basically involved the implantation of experimental materials into a variety of tissue sites and the histologic observation of tissue and cellular responses at varying intervals post-implantation. In view of the clinical usage of Hydron, some of the methodologies described in the studies reviewed in Section 2.4.3 were deemed to be potentially suitable for the aims of the present study, namely to assess the biocompatibility of the endodontic material Hydron.

In clinical use, an endodontic material will have an effect on both soft tissue and bone, thus any experimental model should incorporate both tissues. With respect to bone, the influence the material might have on osseous repair would seem to be of paramount importance. Numerous suitable sites exist in experimental animals for soft tissue implantation. Therefore, the main consideration in these pilot studies was to choose an animal with a suitable site for a meaningful intraosseous implantation.

From the number of models using intraosseous implantation (refer Intraosseous Implantations, Section 2.4.3), the models of Haugen and Mjör (1979), Deemer and Tsaknis (1979), Taylor et al (1971) and Spångberg (1969d) were considered to be potentially suitable with regard to the aims of the present study. It was thus decided to carry out pilot studies employing these methodologies but using Hydron as the test material. The purpose of conducting these pilot studies was to attempt to gauge the practical difficulties associated with the various methods.

3.1.2 SELECTION OF A SUITABLE BONE MODEL

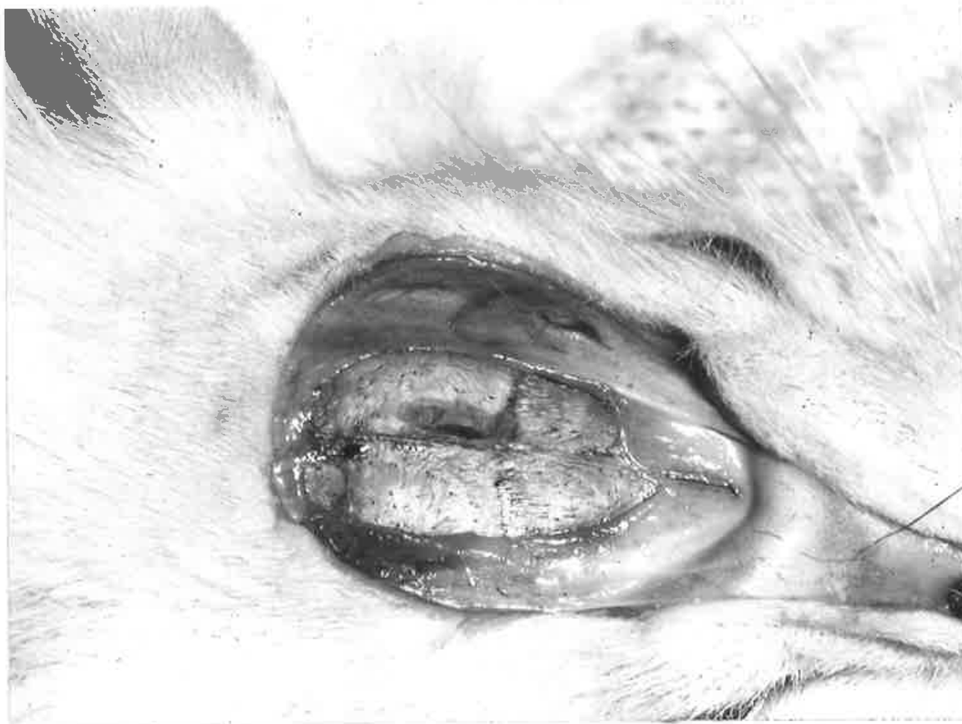
(i) Model of Haugen and Mjör (1979)

This method involves the lifting of a split thickness semilunar flap and creating a groove in the exposed cranial bones of the rat (Fig. 3.1). The test material is placed into this groove and also extended over the adjacent bone and periosteum. The implantation sites are then assessed histologically at various intervals post-implantation.

In the pilot study conducted, it was found that the rat was a most suitable experimental animal because it was not easily stressed, it was easily handled and safely anaesthetized. However, during the surgical procedures involved with cutting the cranial grooves, a major difficulty was encountered. This was that the cranial bones in this area were very thin and were often perforated damaging the brain.

For this reason and for the reasons outlined in the review (Assessment Methods of Intraosseous Implantation Techniques, Section 2.4.3) regarding the histological assessment of material, it was decided that this particular method would not be suitable.

(a)



(b)

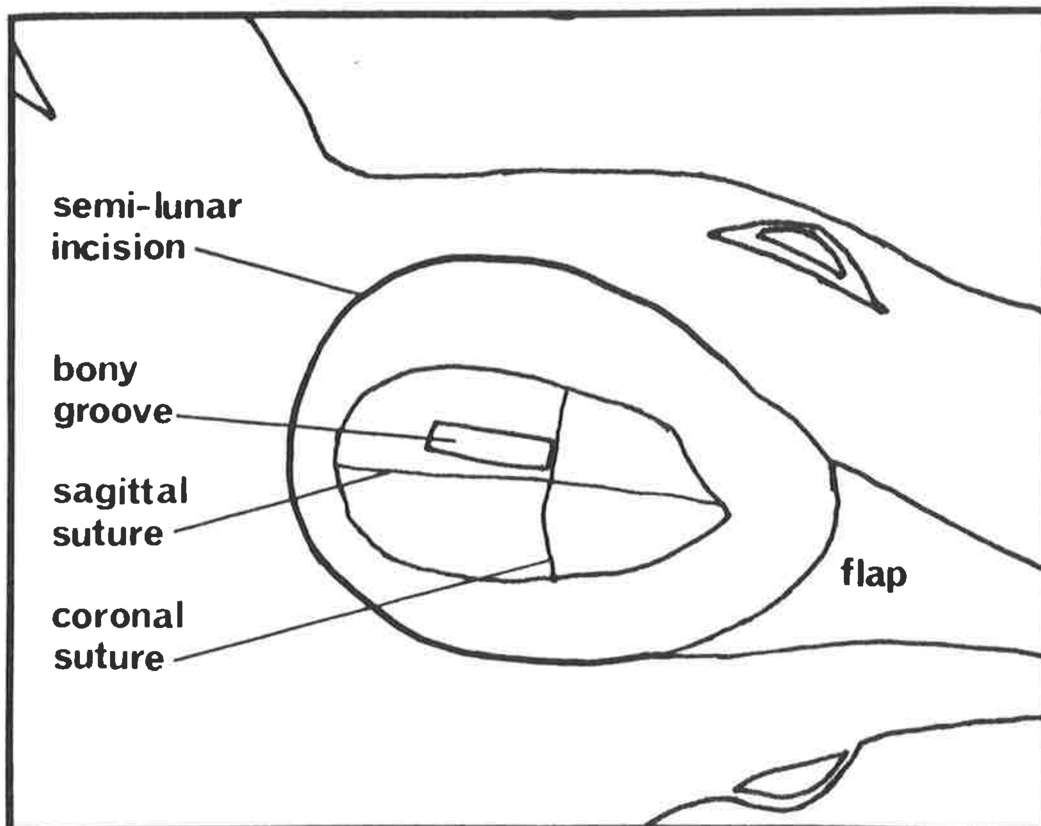


FIGURE 3.1 (a) & (b): Photograph and schematic diagram respectively illustrating the surgical site used in the Hagen and Mjör (1979) method. The features of the clinical photograph are illustrated by reference to the schematic diagram.

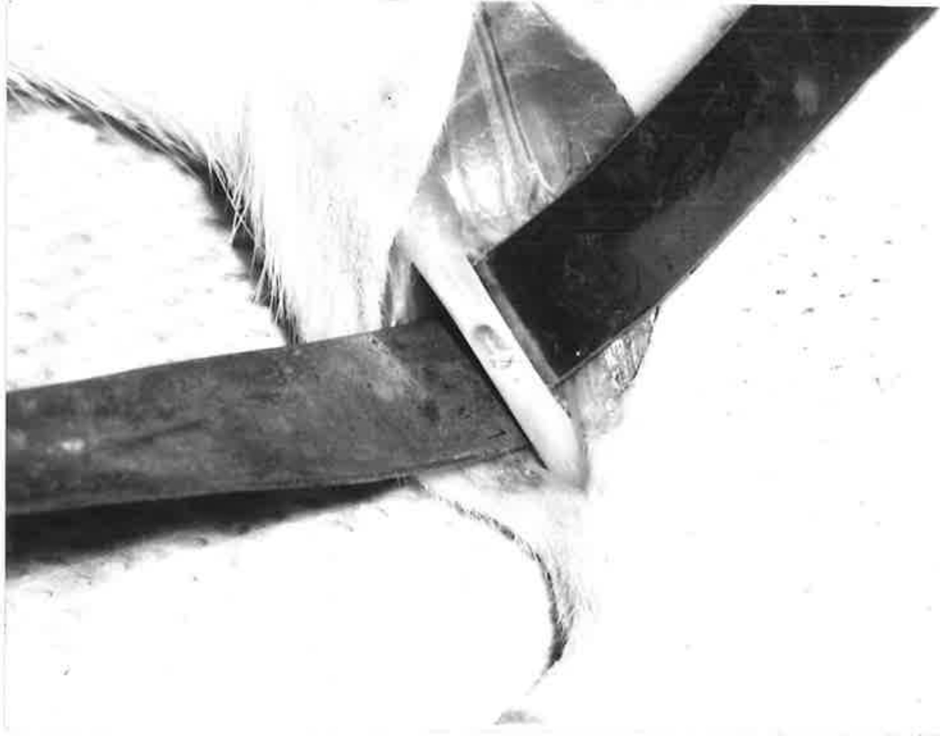
(ii) Model of Deemer and Tsaknis (1979)

In this method the tibia of a rat is exposed and a window of bone removed (Fig. 3.2). Into this defect is placed an inert tube containing the test material. The ensuing reactions at the ends of the tubes are assessed histologically (sections being cut along the long axis of each particular tube) at intervals post-implantation.

Again the rat was found to be a most suitable experimental animal. An advantage of this method compared with that of Hagen and Mjör (1979) was that adequate controls could be readily incorporated in the model. However, the histologic reactions to the test or control materials could only be observed in the bone marrow. After viewing histological sections of the implant sites 1-2 weeks following implantation, it was considered that (in a major study) difficulties could be encountered distinguishing the haemopoietic marrow cells from those of the tissue "reaction" to the implant. This would be particularly so should an inflammatory response occur subsequent to implantation. In addition, the lesion created was large (relative to the size of the bone) and pathological fractures were common.

For these reasons it was decided that this particular method would not be suitable.

(a)



(b)

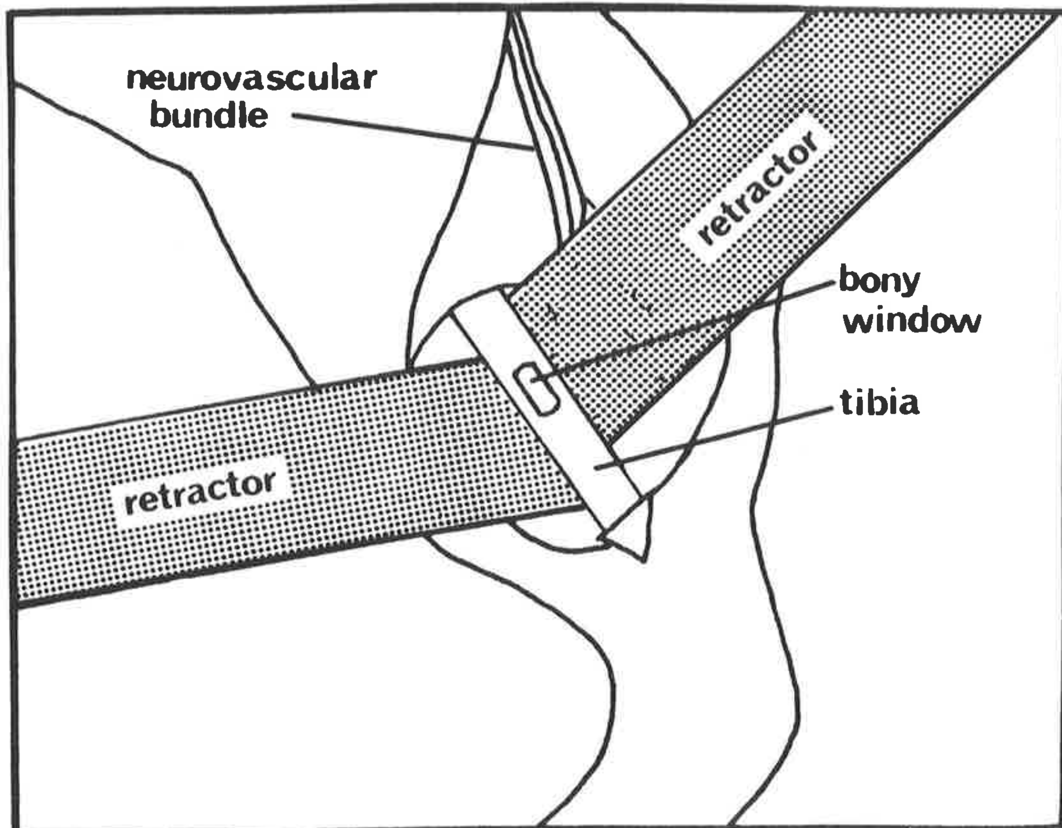


FIGURE 3.2 (a) & (b): Photograph and schematic diagram respectively showing the surgical site of the Deemer and Tsaknis (1979) method. The features of the clinical photograph are illustrated by reference to the schematic diagram.

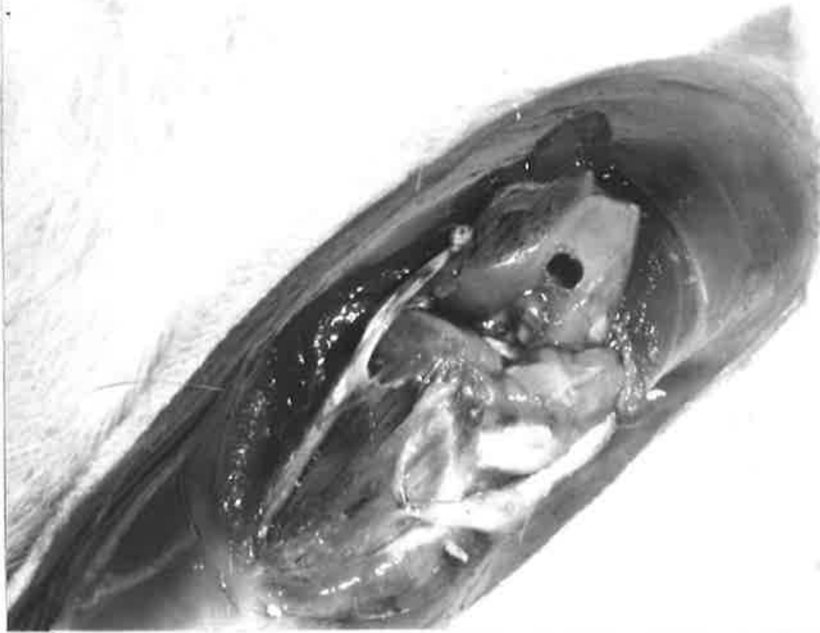
(iii) <u>Model of Taylor et al (1971)</u>

This particular method was also used by Murray and Dow (1975). In this method the tissues overlying the knee of a rat are reflected, and the joint dislocated. A defect is created in the shaft of the femur through its distal head (Fig. 3.3) and into this is placed an inert tube containing the test material. The reaction at the end of the tube is assessed histologically (sections being cut along the long axis of each particular tube) at intervals post-implantation. This method was originally devised to test the suitability of a proposed prosthesis to replace defective cartilage of the articular surface of the distal femur.

In the pilot studies conducted using the method of Taylor et al (1971), again the rat was found to be a most suitable experimental animal and controls could be readily incorporated in the model. However, the end of the tube where the reaction to the test material was assessed, was situated in the same tissue as was the implant in the Deemer and Tsaknis (1979) model, namely haemopoietic marrow. In addition, in the Taylor et al (1971) model, the implant is located near the epiphyseal plate of the distal femur, an area of active bone formation.

For these reasons and the surgical morbidity suffered by the experimental rats, it was decided that this particular method would not be suitable.

(a)



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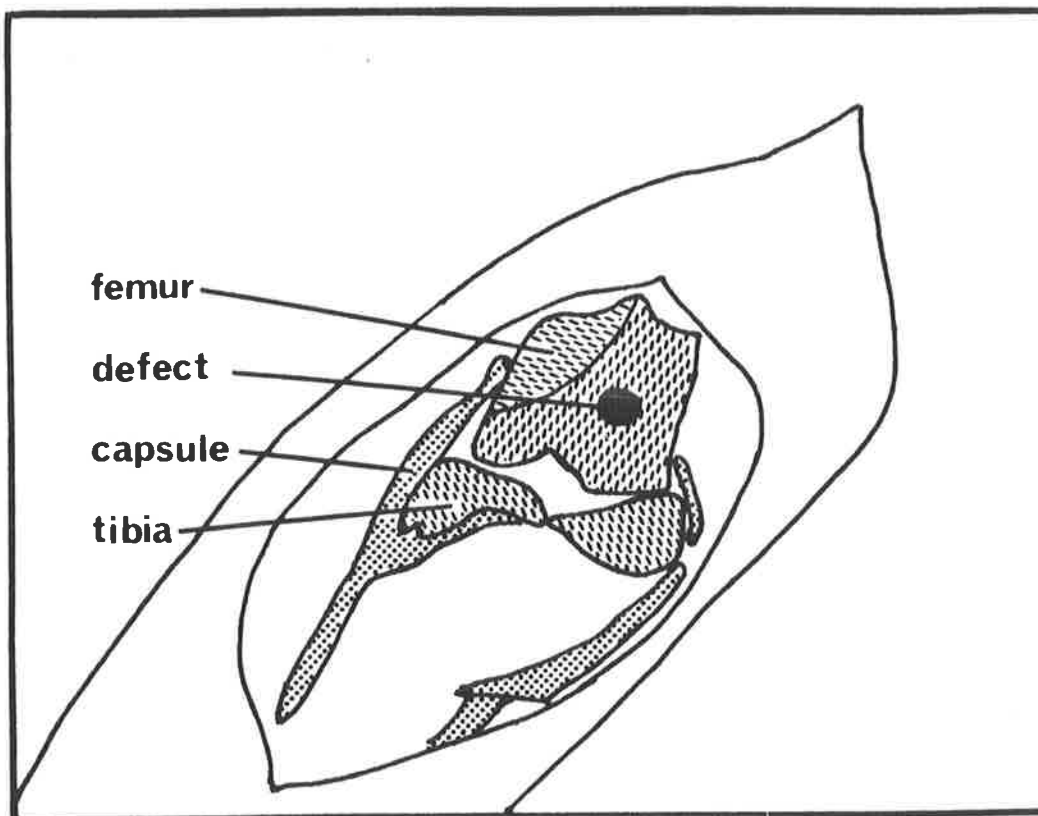

FIGURE 3.3 (a) & (b): Photograph and schematic diagram respectively showing the surgical site of the Taylor et al (1971) method.
The features of the clinical photograph are illustrated by reference to the schematic diagram.

(iv) Model of Spångberg (1969d)

The experimental animal was the guinea pig. The lower border of the mandible in the midline was exposed and the area on either of the symphysis side stripped of periosteum and overlying tissues. A bone defect was created to a depth of approximately 2 mm in the bone on either side of the symphysis, between it and the incisor eminences. Into this defect was inserted a Teflon tube containing the test material. Solid Teflon rods acted as controls. The reaction was assessed by examining any cellular and tissue reactions that might occur at the end of the implant, as well as the inflammatory, degenerative and resorptive reactions in the adjacent bone.

Despite the difficulties anticipated with respect to the nature of the guinea pig, this model was chosen in broad outline.

The advantages this model offered over the other models were considered to be:

- the area assessed in bone was affected by only one variable, i.e. the implanted material;
- access to the area surgically, was relatively uncomplicated and little morbidity resulted;
- suitable controls for the experiment could be readily incorporated;
- the area could be accurately located post-implantation.

3.1.3 MODIFICATIONS TO THE SPÅNGBERG (1969d) MODEL

(i) Introduction

As indicated above, a pilot study was conducted using the basic model of Spångberg (1969d). However, because of the problems that became evident in the course of this pilot study, a number of modifications to the original protocol were thought to be necessary. A number of other aspects of the original protocol were also modified, particularly those related to anaesthesia of the experimental animals.

(ii) Shape of the Bony Defect

In the original model proposed by Spångberg (1969d), the bony defect was virtually the same size as the carrier. Two complications arose from this. Firstly, the implant vehicle virtually completely filled the created bony defect at its base (Fig. 3.4). Consequently, the only means of assessing the influence the Hydron had on bone was the area of dead or dying osteocytes (refer Assessment Methods of Intraosseous Implantation Techniques, Section 2.4.3). Secondly, little could be gauged as to the effect of the test material on osseous repair.

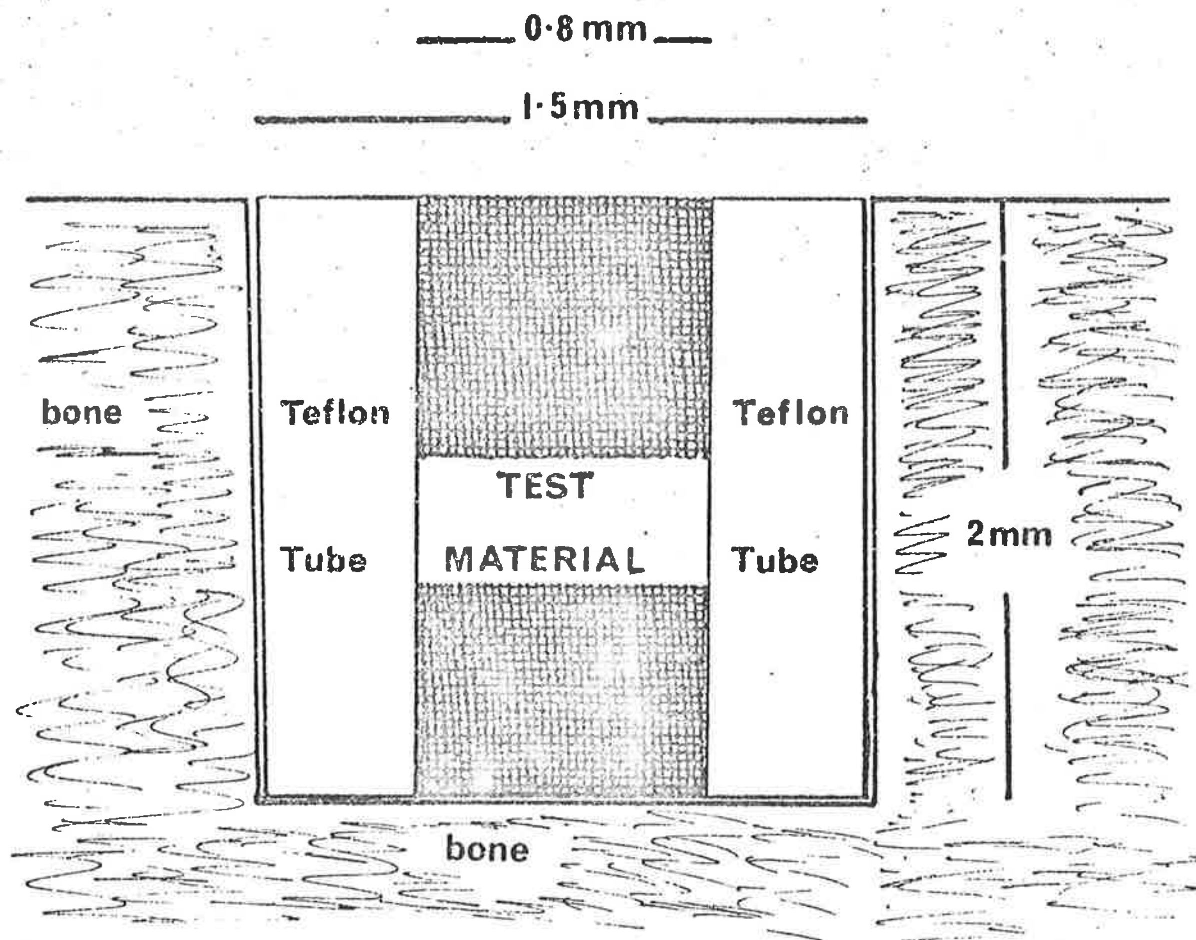

FIGURE 3.4: The shape of the bony defect as proposed by Spångberg (1969d), as seen in sectional view through the guinea pig mandible.

It was therefore decided to create a defect in the bone larger than the carrier of the test material. This defect (Fig. 3.5) allowed uniform and consistent placement of the implant.

The bone defect was produced by making an initial hole with a number 2 round bur to a depth of 2 mm and then enlarging it with a number 4 P.D. tapering reamer*. The influence the material might have on any histologic reaction following implantation and on osseous repair could thus be assessed in the area between the test material and the wall of the bony defect (Fig. 3.5). The small projection at the tip of the P.D. reamer created a convenient reference point for locating the centre of the defect when cutting histological sections. This was readily apparent in sections cut from bone following implantation for up to 2 weeks (the longest implantation time in this pilot study).

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* Products Dentaire S.A., Switzerland.

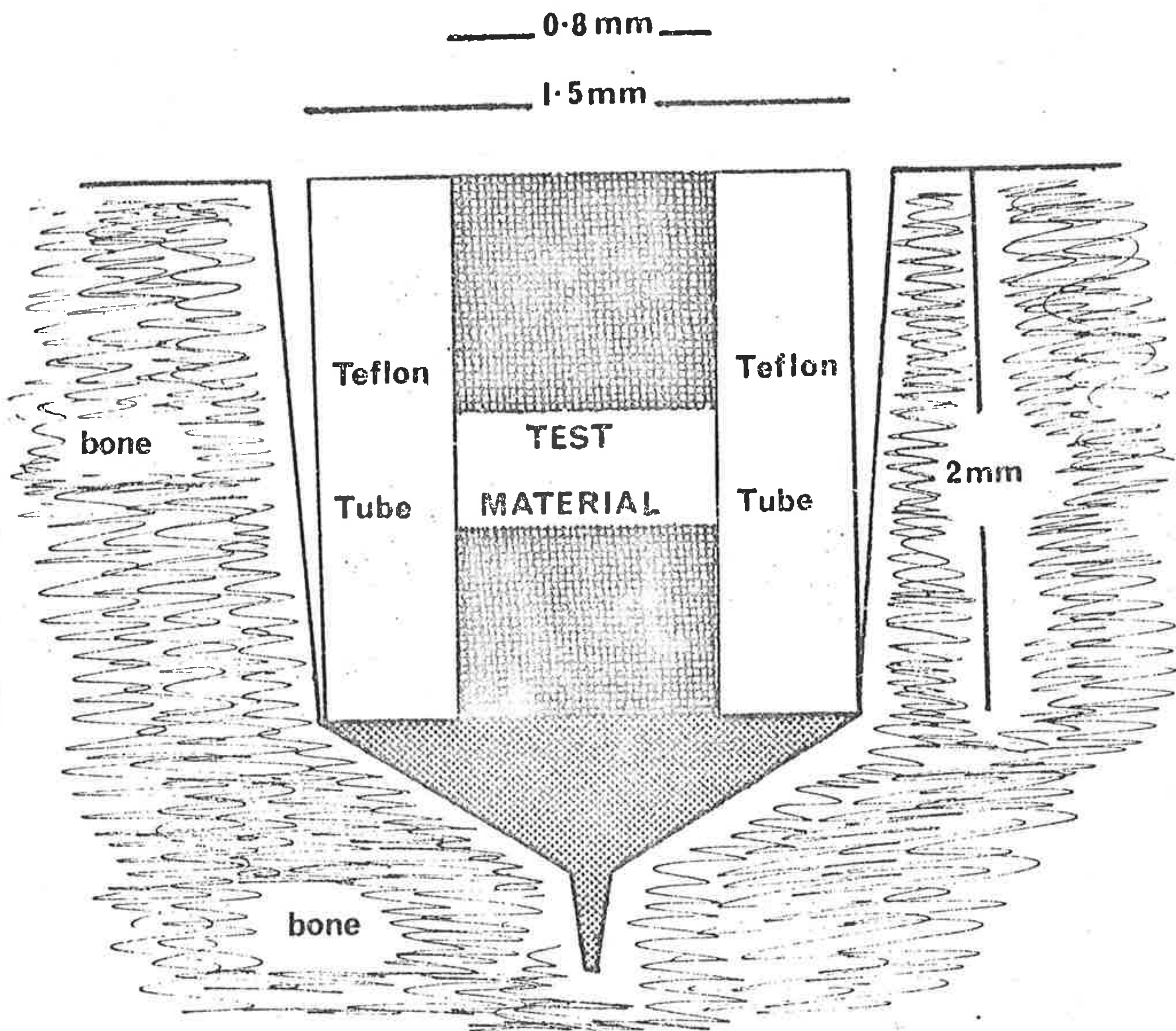

FIGURE 3.5: Illustrating the modifications (as seen in sectional view) to the bony defect compared to the original Spångberg (1969d) model (refer Fig. 3.4). The triangular area between the test material and the wall of the bony defect is the area evaluated histologically to assess the effect the test material might have on any histologic reaction following implantation and on osseous repair.

(iii) The Carrier

Enquiries throughout Australia failed to locate a source from which to obtain the Teflon cups used by Spångberg (1969d). Time constraints unfortunately, did not allow an overseas source of the cups to be located. Consequently, short lengths of Teflon tubing of outside diameter 1.5 mm, inside diameter 0.8 mm, were chosen as the carriers of the Hydron in this experiment. The Teflon tubing was obtained from John Morris Pty. Ltd., Sydney.

Whilst the Teflon tube proved to be an excellent carrier of the Hydron, in the pilot study it was observed histologically that on some occasions unset material was often squeezed out of the tube. In these instances the Hydron completely filled the space between the base of the implant and the walls of the bony defect (refer Fig. 3.5). When the bony defect was filled with Hydron, no assessment was possible of any effect the Hydron might have on osseous repair. For this reason it was decided to employ freshly set Hydron (Fig. 3.6) rather than freshly mixed material in the present study. It was realised that this was not ideal, in as much as this is not the way that Hydron is used clinically. However, it was felt that this compromise was essential to ensure consistency in placement and to avoid the problems created by the Hydron spilling into the defect.

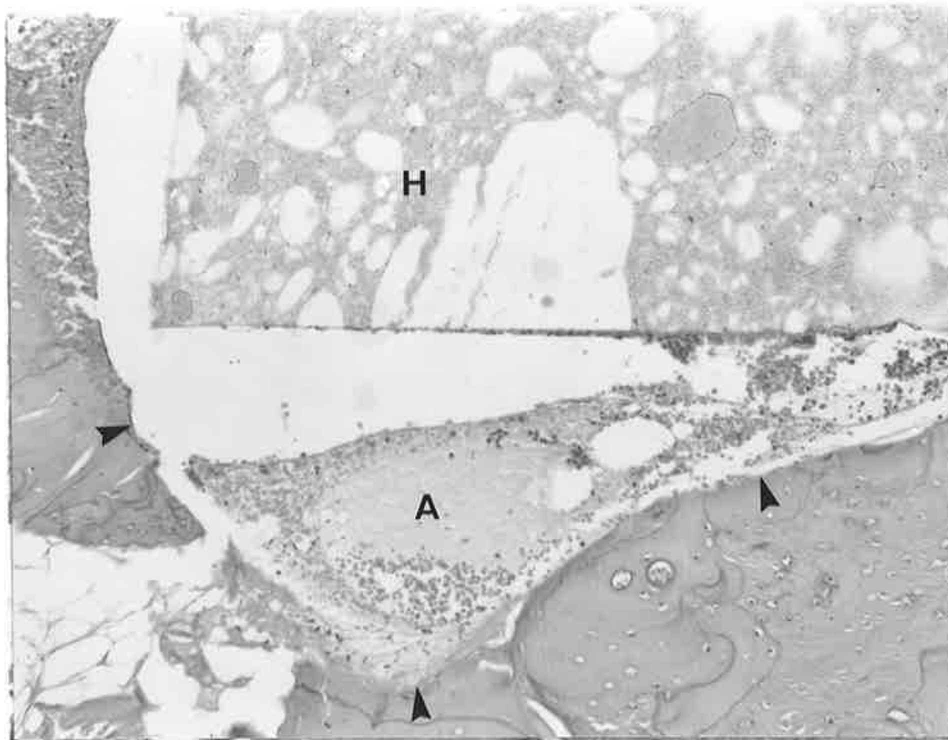

FIGURE 3.6: Freshly set Hydron, 2 days post-implantation in bone.

Photomicrograph illustrating the triangular area (A) between the Hydron implant (H) and the walls of the defect produced in bone (arrows). When unset Hydron was implanted, the triangular defect was often completely filled with Hydron.

Haematoxylin and Eosin (H. & E.).
Original magnification x40.

(iv) Assessment Times

In the original model (Spångberg, 1969d), experimental and control materials were implanted for either 2 weeks or 12 weeks. To assess the histologic reaction at periods of less than 2 weeks post-implantation was felt to be important, not only to assess the initial reaction following implantation, but also to establish the sequence of events following surgery. Ideally, assessment of the histologic reaction would be made at least a year (or more) following implantation of the Hydron. However, the time available for this study made implantation for periods of more than 6 months impracticable. For these reasons the following time periods following implantation were chosen as the most suitable times at which to assess the histologic reaction to the Hydron: 2 days, 1 week, 2 weeks, 4 weeks, 12 weeks and 26 weeks.

(v) Development of a Suitable Anaesthetic Protocol

Following a review of published literature on the subject of guinea pig anaesthesiology, it was evident that guinea pigs are unpredictable in their response to anaesthetic agents. Surprisingly little has been published on this subject and there appears to be a paucity of knowledge regarding this aspect of the guinea pig's physiology (Hoar, 1969).

To develop a workable anaesthetic regime for the guinea pigs involved in this research, a number of methods were tried or reviewed. These are included as an appendix (Appendix I).

(vi) Surgical Technique

Spångberg (1969d) employed an initial incision along the lower border of the mandible, down to bone, then reflected a full thickness flap. This meant that a relatively large flap had to be retracted to give adequate access and as a consequence considerable post-operative swelling resulted.

To overcome this, a skin incision was made in the midline of the neck and undermined by sharp dissection. Suitable retraction exposed the required area of the mandible. An incision was then made along the lower border of the mandible down to bone and a periosteal flap reflected. This was a much thinner flap and more flexible than the previously proposed flap. As such, a smaller flap exposed the required area. Suturing the wound in layers (3) gave a much stronger closure. The post-operative swelling was considerably reduced.

3.1.4 SELECTION OF A SUITABLE SOFT TISSUE MODEL

The main soft tissue sites previously chosen for implantation have been subcutaneous tissue, muscle and the tissue surrounding the peritoneal cavity (refer Soft Tissue Implantation Techniques, Section 2.4.3). Following the selection of the guinea pig as the experimental animal for intraosseous implantation, the various sites were reviewed.

Implantation in the peritoneal space was thought to be unsuitable. The implant would be relatively difficult to locate following its introduction to the area for the time envisaged.

Muscle was preferred to subcutaneous tissue for the following reasons:

the implant would remain much more stable, and therefore be easier to locate;

muscle should be, in theory, a more sensitive indicator of adverse effects from a material than subcutaneous connective tissue;

more research was encountered using quantitative methods for analysis of results in muscle than in subcutaneous tissue.

A pilot study using freshly mixed Hydron as the experimental material contained in 5 mm lengths of 1.5 mm outside diameter Teflon tube was conducted. The thigh was chosen as a suitable site because:

a muscle (the quadriceps muscle) of a suitable size to accommodate the implant could be easily located;

once the main neuro-vascular bundle had been located and retracted, there were no vital structures to complicate the surgery;

the animal was not distressed by the operation and did not suffer any significant impairment to the use of the hind limbs following surgery;

the site was well removed from those of the
intraosseous implantation and the injcctions
for the induction of anaesthesia.

During histologic assessment of tissue harvested in the course of this pilot study, it was observed that often the Hydron had spilled from the Teflon tube into the muscle at the time of implantation (Fig. 3.7).

For this reason it was decided to implant freshly set Hydron as was the case with intraosseous implants.

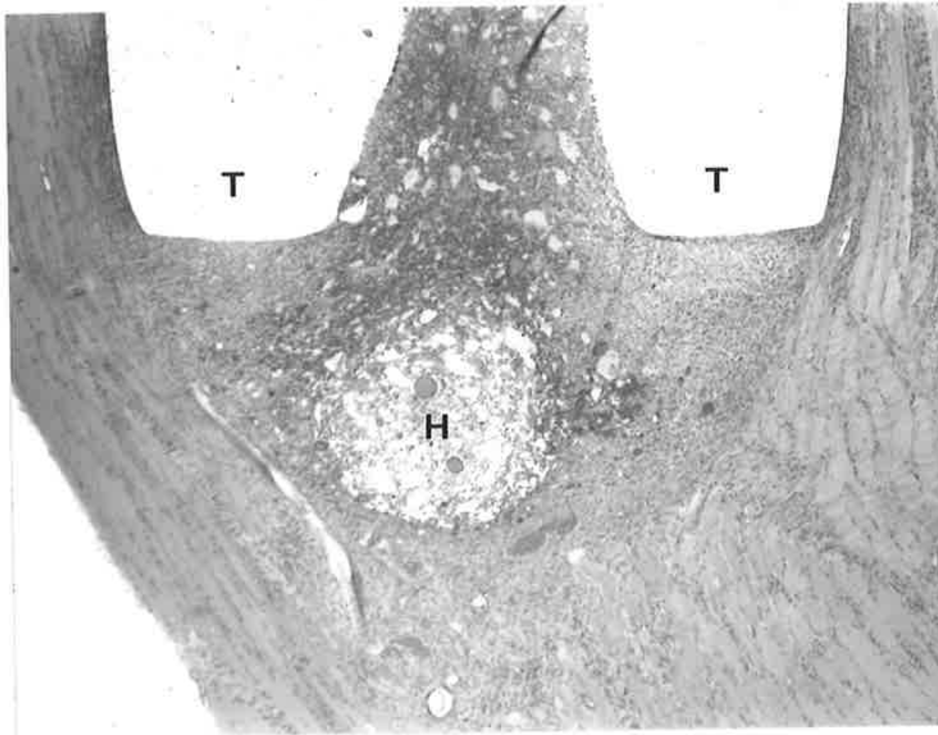

FIGURE 3.7: Freshly mixed Hydron, 2 days post-implantation in muscle.

Photomicrograph illustrating the Hydron (H) that was spilled into the tissue during implantation. Note the relative lack of Hydron contained within the Teflon tube (T).

H. & E. original magnification x100.

3.2 EXPERIMENTAL PROTOCOL - MAIN STUDY

3.2.1 EXPERIMENTAL ANIMALS

The experimental animals used throughout this study were young, healthy, adult male guinea pigs weighing initially 500-900 grams, obtained from an outbred breeding colony at the Central Animal House of the Waite Agricultural Institute, Adelaide, South Australia. Animals were fed a standard diet of M. & V. rabbit pellets (William Charlick Limited, South Australia) supplemented with ascorbic acid in their drinking water. The solid diet was supplemented twice weekly with fresh lucerne hay and fresh carrots. Tap water was supplied *ad libitum*. The guinea pigs were housed 5 per cage in standard rabbit cages (300 x 330 x 500 mm). The rabbit cages were kept in the Animal House of the Dental Department of the Royal Adelaide Hospital during the course of the study.

3.2.2 TEST MATERIAL

(i) Hydron (National Patent Development Corporation, New York)

Freshly set Hydron root canal filling material was prepared according to manufacturer's instructions (Section 3.2.5) for both intramuscular and intraosseous implantation. No specific composition of Hydron or its components has ever been forthcoming from the manufacturer (despite correspondence requesting same). Consequently, a sample of the powder and the paste was submitted for chromatographic analysis: powder - lot 47803, paste - lot 102 from the same Hydron root canal filling material kit, as supplied for clinical use.

The analysis using high pressure liquid chromatography was performed by Sola Optical Australia Pty. Ltd., South Australia.

(ii) Controls

(a) *Positive control*

AH26 (De Treys, Switzerland) an epoxy resin endodontic sealant was chosen since it is the most widely used endodontic sealant in this country.

(b) *Negative control*

Teflon (Du Pont, U.S.A.) was chosen since it was the material used in the original model of Spångberg (1969d) and was readily available.

3.2.3 POST-IMPLANTATION OBSERVATION TIMES

The experimental implant sites in both hard and soft tissues were harvested at 2 days, 1 week, 2 weeks, 4 weeks, 12 weeks and 26 weeks post-implantation.

3.2.4 DISTRIBUTION OF IMPLANTS IN EXPERIMENTAL ANIMALS

In this study both the bone and soft tissue implants were placed in all the individual guinea pigs as indicated in Fig. 3.8. A total of 54 animals (i.e. 9 per post-implantation observation period) were used in this study.

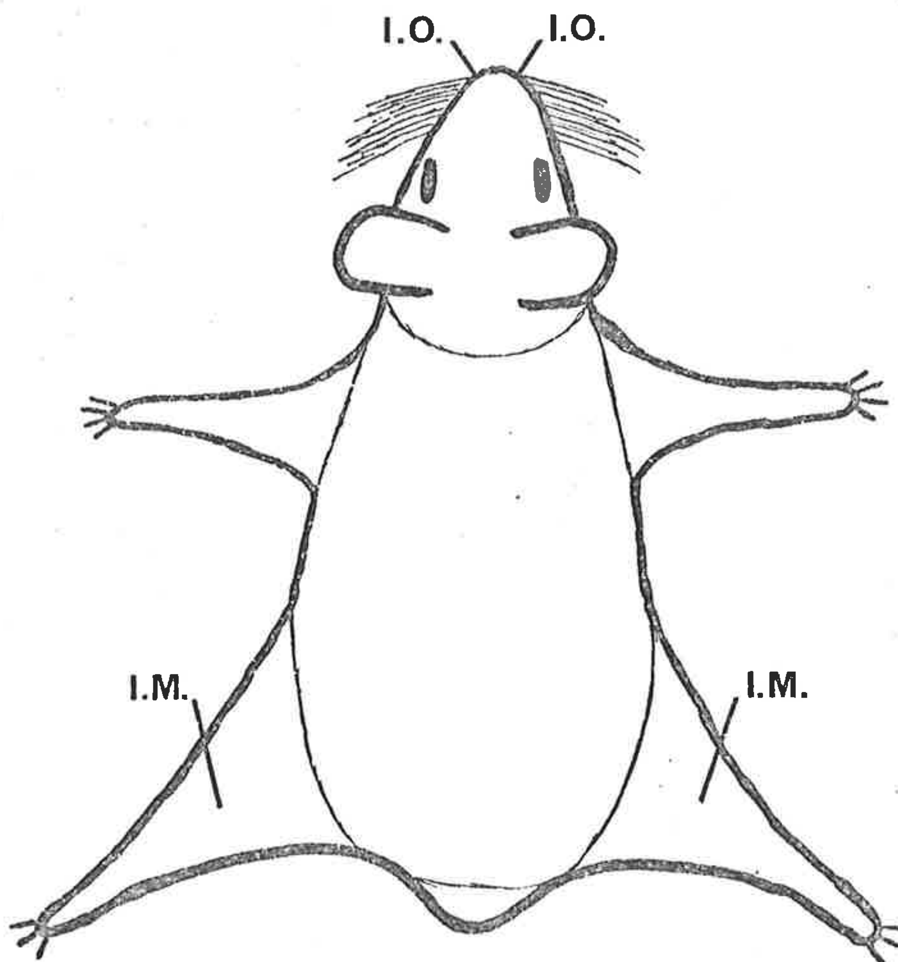

FIGURE 3.8: Schematic drawing of a guinea pig indicating the two intraosseous implantation sites in the anterior mandible and the two intramuscular implantation sites in the thighs.

The Hydron and the control implant materials were distributed in each group of 9 animals as follows:

three guinea pigs - Hydron one side, AH26 the other (both in bone and muscle);

three guinea pigs - Hydron one side, solid Teflon tube the other (both in bone and muscle);

three guinea pigs - AH26 one side, solid Teflon tube the other (both in bone and muscle).

This resulted in 6 implants of Hydron, 6 implants of AH26 (positive control) and 6 implants of solid Teflon tube (negative control) being available for histologic assessment per time period.

For each of the 7 groups of animals, whether a particular material was implanted on the right or left side was decided by tossing a coin. Thereafter, during each subsequent operation, the side (right or left) of the animal where the particular materials were implanted was alternated.

3.2.5 PREPARATION OF IMPLANTS

As required, immediately prior to preparation of implants, lengths of Teflon tube of outside diameter 1.5 mm and inside diameter 0.8 mm, were washed in alcohol, rinsed in distilled water and then autoclaved. This tubing was obtained from John Morris Pty. Ltd., Sydney - cat. no. T 21195-0034.

(i) Hydron

A clean glass mixing slab and a stainless steel spatula were autoclaved. Prior to actual mixing, these, along with a length of sterile Teflon tube were laid on a sterile drape, along with a sachet of Hydron liquid and a capsule of Hydron powder (Fig. 3.9).

FIGURE 3.9: Instruments for mixing Hydron along with Teflon tube (left) and prepackaged Hydron (right). The sachet of Hydron (far right) contains the Hydron liquid and next to it is the capsule containing Hydron powder.

The contents of the sachet and the capsule were transferred to the sterile glass slab (Fig. 3.10) ensuring the slab's sterility was not compromised. The "lumps" in the powder were then broken up as much as possible.

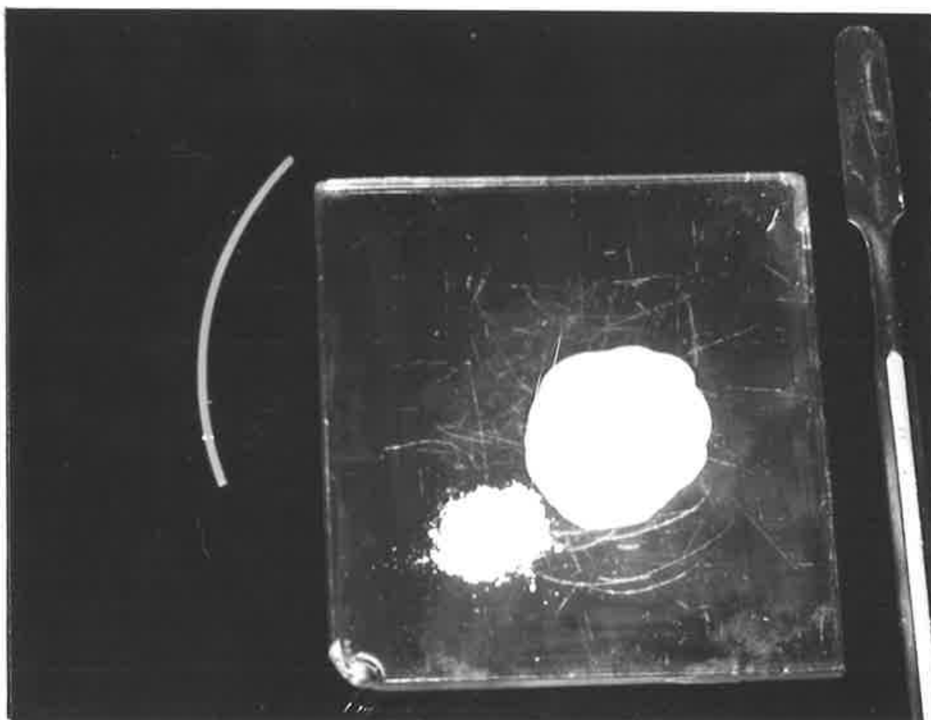

FIGURE 3.10: Hydron liquid (right) and powder (left) have been transferred to the sterile glass mixing slab.

The Hydron liquid and powder were then spatulated for 60 seconds. The resultant paste was subsequently aspirated into a 1 ml plastic syringe (Fig. 3.11). The syringe was that supplied as part of the Hydron root canal filling material kit.

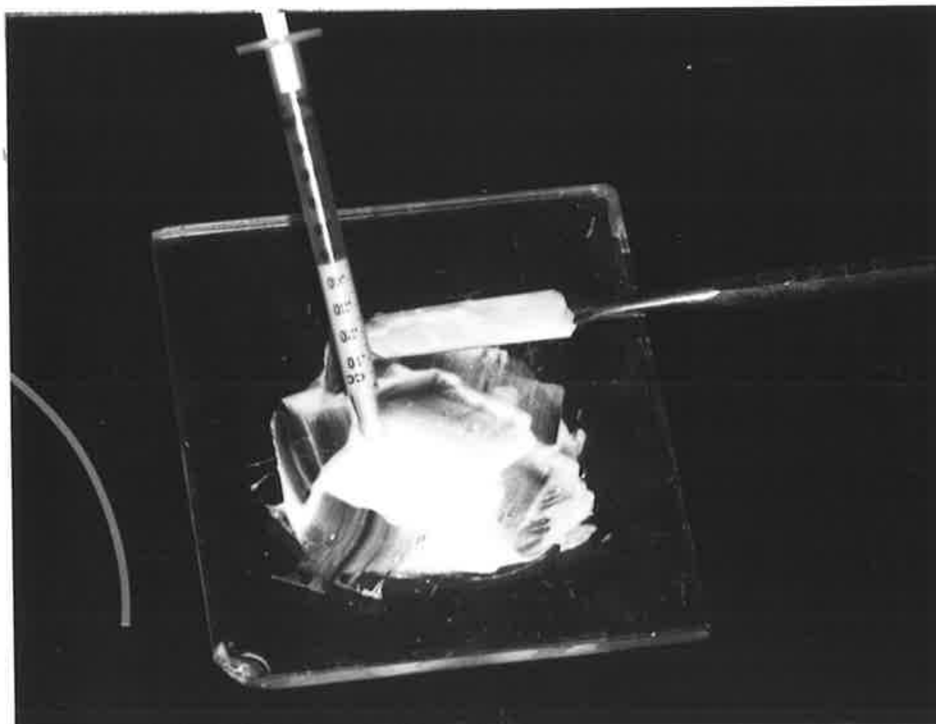

FIGURE 3.11: Spatulated Hydron being aspirated into the provided 1 ml syringe.

The length of sterile Teflon tube held in a pair of modified artery forceps was then inserted into the nozzle of the 1 ml syringe. Hydron paste was injected into this Teflon tube (Fig. 3.12) ensuring that none of the Hydron contaminated the outside of the tube. The artery forceps referred to above had been modified by cutting half a circle from each beak so that when clamped the Teflon tube would be held firmly, but not be excessively compressed.

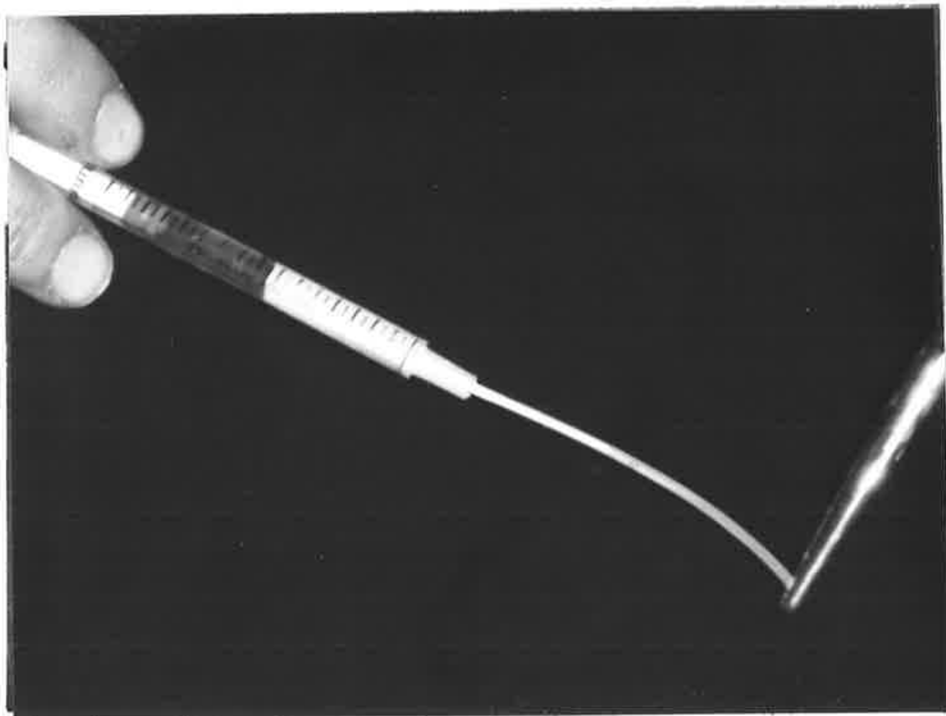

FIGURE 3.12: Hydron being injected into a length of Teflon tube held in a pair of artery forceps.

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The Hydron in the Teflon tube was allowed to set on the sterile drape. This took 7-10 minutes. In some instances, the length of Teflon tube containing the freshly set Hydron was retained as such, and these were subsequently used as the intraosseous implants. In other instances, the Teflon tube containing the freshly set Hydron was cut on a sterile drape with a sterile scalpel into 5 mm lengths. These lengths of Teflon tube containing the Hydron were subsequently used as the intramuscular implants. All tubing containing the Hydron was stored in a sterile petri dish prior to implantation.

(ii) AH26

Manufacturer's instructions were followed while mixing the AH26. The AH26 paste was then transferred to the same sterile Teflon tubing, in a similar manner to that used with the Hydron. The AH26 took at least 24 hours and up to 36 hours before it set. The setting process took place in a sterile petri dish and freshly set material (contained in Teflon tubing) was stored in a similar vessel.

(iii) Teflon Controls

Slivers of Teflon were fed into lengths of the 1.5 mm diameter Teflon tube to form a solid rod. Some of the filled Teflon tubing was left uncut for intraosseous implantation and some was cut into 5 mm lengths for intramuscular implantation. These implants were autoclaved prior to implantation and then stored in a sterile petri dish.

3.2.6 STERILITY CONTROL

During the course of the experiment, both intraosseous and intramuscular implants were sampled to test their sterility, prior to implantation. A sample implant of each material being implanted on any particular day, either Hydron and/or AH26, and/or Teflon was randomly selected, placed in Todd-Hewitt broth and yeast extract and incubated aerobically for 4 days at 35°C.

3.2.7 ANAESTHESIA

The guinea pig was injected pre-operatively with 0.5 ml (2.5 mgm) of atropine sulphate, subcutaneously in the shoulder region of the back. Surgical anaesthesia was induced by an injection of Ketalar at the dosage of 50 mgm/kg body weight (see Appendix I), into the deltoid muscle (Fig. 3.13). Anaesthesia in the surgical field was supplemented with 2% lignocaine containing 1:80,000 adrenaline

During the course of this study a record was kept of the duration of the operation and the duration of anaesthesia. The latter was analysed using a correlation of variance (Section 3.2.14) to test the validity of the method of calculating anaesthetic dosage.

FIGURE 3.13: Photograph illustrating the injection of Ketalar into the deltoid muscle for surgical anaesthesia.

3.2.8 ASEPSIS

All surgical instruments were cleaned and autoclaved prior to use and the operation was performed on sterile drapes. Throughout the operation the operators were masked, gowned and gloved.

3.2.9 SURGICAL TECHNIQUE

Prior to all surgery the appropriate fields were shaved and swabbed with a solution containing chlorhexadine 0.05%, cetrimide 0.5%, alcohol 70% (as supplied by the Pharmacy of the Royal Adelaide Hospital).

(i) Intraosseous Implantation

An incision was made through the skin in the midline of the neck. This skin was undermined by a sharp dissection to allow access to the mandible in the midline. An incision was then made to bone, along the lower border of the mandible (Fig. 3.14).

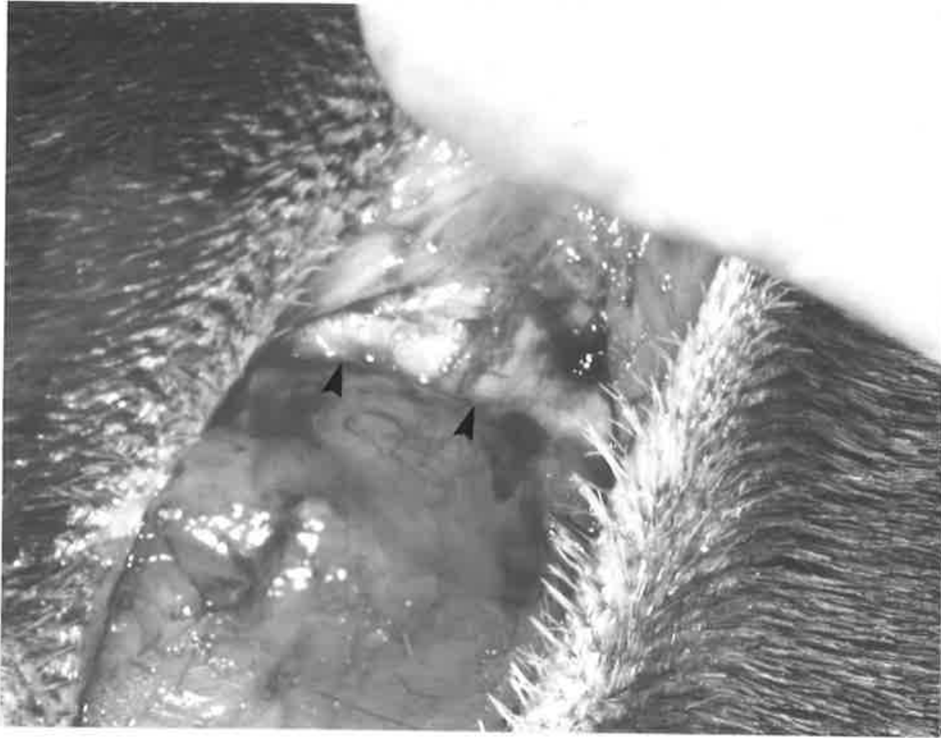

FIGURE 3.14: Photograph illustrating the initial surgical stage in the preparation of the intraosseous implantation sites. The lower border of the mandible (arrows) has been exposed and the area between the incisor eminences can be seen.

A periosteal flap was then retracted to expose the site of the implantation (Fig. 3.15).

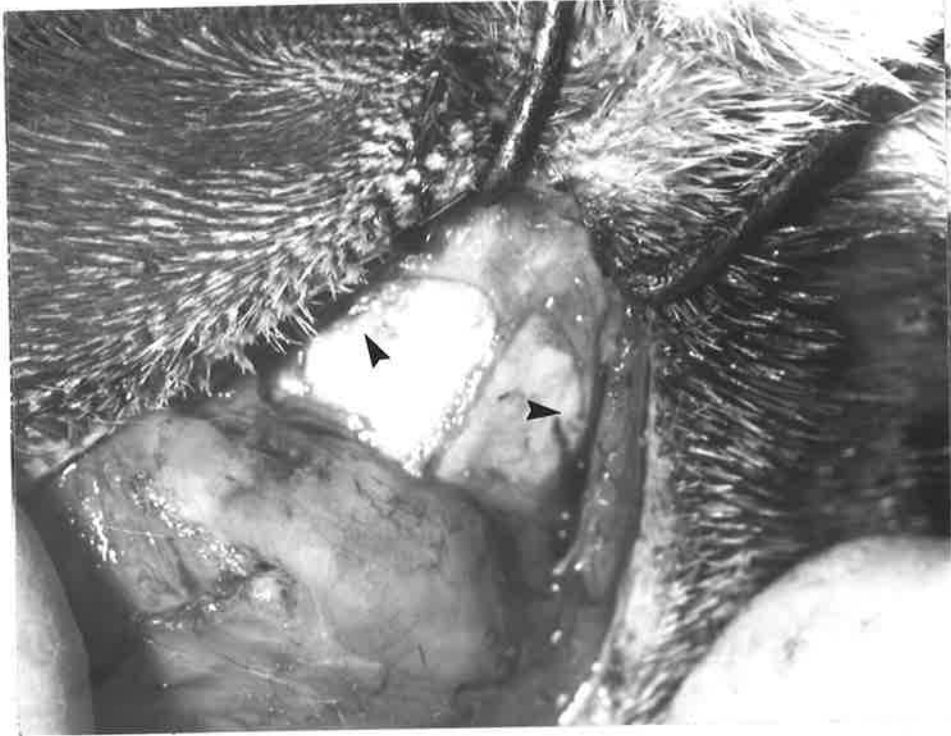

FIGURE 3.15: Photograph illustrating the initial surgical stage in the preparation of the intraosseous implantation sites. Between the incisor eminences (arrows) the bone has been stripped of periosteum.

A hole was created in the bone, in the area bounded by the incisor eminence, the symphysis and the lower border of the mandible (one each side) using a number 3 round dental bur in a slow speed dental handpiece. This hole was created to a depth of 2 mm and then enlarged with a number 4 P.D. tapering reamer (also in a slow speed dental handpiece) to the desired dimensions (refer Fig. 3.5). A piece of plastic tube placed on tapering reamer ensured the defect was created to the correct depth (Fig. 3.6). Isotonic physiological saline was used as a coolant at all stages involving the cutting of bone.

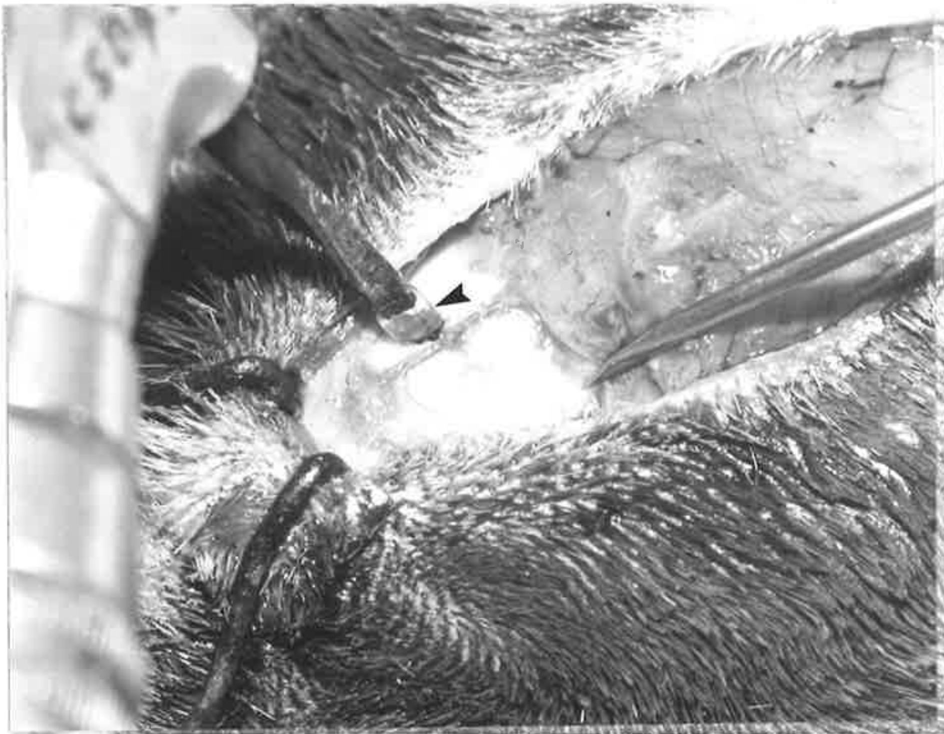

FIGURE 3.16: Photograph illustrating the cutting of the bony defect into which the intraosseous implant is placed. The intraosseous defect on the left side of the guinea pig is being enlarged with a number 4 P.D. reamer. Note the depth marker on the reamer (arrow). The implant on the right side of the guinea pig is *in situ*.

The wound was then washed with sterile Ringer's solution and the implant, held in a pair of modified artery forceps (referred to previously) inserted into the defect.While still holding the implant in place, it was cut level with the surface of the mandible (Figs. 3.17 and 3.18)

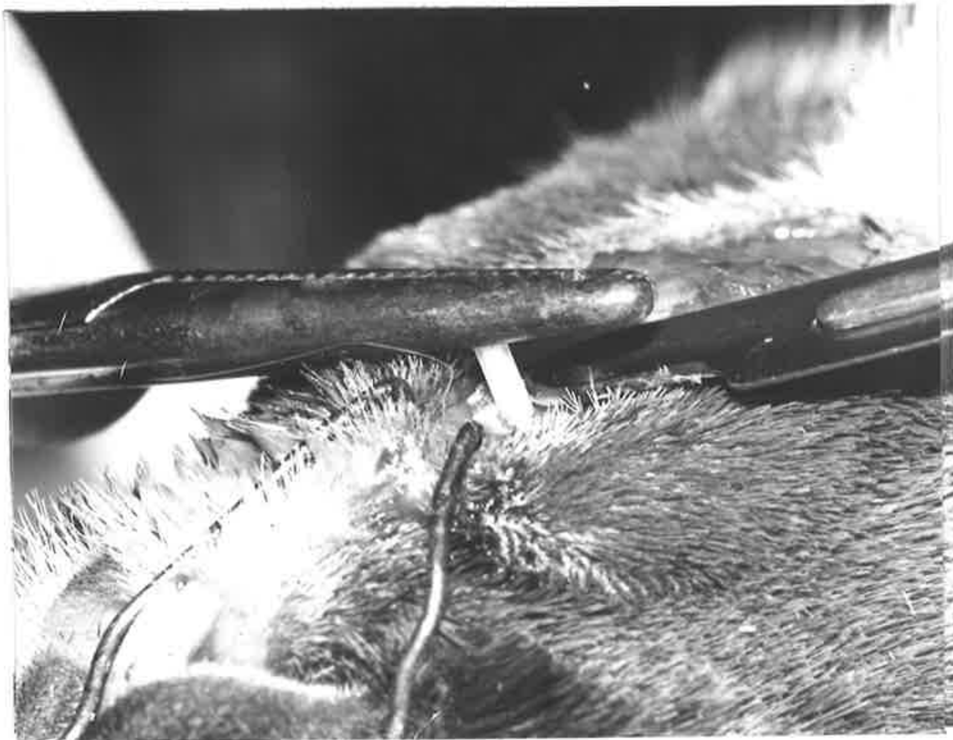

FIGURE 3.17: Photograph illustrating the intraosseous implanting technique. The implant held in the modified artery forceps, has been inserted into the bony defect and is being cut level with the surface of the mandible.

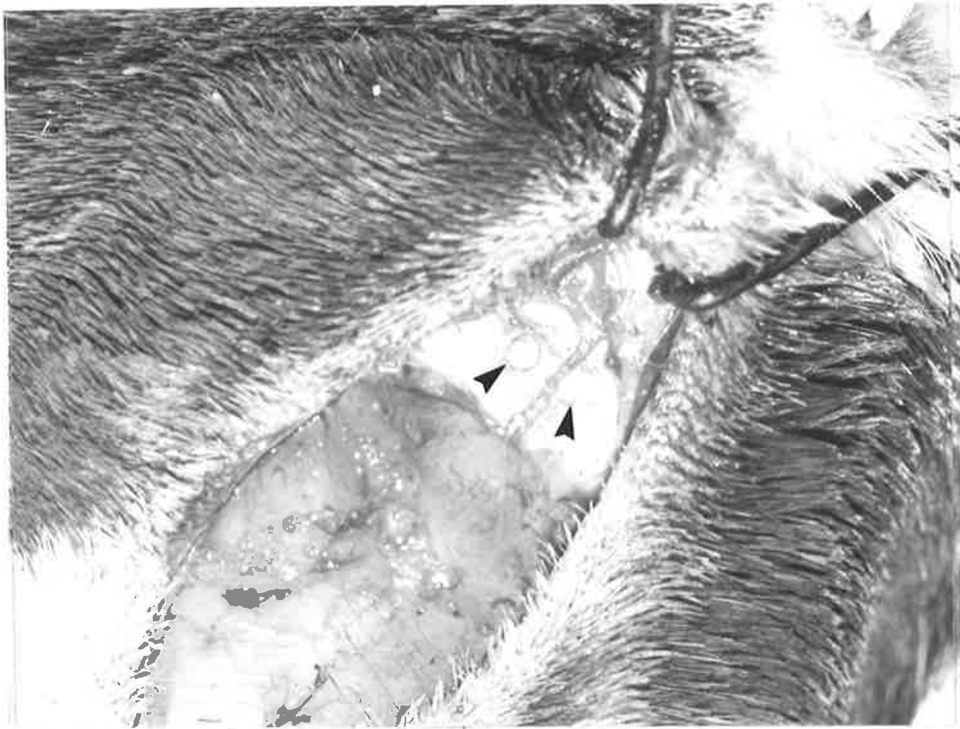

FIGURE 3.18: Photograph illustrating the final stages of the intraosseous implantation technique prior to closure. The implants (arrows) on both sides are *in situ* having been cut level with the surface of the bone.

The wound was then closed in layers using 6/0 silk for internal sutures and 4/0 silk for external sutures. In the early stages of this experiment some internal suturing was with Dexon. Use of the latter had to be discontinued because of lack of supply.

(ii) Intramuscular Implantation

An incision was made through the skin on the lateral aspect of the thigh, exposing the underlying ilio-tibial muscle (Fig. 3.19).

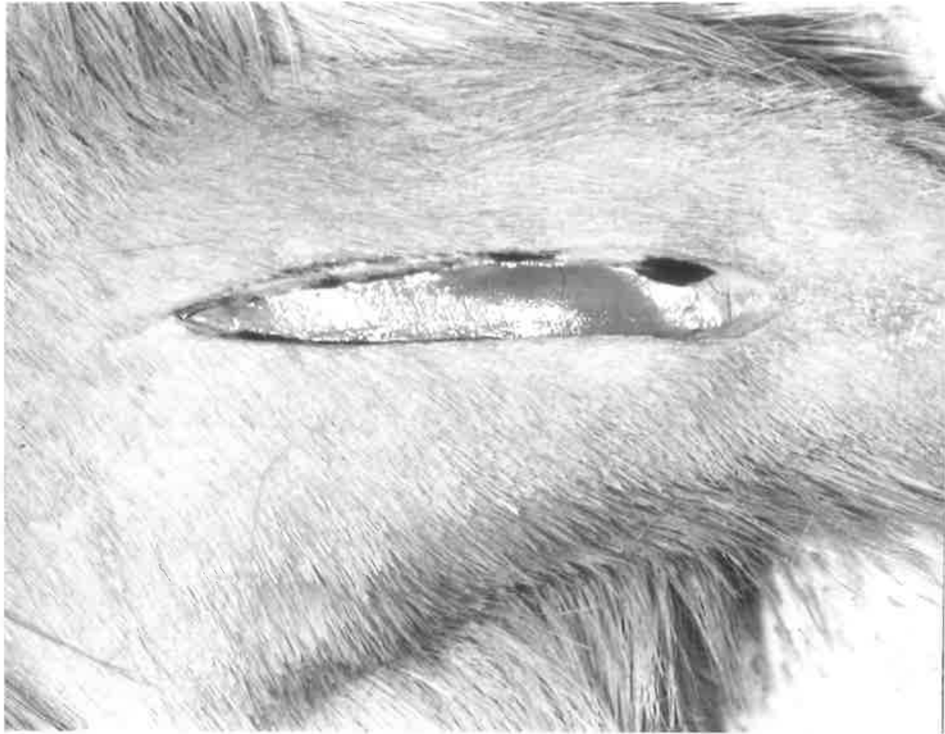

FIGURE 3.19: Photograph illustrating the initial surgical incision in the skin of the thigh, in preparation for the intramuscular implantation. The underlying ilio-tibial muscle has been exposed by this incision.

The ilio-tibial muscle layer was then incised revealing the quadriceps muscle and the major neuro-vascular bundle (Fig. 3.20).

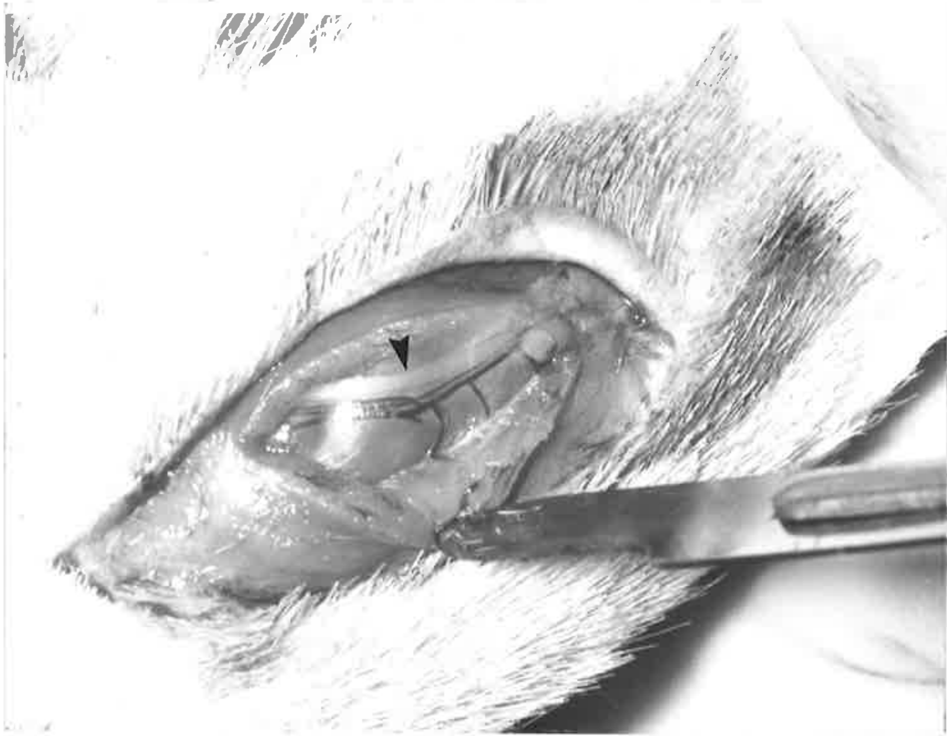

FIGURE 3.20: Photograph illustrating the initial surgical stage in the preparation of the intramuscular implantation site. The ilio-tibial muscle has been incised revealing the major neuro-vascular bundle (arrow) and the quadriceps muscle.

Taking care not to damage the neuro-vascular bundle, a small incision was then made in the exposed quadriceps muscle. The incision was made parallel to the muscle fibres and deep enought to accommodate the implant (Fig. 3.21).

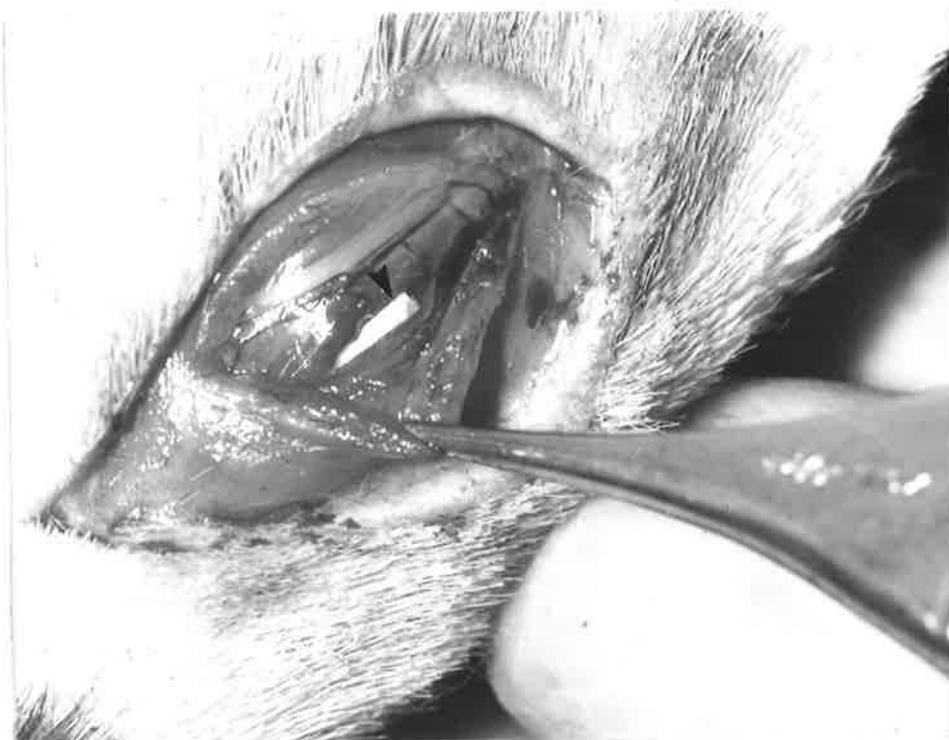

FIGURE 3.21: Photograph illustrating the site of the intramuscular implantation. A small pocket has been created in the quadriceps muscle adjacent to the neuro-vascular bundle. The intramuscular implant (arrow) has been placed into this pocket.

While ensuring that the implant is in the correct position, the incision in the quadriceps muscle was closed with a 6/0 silk suture (Fig. 3.22).

FIGURE 3.22: Photograph illustrating the initial closure of the wound following intramuscular implantation. With the intramuscular implant in position the incision in the quadriceps muscle is closed with a single suture.

The incision in the ilio-tibial tract of muscle was subsequently closed with interrupted 4/0 silk sutures. Following this, the skin incision was closed with a continuous 4/0 silk suture (Fig. 3.23).

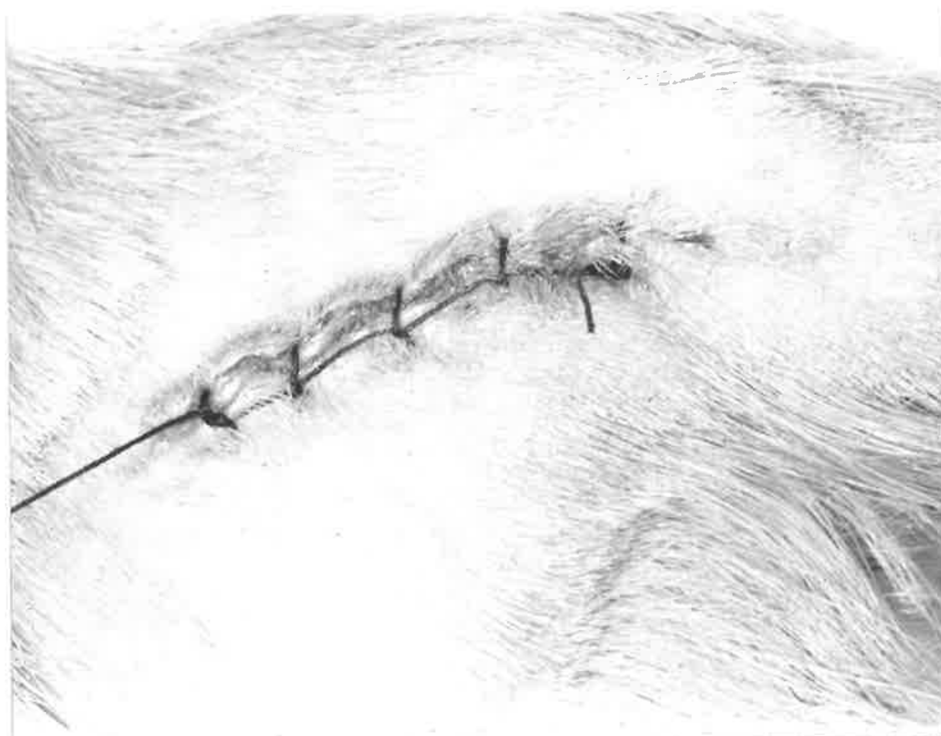

FIGURE 3.23: Photograph illustrating the final closure of the wound following intramuscular implantation. The skin incision has been closed with a continuous 4/0 silk suture as was the cutaneous wound in the neck.

3.2.10 HARVESTING OF TISSUES

At the pre-designated times post-implantation (Section 3.2.3) the animals were sacrificed with an overdose of barbiturate, injected into the heart. Blocks of both hard and soft tissue containing the implants were immediately dissected out and fixed in neutral buffered formalin (Appendix II) for at least 2 days. A total of 101 intraosseous implant sites and 100 intramuscular implant sites were harvested for histologic processing.

3.2.11 HISTOLOGICAL TECHNIQUE

(i) Decalcification of Bone

Each piece of bone containing an implant was decalcified following fixation. The decalcifying solution was Decal* diluted with an equal volume of distilled water. Progress with the decalcification process was monitored from time to time by radiographing the tissue. Exposures were made for 0.15 seconds. The tube settings were 44 kv and 100 mAmps. High resolution, single emulsion film was used in conjunction with an intensifying screen. Decalcification was usually completed after 30 hours in the diluted Decal solution.

(ii) Embedding

Both hard and soft tissue specimens were double embedded in paraffin wax (Appendix III).

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* Omega Company, U.S.A.

(iii) Sectioning

Following location of the implant site, in both hard and soft tissue, the block was aligned in a Leitz* rotary microtome in such a manner that the long axis of Teflon tube containing the implant material was parallel to the cutting plane. Serial 7μ sections were cut with a sharp steel microtome knife. Sections were flattened in a 40°C water bath and baked onto clean glass microscope slides at 60°C.

(iv) Selection of Sections for Assessment

(a) Intraosseous implant specimens

A total of 101 blocks of bone, each containing an implant of either Hydron, AH26 or Teflon were recovered (Table 3.1) and processed. Serial sections cut from these blocks were mounted on glass microscope slides in two rows of 6 sections per slide. An average of 8 slides were prepared in this manner from each block, i.e. a total of 800 slides containing upwards of 9,000 sections from the 101 blocks. All slides were stained with haematoxylin and eosin (Appendix IV). These stained sections were then examined using a light microscope, to select those sections in which the central mark produced by the P.D. tapering reamer could be distinguished (refer Figs. 3.5 and 3.6). When this mark could be readily distinguished, the best slide was identified and subsequently used for assessment. It was not uncommon with sections from the longer post-implantation time

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* Wild Leitz, Wetzlar, West Germany.

	HYDRON	AH26	TEFLON
2 days	6	6	6
1 week	6	6	6
2 weeks	5	7	4
4 weeks	5	5	6
12 weeks	6	5	3
26 weeks	7	6	6

TABLE 3.1: The number of blocks of bone, each containing either experimental or control material recovered from the various time periods post-implantation.

periods for the mark to be poorly distinguished or be in-distinguishable (because of bone remodelling). However, when the series of sections from a particular block was viewed, a section from the centre of the tube could be readily distinguished by changes in the shape of the original defect. In this case, the best section from this area was identified and used for future assessments.

(b) Intramuscular implant sites

A total of 100 blocks of muscle each containing an implant of either Hydron, AH26, or Teflon were recovered (Table 3.2). Serial sections cut from these blocks were mounted on glass microscope slides (5 per slide) and sections from the central area of the tube selected for future assessments. An average of 20 slides were prepared in this manner from each block, i.e. a total of 2,000 slides containing 10,000 sections. Six consecutive slides containing sections from the central area of the tube (implant) were selected and stained according to the following schedule:

the first, third and sixth - haematoxylin and eosin (Appendix IV);

the second and fifth - Van Gieson's stain (Appendix V);

the fourth - P.T.A.H. (Appendix VI).

	HYDRON	AH26	TEFLON
2 days	5	6	6
1 week	6	3	6
2 weeks	5	6	6
4 weeks	6	6	7
12 weeks	5	6	4
26 weeks	6	6	5

TABLE 3.2: The number of blocks of muscle, each containing either experimental or control material recovered from the various time periods post-implantation.

Following examination of sections stained with H. & E. during assessment, it appeared that some form of mineralization was occurring at the interface of the Hydron and the soft tissues (Fig. 4.42). A section from each block of muscle containing a Hydron implant was subsequently stained using a modified von Kossa stain (Appendix VII).

Selected sections were stained with Prussian Blue (Appendix VIII) for the purpose of distinguishing particles of haemosiderin from other phagocytosed material in macrophages.

Other sections from the early time periods post-implantation were stained with Lendrum's Martius-Scarlet-Blue method for fibrin (Appendix IX) for the purpose of distinguishing the fibrin.

To confirm the validity of the above selection method, the whole series of sections from 6 blocks were stained with H. & E. The area immediately adjacent to the implant was photographed at x400 magnification and 8" x 10" black and white prints prepared. The total cell population in these photographs were counted and the results expressed graphically. An example of the results from the above procedure is shown in Fig. 3.24. While there was considerable variation between both ends, the total cell population in the central area of the tube, at each end remained relatively constant. This confirmed the validity of the above method for selecting sections for assessment.

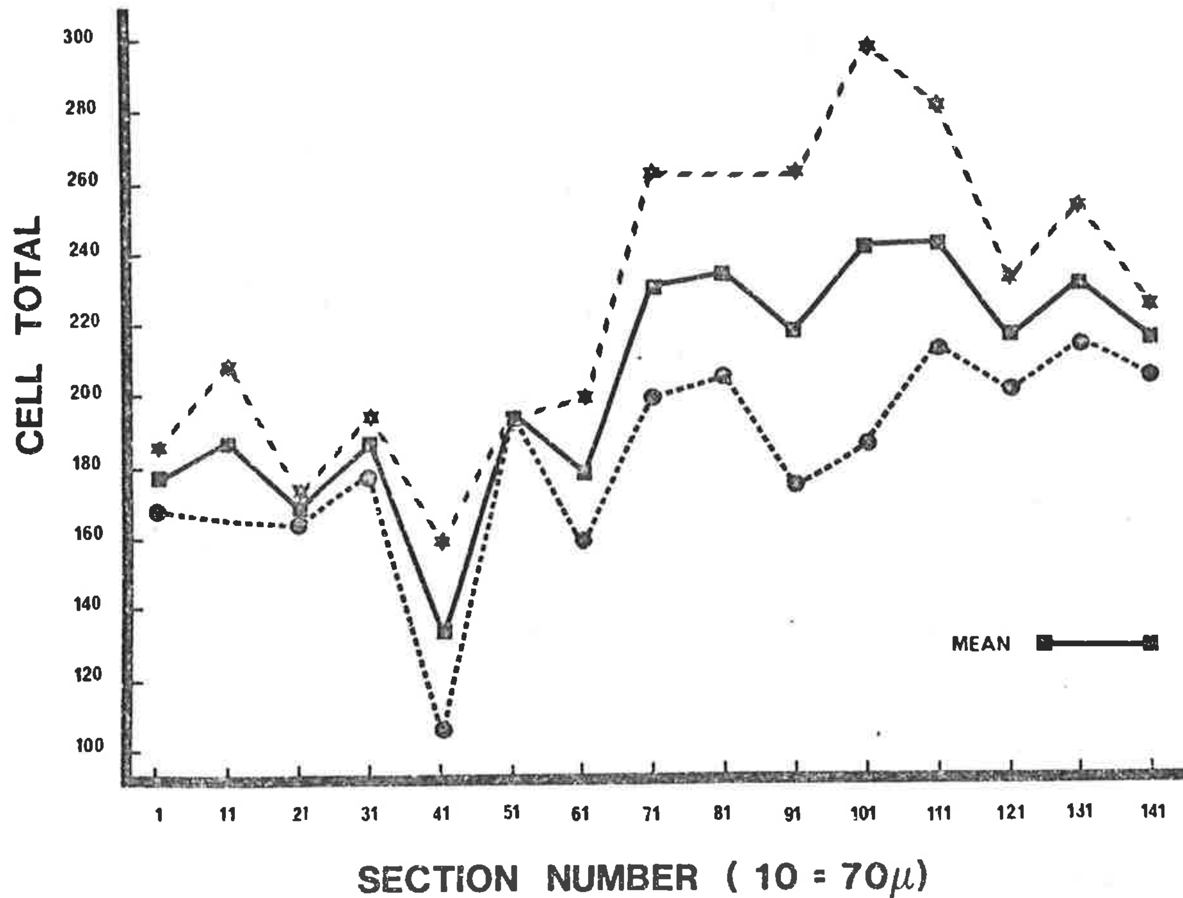

FIGURE 3.24: Graph illustrating the total number of cells counted in x400 fields each 70μ deeper into the block of tissue surrounding the implant. The data shown is from a Hydron implant, 12 week post-implantation. A plateau is reached corresponding to the central area of the tube.

It was the norm to have considerable variation between the reactions seen at both the ends of each tube. This possibly reflected the slight differences in the orientation of the tube ends in the muscle. However, since it occurred in virtually all cases, and at all time periods, all results should have been equally affected.

3.2.12 SCANNING ELECTRON MICROSCOPE TECHNIQUE

As was stated in Section 3.2.11, some sections were stained with Prussian Blue. In the muscle sections containing Hydron implants (Fig. 3.25) it was noticed that some stain was absorbed in an area corresponding to the zone of apparent mineralization noted in the H. & E. sections. As a result of this observation, there was some doubt as to the composition of the small apparent calcifications. It was therefore decided to analyse these entities and the adjacent area using a scanning electron probe.

(i) Preparation of Specimens

Dewaxed and dehydrated histological sections mounted on a clean glass microscope slide were prepared for probe examination. A carbon coating of approximately 100 Å was deposited over the sections by vacuum evaporation. The particular sections analysed contained Hydron and adjacent soft tissues 4 weeks post-implantation.

FIGURE 3.25: Hydron implant 4 weeks post-implantation in muscle.

Photomicrograph illustrating several areas within the Hydron implant (arrows) adjacent to the interface with the soft tissues, have absorbed some stain.

Prussian Blue. Original magnification x400.

(ii) S.E.M. Equipment

This analysis was conducted with a JOEL 733 superprobe* with an ORTEC EDDS 11 energy dispersive analysis system in conjunction with a scanning electron microscope (Fig. 3.26). Operating conditions were 15 kv and 5 nano Amps. This equipment was located at the Electron Optical Centre at the University of Adelaide and was operated by staff of the Centre.

3.2.13 HISTOLOGIC ASSESSMENT OF RESULTS

(i) Assessment Following Intraosseous Implantation

The tissue harvested at the designated time periods post-implantation (Section 3.2.3) containing implants of Hydron, AH26 and Teflon were assessed histologically. This study had been planned with a view to studying specifically what effect the implanted material might have on osseous repair as well as cellular responses to the material in general, following its implantation in bone. It was hoped that in some way, the effect of the material on osseous repair might be able to be quantitated. In fact, this proved not to be the case. The assessment methodology employed was a qualitative analysis of the histologic process evident following implantation of the various materials for the pre-designated times. Special attention has been given to the initiation and extent of osseous repair and the relationship between the regenerated bone and the implant.

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* JOEL, Japan.

FIGURE 3.26: Photograph showing the scanning electron microscope and probe used in this analysis. The unit on the right is the JOEL 733 superprobe. The unit on the left is the actual electron microscope while the unit in the centre contains the controls and display screen of the scanning electron microscope.

(ii) Assessment Following Intramuscular Implantation

The tissues harvested at the designated time periods post-implantation (Section 3.2.3) containing implants of Hydron, AH26 and Teflon were assessed histologically. The method of Gourlay et al (1978) was considered the most comprehensive assessment method. It was hoped, when this experiment was being planned, that a method similar to that employed by Gourlay could be used for the assessment of results.

The quantitation of differences in inflammatory cell populations formed the basis of Gourlay's assessment. However, since there were virtually no inflammatory cells present in the tissues adjacent to the implant at 2 weeks post-implantation and time periods thereafter, this method was thus not entirely applicable to the present study. It was therefore decided that as many indicators as possible would be investigated in order to assess the biocompatibility of the experimental and control materials used in the present study. The following parameters were investigated in the tissues adjacent to both ends of the various implants:

(a) the nature and extent of the capsular zone (including the inflammatory responses);

(b) the foreign body giant cell response;

(c) the macrophage response;

(d) the presence and extent of adipose fat cells;

(e) the nature and extent of any mineralization that might occur.

Where possible these results were quantitated. However, where this was not possible, a description of the histologic processes following implantation formed the assessment. Quantitation of results was possible in *(c)* and *(d)*. The quantitative assessments were carried out by placing 1 cm square gradicule over the eyepiece of a light microscope. The lens was rotated until one side of the counting square was aligned with the surface of the implant. The counting square is divided into 100 equal smaller squares. Total counts were obtained by the summation of the counts made per small square.

The significance of differences between the amounts of adipose fat associated with the various implants was tested using Student t-tests (Section 3.2.14). The distribution of macrophages was tested using the Poisson distribution (Section 3.2.14).

3.2.14 STATISTICAL METHODS

(i) Student's t-test

The significance of differences between the variances was assessed by Snedecor's F-test and the significance of differences between the means was tested by Student's t-test *(1)*. If the variances differed significantly, the values of t were calculated according to *(2)* (Hald, 1952).

Snedecor's F-test $\frac{S^2 \text{max}}{S^2 \text{min}}$

(1) $$\frac{(\bar{x}_1-\bar{x}_2)}{\sqrt{\left(\frac{1}{N_1}+\frac{1}{N_2}\right)\frac{(N_1-1)s_1^2+(N_2-1)s_2^2}{N_1+N_2-2}}}$$

(2) $$\frac{(\bar{x}_1-\bar{x}_2)}{\sqrt{\frac{s_1^2}{N_1}+\frac{s_2^2}{N_2}}}$$

The levels of significance were 1% and 5%.

(ii) Poisson Distribution

(a) *Introduction*

The distribution of macrophages phagocytosing Hydron was determined by using the Poisson distribution. To be distributed in a Poisson fashion a variable must have the following properties:

the mean must be small relative to the maximum possible number of events per sampling unit, i.e. the event must be rare;

the occurrence of the event must be independent of prior occurrences within the sampling unit.

The purpose of fitting a Poisson distribution to numbers of rare events is to test whether the events occur independently, with respect to each other. If they do, they will follow the Poisson distribution. If the occurrence of one event enhances the probability of a second such event, a clumped distribution is obtained. If the occurrence of one event impeded that of a second such event in the sampling unit a repulsed distribution is obtained (Sokal and Rohlf, 1969a).

(b) *Coefficient of dispersion*

In a Poisson distribution the variance is equal to the mean. The value obtained by dividing the variance by the mean is known as the coefficient of dispersion:

$$C.D. = \frac{S^2}{\bar{Y}}$$

As stated above, the coefficient of dispersion in a true Poisson distribution = 1. A coefficient of dispersion value > 1 will indicate a clumping of samples, and a value < 1 will indicate repulsion between samples (Sokal and Rohlf, 1969a).

The coefficient of dispersion is an easy computation to make to see if observed data might fit a Poisson distribution, before calculating expected occurrences and determining how good the fit is between observed and expected occurrences.

(c) Calculation of a Poisson distribution

The Poisson distribution is an approximation of the Binomial distribution which is usually expressed as: $(r + \pi)^N$. Should either of the values π (or r) become very small while N approaches infinity, it can be shown (Wilks, 1948) that the probability of an event Y occurring r times is:

$$\frac{\mu^r}{r!\, e^{\mu}}, \text{ where:}$$

e is the base of the natural logarithm and is a constant = 2.71828, accurate to 5 decimal places;

μ is the parametric mean of the distribution and is constant for any given example;

r is a discrete value and represents the number of events of Y that occurred in any particular example, i.e. 0, 1, 2, 3, 4 ..... r.

For the calculation of expected occurrences it is more convenient to write the above formula as:

$$\frac{1}{e^{\bar{Y}}},\ \frac{1}{e^{\bar{Y}}}\left(\frac{\bar{Y}}{1}\right),\ \frac{\bar{Y}}{e^{\bar{Y}}}\left(\frac{\bar{Y}}{2}\right),\ \frac{\bar{Y}^2}{2e^{\bar{Y}}}\left(\frac{\bar{Y}}{3}\right),\ \frac{\bar{Y}^3}{2x3e^{\bar{Y}}}\left(\frac{\bar{Y}}{4}\right),\ \ldots\ldots\ \frac{\bar{Y}^{(r-1)}}{(r-1)!e^{\bar{Y}}}\left(\frac{\bar{Y}}{r}\right)$$

i.e. for the purpose of calculation, the first number $\frac{1}{e^{\bar{Y}}}$ is calculated. The second number of occurrences is obtained by multiplying the second number by $\frac{\bar{Y}}{1}$. The third number of occurrences by multiplying the second number by $\frac{\bar{Y}}{2}$ and so on.

Thus, the number of occurrences can be calculated so long as the mean of the sample is known. These values can also be obtained from tables (Pearson and Hartley, 1958; Molina, 1942).

(d) χ^2 *test for goodness of fit*

Observed numbers of macrophages were compared statistically with those expected assuming a Poisson distribution by mean of the Chi-square test for goodness of fit (Sokal and Rohlf, 1969b), where:

$$\chi^2 = \frac{\Sigma\,(O-E)^2}{E}$$

O = observed number of macrophages;

E = expected number of macrophages (Section *c*).

The level of significance was 5%.

(iii) Errors

To ensure the validity of each series following quantitation, on a different day, randomly selected samples were recounted as per the original method. Discrepancies between first and second counts were small and showed no systematic trends. The interpretation of results was not altered whether the first or the second quantitation was used.

(iv) Correlation of Variance

The formula used for the correlation of variance was:

$$r = \frac{\Sigma xy - \frac{(\Sigma x)\,(\Sigma y)}{N}}{\sqrt{\left(\Sigma x^2 - \frac{(\Sigma x)^2}{N}\right)\left(\Sigma y^2 - \frac{(\Sigma y)^2}{N}\right)}}$$

where: r = correlation coefficient;

x and y = are the two variables respectively;

N = number of observations involving both variables.

(Croxton, 1953)

CHAPTER 4.

RESULTS.

4.1 GENERAL OBSERVATIONS

A total of fifty-seven guinea pigs were implanted, of which fifty-four received two intraosseous and two intramuscular implants. In the three remaining animals, where the mandible on one side was damaged during the preparation of the defect, intraosseous implants were placed on one side only and intramuscular implants bilaterally.

All animals recovered uneventfully from the intramuscular Ketamine anaesthetic. The immediate post-operative phase of recovery was uneventful in all cases.

In the early stages of the experiment, four deaths occurred at times ranging from one to four weeks post-operatively. The body of one of these animals was submitted to the Institute of Medical and Veterinary Science for an autopsy. Exhaustive microbiological and histological investigations of numerous tissues and organs failed to determine a cause of death.

In the post-operative healing phase, a number of thigh wounds ruptured. The damage appeared to be superficial, not involving the underlying muscle. These animals were re-anaesthetized, the wound debrided and the skin resutured. Healing in all cases was subsequently uneventful.

The recovery of bony tissue containing the implants was found to be a relatively simple procedure. In a few instances the implant had moved from its original position and the resulting specimens proved unsuitable for assessment. Notwithstanding this, 101 blocks of bone containing implants of either Hydron, AH26 or Teflon were recovered (Table 3.1) and prepared for histological assessment.

The recovery of tissue containing the implant from some intramuscular sites proved to be impossible. At 12 and 26 weeks, some intramuscular locating sutures could not be found. This made location of the implant within the tissues difficult if not impossible. In other instances, even though the sutures could be located, no implant could be found. Despite this, 100 blocks of tissue containing implants were recovered (Table 3.2), and prepared for histological assessment.

No bacterial growth was observed after incubation of selected implants of Hydron, AH26 and Teflon, indicating the sterility of implants at the time of implantation.

4.2 HISTOLOGICAL OBSERVATIONS AND RESULTS

4.2.1 BONE

(i) Two Days Post-Implantation

(a) Hydron

Low power microscopic observations revealed that in all cases the outline of the bony defect was readily apparent (Fig. 4.1). The bone immediately surrounding this defect contained either empty lacunae or lacunae with karyolytic osteocytes (Fig. 4.2). Some bone stromal tissues surrounding the defect were acutely inflammed.

High power microscopic observations of the Hydron implant specimens revealed that the triangular defect between the Hydron and the bone was characterized by the presence of fibrin, extravasated erythrocytes and polymorphonucler leukocytes (similar to that seen in Figs. 4.3 and 4.5). Occasional eosinophils and lymphocytes were also noted. Bone chips were numerous (similar to that observed in Fig. 4.5) especially near the apex of the triangular defect (i.e. farthest from the implant).

(b) Controls

Both low and high power microscopic observations of specimens containing AH26 and Teflon implants 2 days post-implantation (Figs. 4.3, 4.4 and 4.5) were characterized by essentially the same features as those observed in specimens containing Hydron implants 2 days post-implantation.

FIGURE 4.1: HYDRON, 2 DAYS POST-IMPLANTATION IN BONE.

Photomicrograph illustrating the outline of the defect in bone (arrows). The Hydron implant (H) has been lost during processing as has the Telfon tube (T) surrounding it. Between the Hydron implant and the walls of the bony defect are large numbers of extravasated erythrocytes (E) and fibrin (F). The structure at the bottom centre of this photomicrograph (P.L.) is the periodontal ligament of the guinea pig incisor.

H. & E. original magnification x40

FIGURE 4.2: HYDRON, 2 DAYS POST-IMPLANTATION IN BONE.

Photomicrograph illustrating the bone and stromal tissues adjacent to the walls of the bony defect. Some lacunae contain apparently normal osteocytes while others are empty. Nuclear dust (D) is noted in some lacunae.

H. & E. original magnification x400.

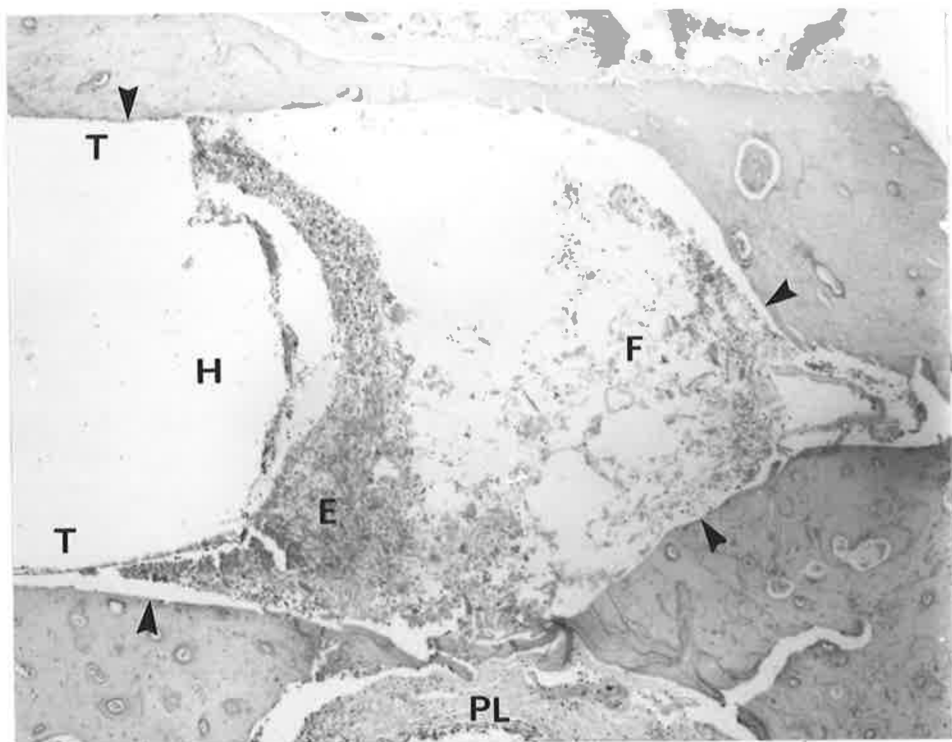

FIGURE 4.1

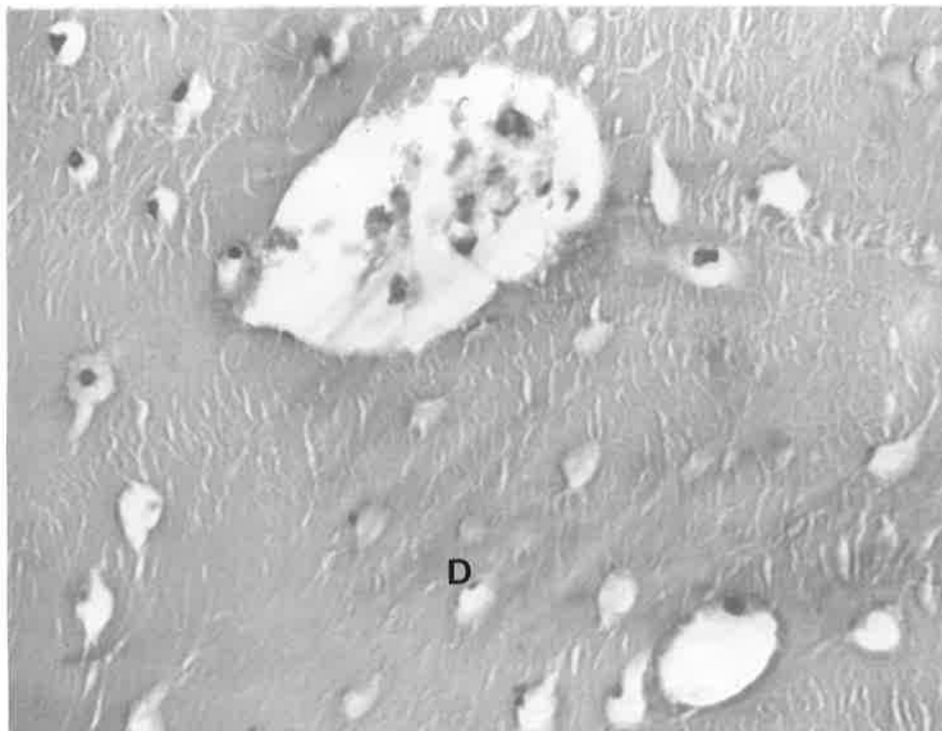

FIGURE 4.2

FIGURE 4.3: AH26, 2 DAYS POST-IMPLANTATION IN BONE.

Photomicrograph illustrating fibrin and extravasated erythrocytes in the area between the implant and the the bone. Note the empty lacunae in the bone, on the right side of this photomicrograph.

H. & E. original magnification x400.

FIGURE 4.4: TEFLON, 2 DAYS POST-IMPLANTATION IN BONE.

Photomicrograph illustrating the outline of the bony defect (arrows). The Teflon implant (T) was normally lost during processing as was the Teflon tube surrounding it. Between the Teflon implant and the walls of the bony defect are large numbers of extravasated erythrocytes (E) and fibrin (F).

H. & E. original magnification x40

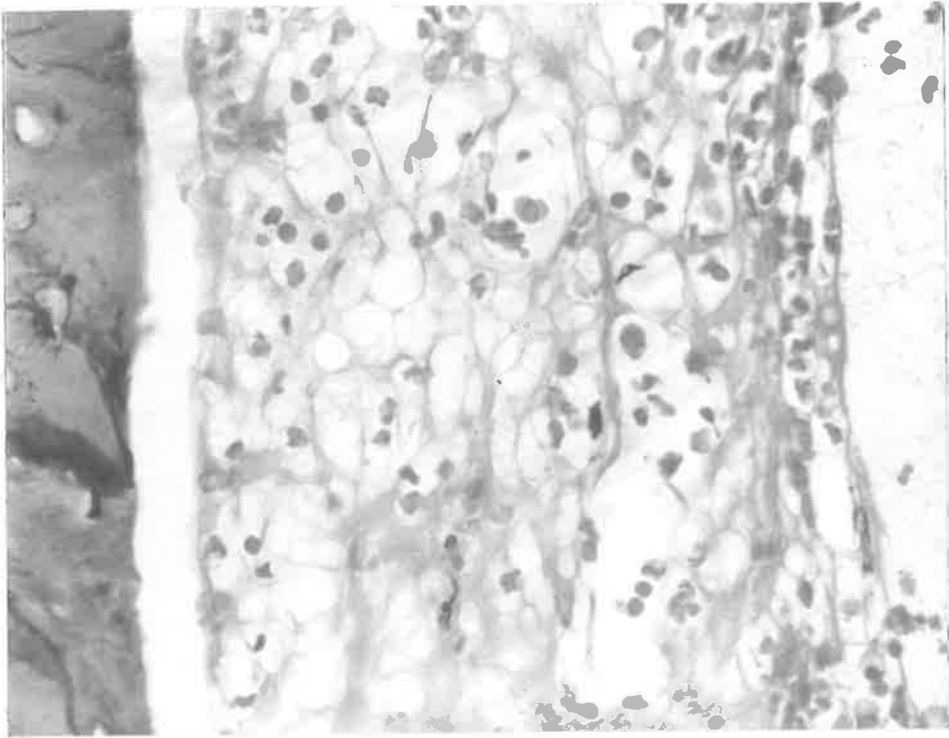

FIGURE 4.3

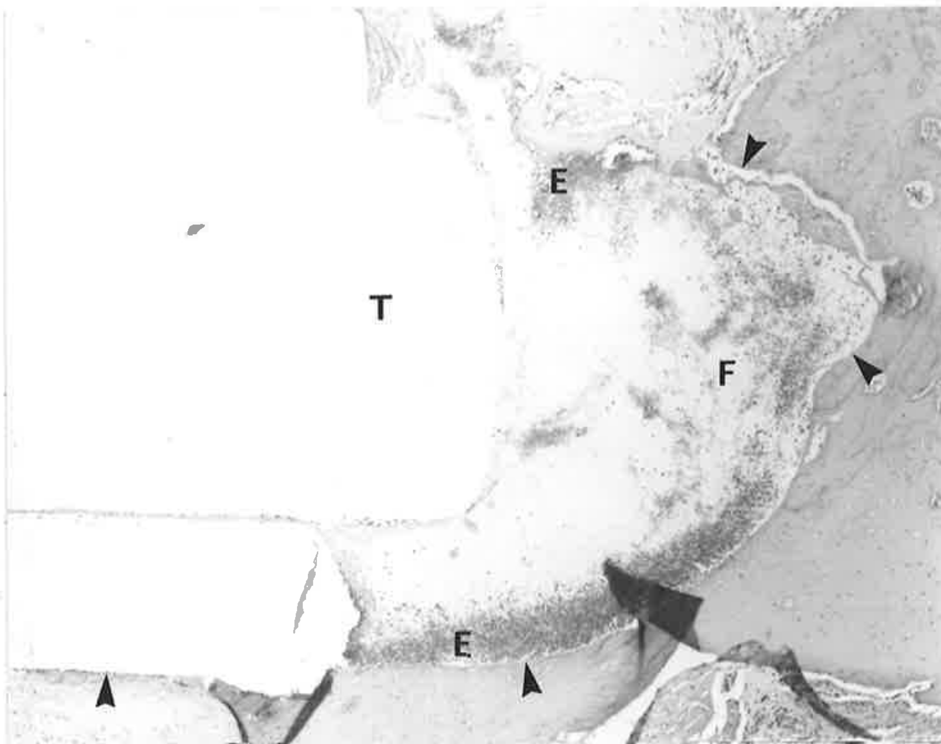

FIGURE 4.4

FIGURE 4.5: TEFLON, 2 DAYS POST-IMPLANTATION IN BONE.

Photomicrograph illustrating the contents of the defect. Polymorphonuclear leukocytes, extravasated erythrocytes and quantities of fibrin are apparent within the defect. The bone (B) of the wall of the defect is shown in the lower portion of this photomicrograph. Note the bone chips (arrows) within the defect.

H. & E. original magnification x 400

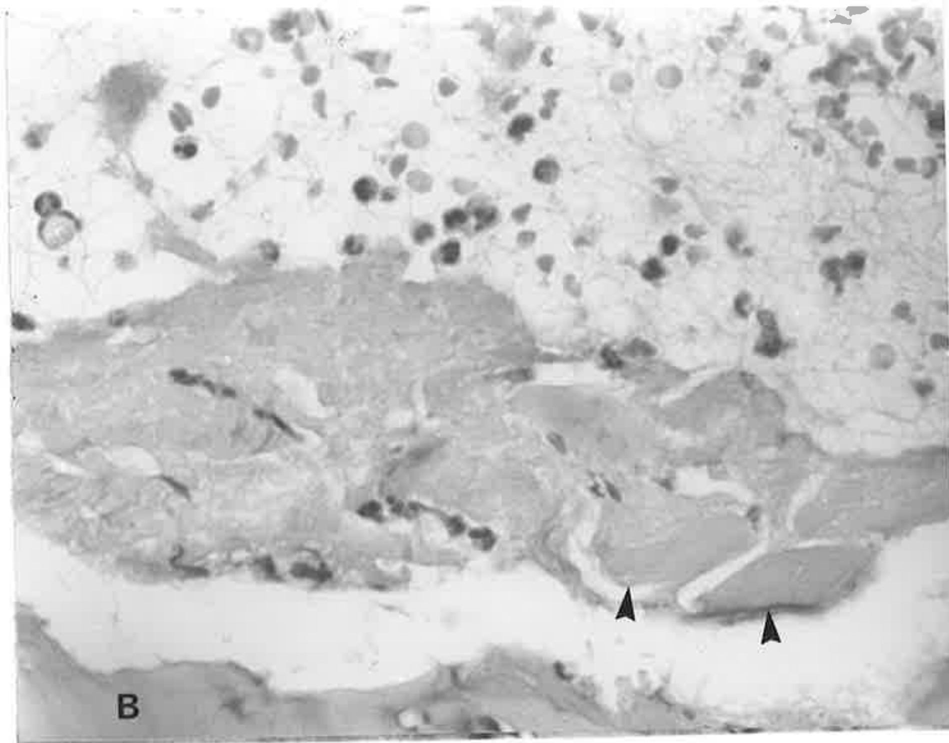

FIGURE 4.5

(ii) One Week Post-Implantation

(a) Hydron

Low power microscopic observations revealed that in all cases the outline of the bony defect was readily apparent (Fig. 4.6). The bone surrounding the defect contained many empty lacunae (Fig. 4.8). The marrow tissues were inflamed except in the cases referred to below where there was no inflammation.

High power observation of Hydron implant specimens revealed that the triangular defects between the Hydron and the bone were characterized, in 4 of the 6 specimens, by the presence of fibrin (Fig. 4.8) and extravasated erythrocytes. Acute and chronic inflammatory cells were evident and occurred either in clumps or scattered through the area (Fig. 4.8). The remaining two specimens were characterized by the present of immature fibrous connective tissue consisting of fibroblasts, small amounts of collagen and numerous macrophages. Some of these macrophages contained phagocytosed particles of Hydron similar to those observed 2 weeks post-implantation (Refer Fig. 4.12) and occurred in proximity to the Hydron implant. The marrow in the adjacent bone of these 2 specimens exhibited no overt signs of inflammation.

There was osteoclastic activity in the marrow of the bone in all 6 cases; as evidenced by the presence of Howships lacunae and osteoclasts (Fig. 4.7).

(b) Controls

AH26

Observations of AH26 implant specimens revealed a picture similar to that of the 2 Hydron specimens 1 week post-implantation which exhibited granulation tissue - immature fibrous connective tissue filling the defect. There was intense osteoclastic activity evident in the bone associated with all AH26 specimens.

Teflon

Low power observations of Teflon specimens revealed features similar to those exhibited by Hydron and AH26 specimens in that the outline of the bony defect could be clearly identified (Fig. 4.9).

Microscopic observations, at higher power, revealed that the triangular defect was characterized by immature fibrous connective tissue (Fig. 4.9). This tissue consisted of fibroblasts, collagen, large "foamy" macrophages, extravasated erythrocytes and the occasional foreign body giant cell. The latter appeared to be associated with the surface of the Teflon implant. In 4 of the 6 specimens small spicules of woven bone were noted in the triangular area (Figs. 4.9 and 4.10) away from both the bone of the wall and the Teflon implant.

Inflammatory cells were not observed in the 4 Teflon specimens which exhibited woven bone formation in the defect. Signs of inflammation,as evidenced by the presence of polymorphonuclear leukocytes,were noted in the marrow tissues of the remaining 2 Teflon implant specimens.

FIGURE 4.6: HYDRON, 1 WEEK POST-IMPLANTATION IN BONE.

Photomicrograph illustrating the inflammatory exudate and extravasated erythrocytes that are present between the Hydron implant (H) and the readily apparent walls of the defect. Note the Howships lacunae (arrows) and osteoclasts associated with the bone adjacent to the defect.

H. & E. original magnification x40

FIGURE 4.7: HYDRON, 1 WEEK POST-IMPLANTATION IN BONE.

Photomicrograph illustrating a higher power view of the area of osteoclastic activity noted in Fig. 4.6. Multinucleated osteoclasts (M) are located within Howships lacunae. Extravasated erythrocytes and polymorphonuclear leukocytes are evident in the immature fibrous connective tissue. Note the bone lacunae of the defect wall are empty.

H. & E. original magnification x400

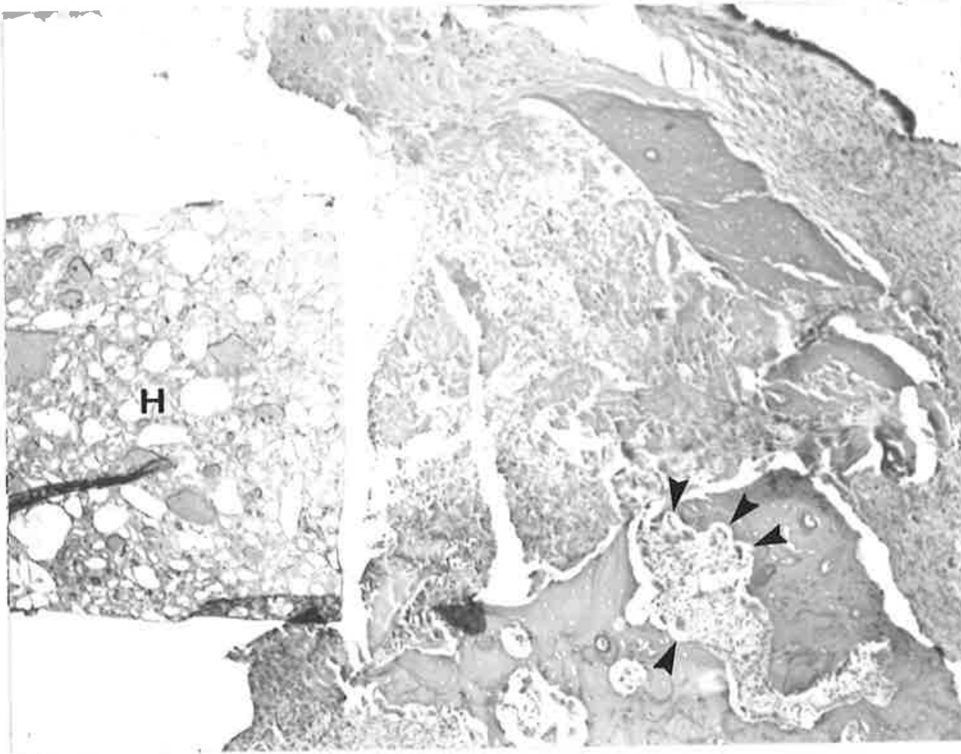

FIGURE 4.6

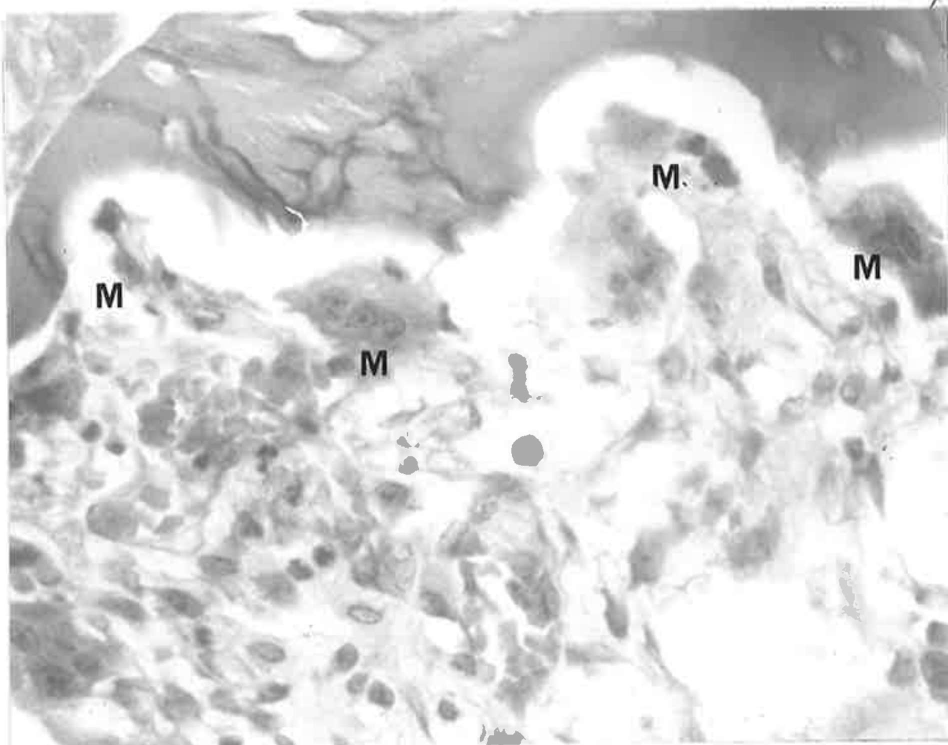

FIGURE 4.7

FIGURE 4.8: HYDRON, 1 WEEK POST-IMPLANTATION IN BONE.

Photomicrograph illustrating an area of the wall of the defect. The marrow (M) is acutely inflamed as evidenced by the presence of numerous polymorphonuclear leukocytes. Polymorphonuclear leukocytes are also present between walls of the defect and the implant and here are present in clumps or scattered through the fibrin (F). Note the numerous empty lacunae in the bone.

H. & E. original magnification x 400

FIGURE 4.9: TEFLON, 1 WEEK POST-IMPLANTATION IN BONE.

Photomicrograph illustrating immature fibrous connective tissue between the Teflon implant (T) and the acellular bone (A) of the wall of the defect. Several small spicules of bone (S) are located within this immature fibrous connective tissue.

H. & E. original magnification x40.

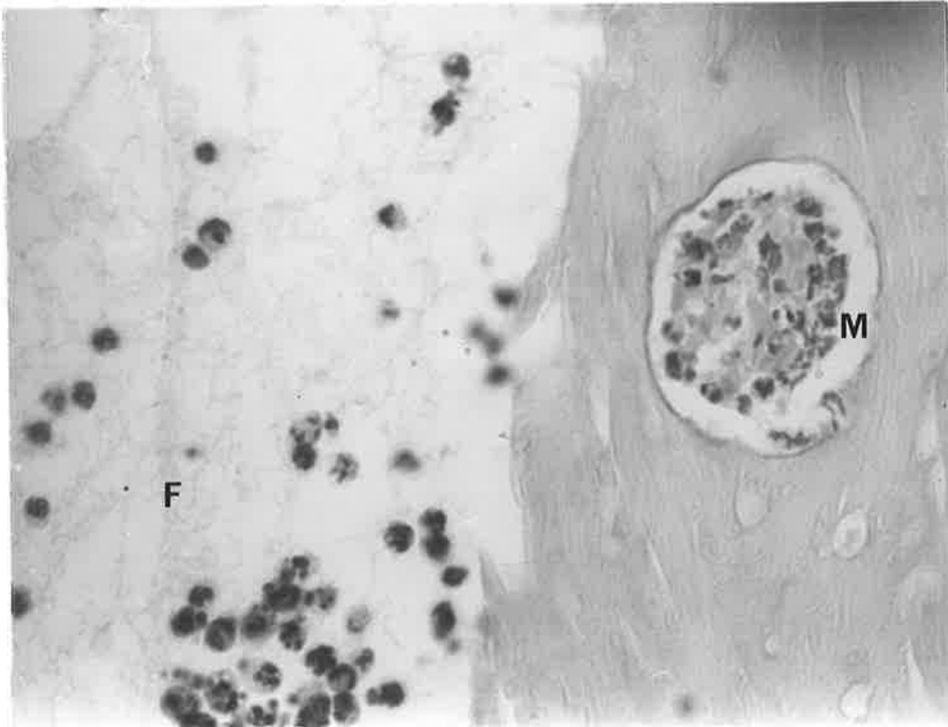

FIGURE 4.8

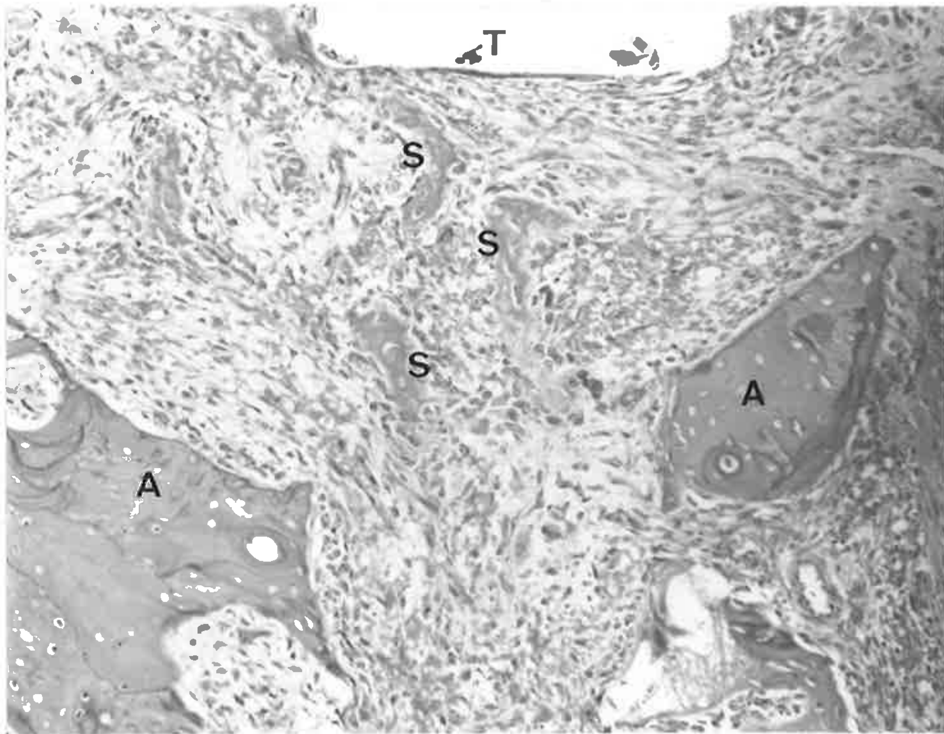

FIGURE 4.9

FIGURE 4.10: TEFLON, 1 WEEK POST-IMPLANTATION IN BONE.

Photomicrograph illustrating at higher magnification the spicules of bone apparent in Fig. 4.9. The bone is woven bone. Note the large immature osteoblasts. Note also the numerous extravasated erythrocytes in the surrounding immature fibrous connective tissue.

H. & E. original magnification x400

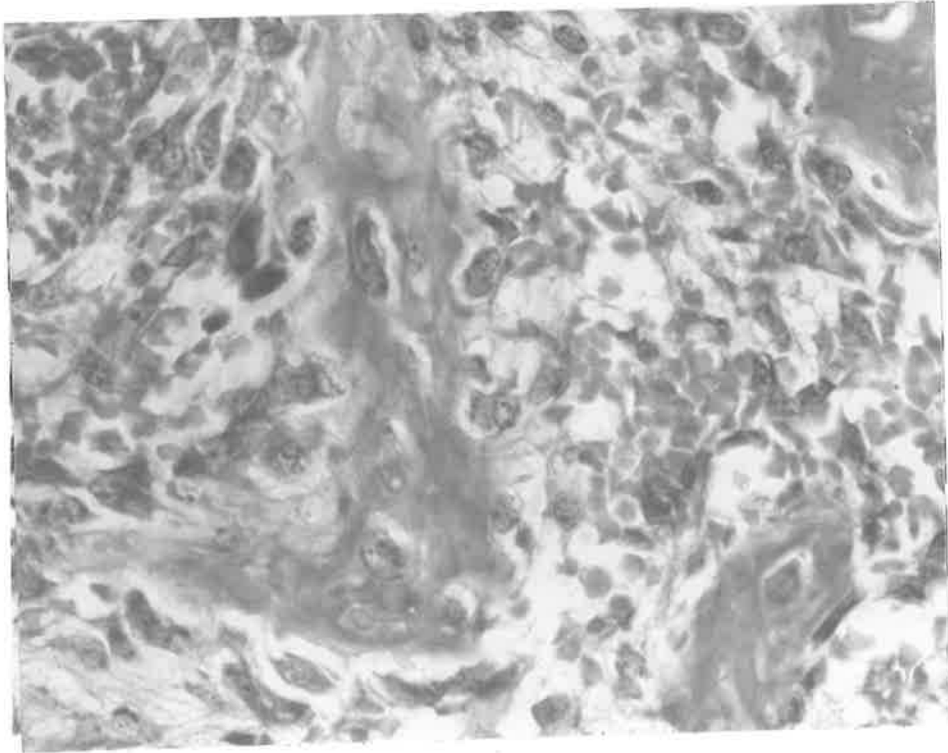

FIGURE 4.10

(iii) Two Weeks Post-Implantation

(a) Hydron

Low power microscopic observations revealed that the outline of the original defect could not be distinguished in its entirity in any of the specimens from this time period. However, the major portion of the outline could usually be distinguished (Fig. 4.11). The old bone forming the walls of the defects was largely devoid of osteocytes and new bone had been deposited on this old apparently cellular bone.

Signs of overt inflammation were not observed.

High power microscopic observations of the Hydron implant specimens revealed that the triangular defect between the implant and the bone was characterized by the presence of new bone and immature fibrous connective tissue. The fibrous connective tissue contained numerous macrophages (Fig. 4.12). Many of the macrophages contained phagocytosed Hydron (Fig. 4.12). In all specimens woven bone had been deposited in apparent contact with the Hydron (Fig. 4.12). The areas of the Hydron implant not in contact with bone were separated from the immature connective tissue by a thin fibrous "capsule" containing fibroblasts, collagen and macrophages.

In one specimen haemopoietic marrow was noted in the area between the Hydron and the walls of the defect.

(b) Controls

Low power microscopic observations of specimens containing either AH26 or Teflon implants were similar to those made when Hydron had been implanted for the same period.

Signs of overt inflammation were not observed.

High power microscopic observations of implant specimens of both AH26 and Teflon revealed that the triangular defect between the implant and the bone was characterized by essentially the same features as those observed in the two week Hydron specimens. However, bone did not contact the implant in either Teflon or AH26 specimens. In all instances, the bone was separated from the implant material by a fibrous connective tissue "capsule" consisting of fibroblasts, collagen, macrophages and a variable number of foreign body giant cells (Figs. 4.14 and 4.15). In general, the capsule between the AH26 and the bone was thicker than that between the Teflon and the bone and also contained more foreign body giant cells. The foreign body giant cells appeared to be mainly associated with irregularities of the surface of the implant. The surfaces of the AH26 implants appeared to be more irregular than those of either Teflon or Hydron implants. Macrophages in tissues associated with implants of AH26 often contained phagocytosed particles of the AH26, although the number of phagocytosing macrophages did not appear as great as the number of these cells associated with Hydron implants.

FIGURE 4.11: HYDRON, 2 WEEKS POST-IMPLANTATION IN BONE.

Photomicrograph illustrating the tissues between the Hydron implant (H) and the walls of the original bony defect. The outline of the defect while apparent is not discernible in some areas. The old bone (O) of the defect wall contains numerous empty lacunae. New bone (arrows) has been deposited on the defect walls. The junction between the new and old bone (J) is readily apparent. Several spicules of bone (S), one of which appears to be in contact with the Hydron implant, are apparent between the implant and the defect walls.

H. & E. original magnification x40

FIGURE 4.12: HYDRON, 2 WEEKS POST-IMPLANTATION IN BONE.

Photomicrograph illustrating the bone spicule (B) mentioned in Fig. 4.12 apparently in contact with the Hydron implant (H). The adjacent immature fibrous connective tissue consists of numerous mononuclear macrophages (many of which contain phagocytosed particles of Hydron), fibroblasts and collagen.

H. & E. original magnification x400

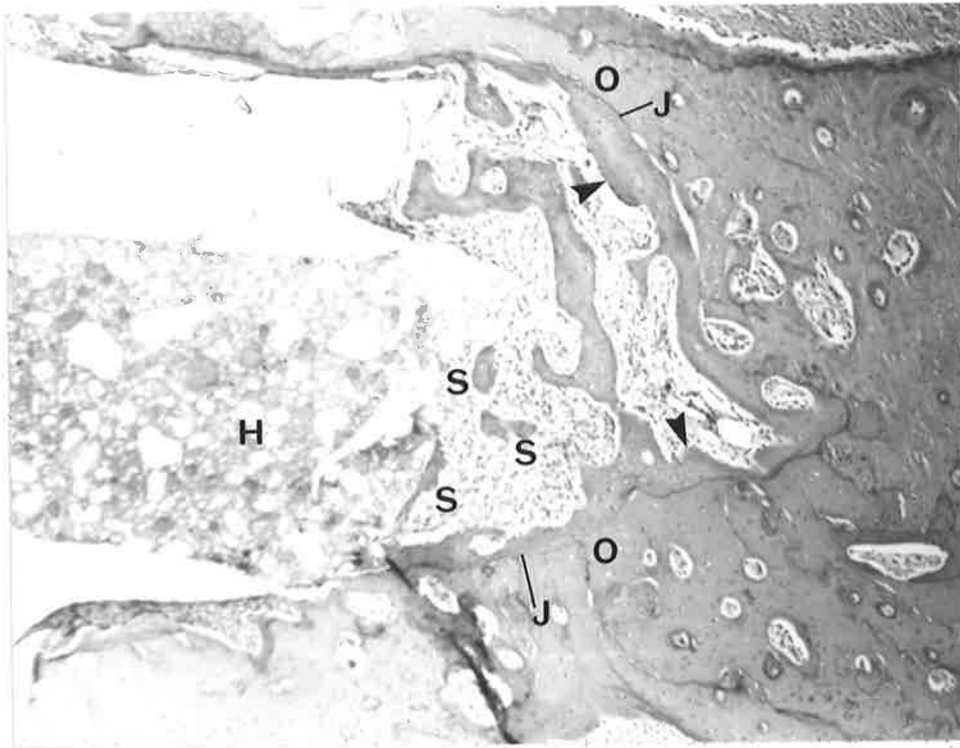

FIGURE 4.11

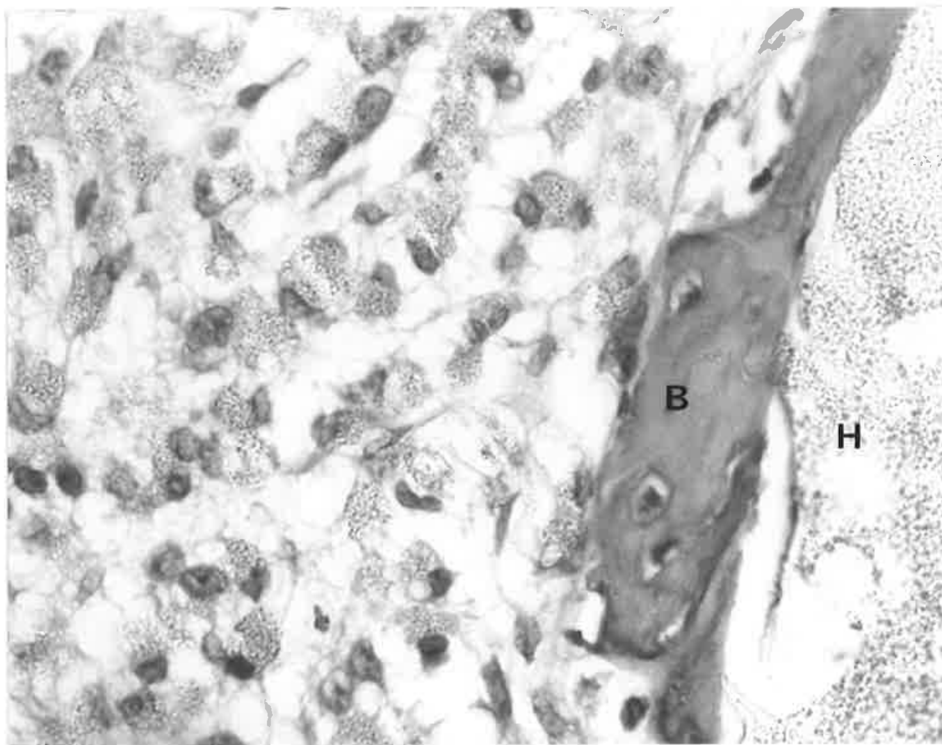

FIGURE 4.12

FIGURE 4.13: HYDRON, 2 WEEKS POST-IMPLANTATION IN BONE.

Photomicrograph illustrating the junction between the older acellular bone (A) and the newer cellular bone (N). The adjacent stromal tissues (S) consists of mononuclear macrophages, fibroblasts and collagen. The spaces between the older and newer bone would appear to be a processing artifact.

H. & E. original magnification x400

FIGURE 4.14: AH26, 2 WEEKS POST-IMPLANTATION IN BONE.

Photomicrograph illustrating the tissues adjacent to AH26 implant (A). A fibrous connective tissue capsule (C) is present between the immature woven bone (B) and the implant. This capsule consists of fibroblasts, collagen and macrophages. Evident within the capsule and associated with the surface of the AH26 implant is a multinucleated foreign body giant cell (F). The stromal tissues (S) consists of macrophages, fibroblasts and collagen.

H. & E. original magnification x400.

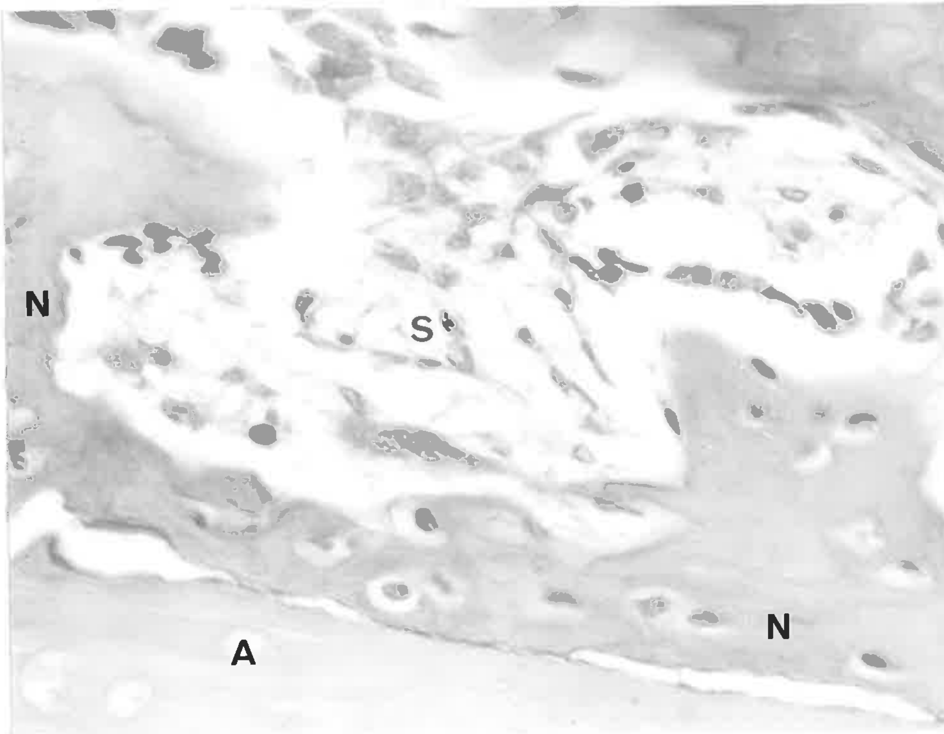

FIGURE 4.13

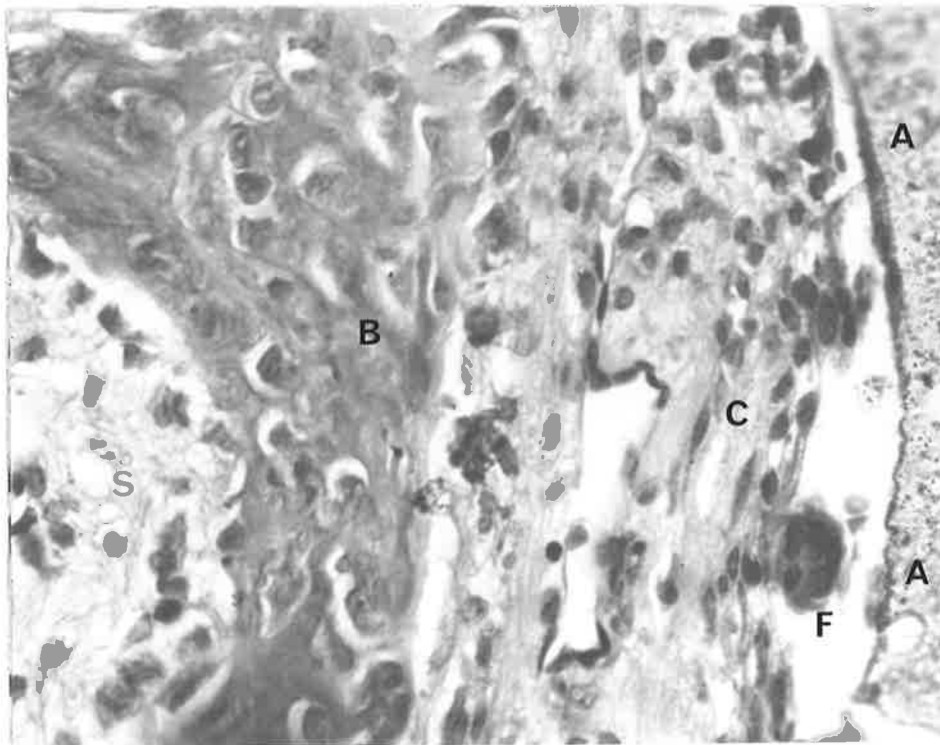

FIGURE 4.14

FIGURE 4.15: TEFLON, 2 WEEKS POST-IMPLANTATION IN BONE.

Photomicrograph illustrating the tissues adjacent to Teflon implant (T). There is a fibrous connective tissue capsule (C) between the Teflon implant and the osseous tissues. These tissues are mature woven bone (M) and immature woven bone (I).

H. & E. original magnification x400

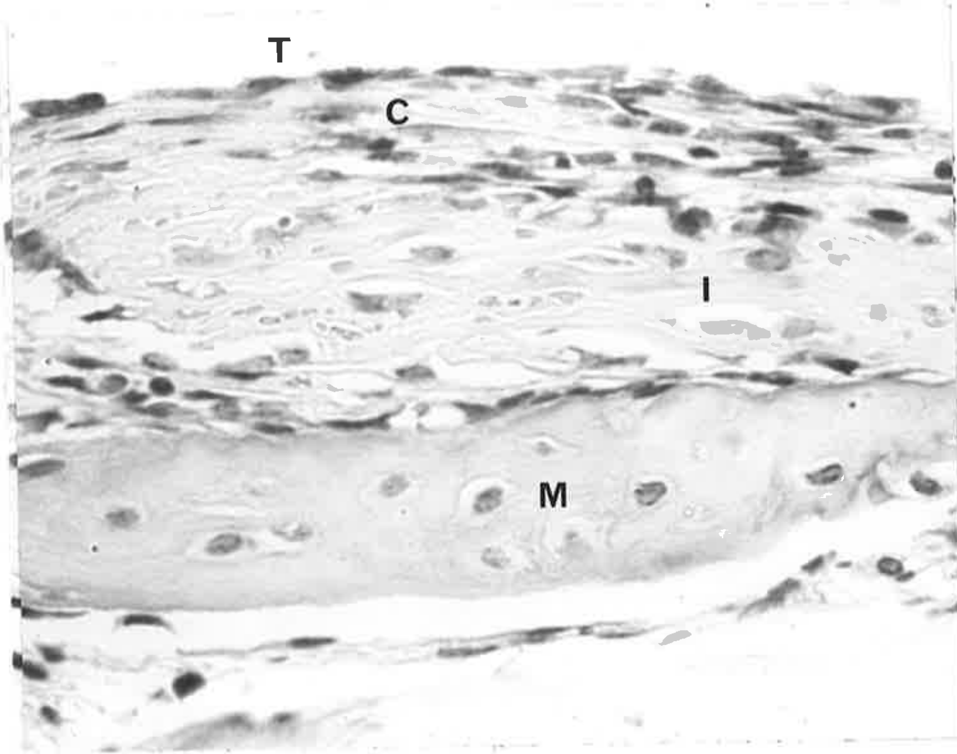

FIGURE 4.15

(iv) Four Weeks Post-Implantation

(a) Hydron

Low power microscopic observations revealed that in 2 of the 5 specimens studied, the outline of the original bony defect was apparent (similar to Fig. 4.16). In the remaining 3 specimens, only a small portion of the outline could be detected and often this was indistinct. New bone had been deposited on the older, acellular bone forming the walls of the original defect.

Signs of overt inflammation were not apparent.

High power microscopic observations of the Hydron implant specimens revealed that the triangular defect between the implant and the walls of the defect was characterized by the presence of bone and immature fibrous connective tissue (Fig. 4.17). Hydron containing macrophages were noted. The repair bone exhibited varying degrees of maturity. In half of the specimens from this post-implantation period, bone contacted the Hydron implant. A minimal thickness of fibrous connective tissue separated bone from the implant in the remaining specimens.

In one specimen, the deepest region of the original defect could be distinguished. This area was characterized by the presence of fibrin and necrotic bone chips. Associated with these features were acute and chronic inflammatory cells, multinucleated giant cells and macrophages.

(b) Controls

Low power microscopic observations of specimens containing implants of both AH26 (Fig. 4.16) and Teflon revealed a similar histological picture to that described for the Hydron implants from the same post-implantation period.

Signs of overt inflammation were not present.

High power observations of specimens containing implants of both AH26 and Teflon revealed similar features to those observed in 4 week Hydron specimens. The triangular area was characterized by the presence of bone and immature fibrous connective tissue (Fig. 4.18). However, in specimens containing implanted AH26 or Teflon, a fibrous connective tissue capsule was always interposed between the implant and the regenerated bone. This capsule consisted of fibroblasts, collagen, macrophages and a variable number of foreign body giant cells which appeared to be associated with the interface of the particular implant. Many macrophages in the tissues surrounding AH26 implants contained phagocytosed particles of AH26. In all control specimens, the capsule appeared to be thicker than any capsule associated with Hydron implants (Fig. 4.16). Osteoclastic resorption of the new bone was evident in all specimens (Fig. 4.18).

FIGURE 4.16: AH26, 4 WEEKS POST-IMPLANTATION IN BONE.

Photomicrograph illustrating tissues adjacent to AH26 implant (A). Part of the outline of the original bony defect (arrows) is clearly distinguishable. Bone (B) and stromal tissue characterize the area between the implant and the walls of the bony defect. Interposed between the new bone and the implant is a capsule (C).

H. & E. original magnification x40

FIGURE 4.17: HYDRON, 4 WEEKS POST-IMPLANTATION IN BONE.

Photomicrograph illustrating relatively more mature bone (M) and immature bone (I) adjacent to the space originally occupied by Hydron implant (H). The erythrocytes at the periphery of the immature bone have resulted from the surgery involved in recovering this tissue. Note the numerous particles of phagocytosed Hydron contained within macrophages in the stromal tissues.

H. & E. original magnification x200

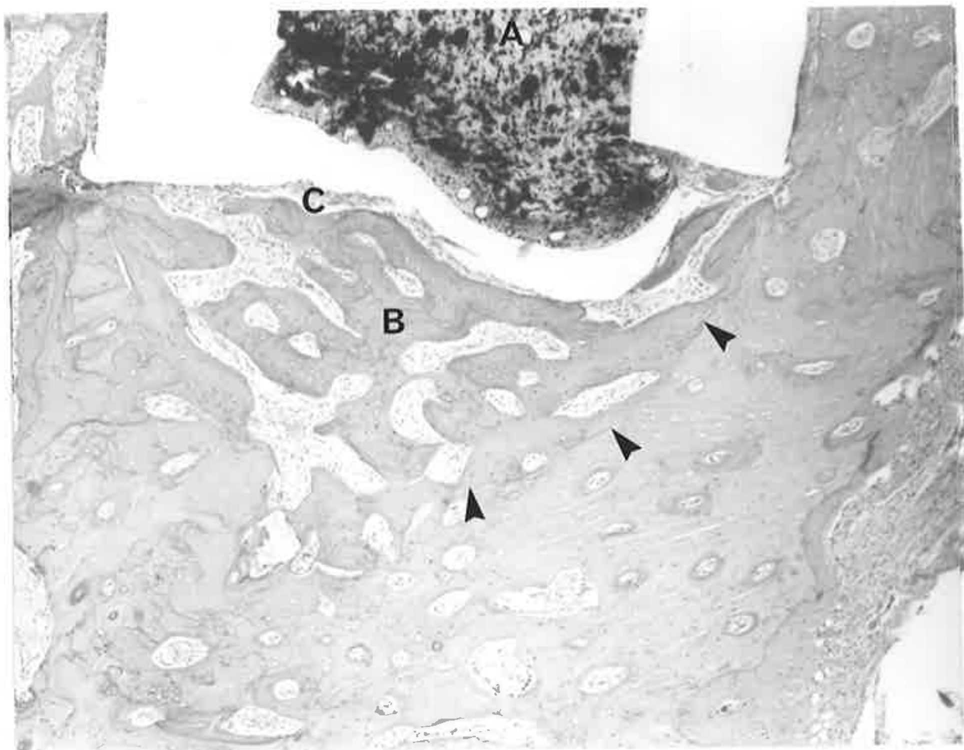

FIGURE 4.16

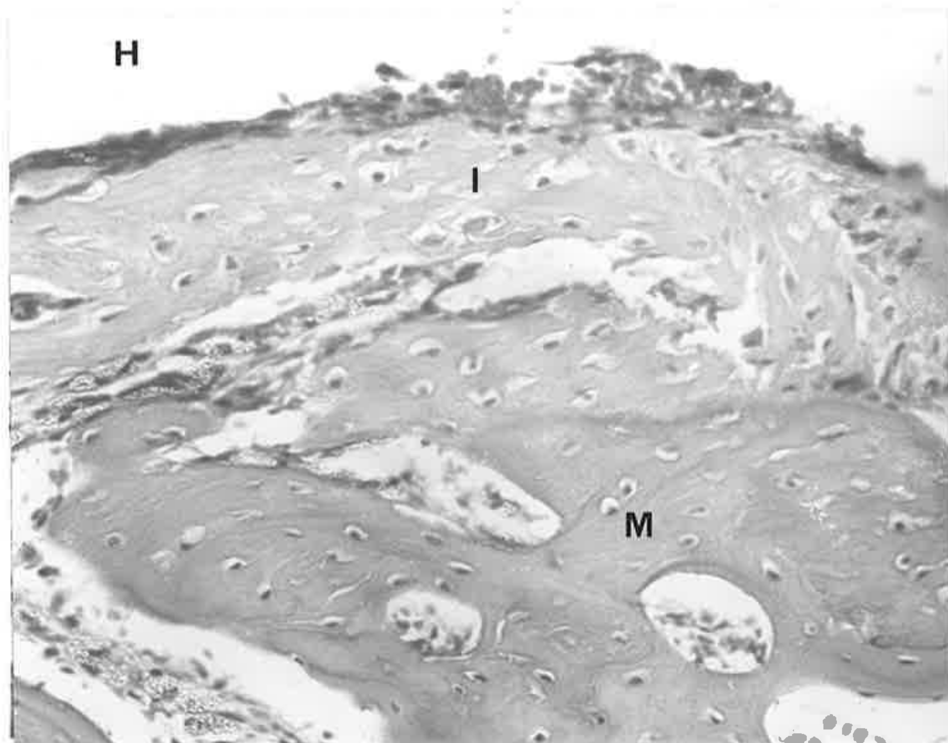

FIGURE 4.17

FIGURE 4.18: TEFLON, 4 WEEKS POST-IMPLANTATION IN BONE.

Photomicrograph illustrating the bone (B) and stromal tissues between the Teflon implant (T) and the walls of the original bony defect. A fibrous connective tissue capsule (C) is interposed between the implant and the bone. Note the Howships lacunae (arrows) and associated osteoclasts indicating active bone resorption in the bottom left area of this photomicrograph.

H. & E. original magnification x200

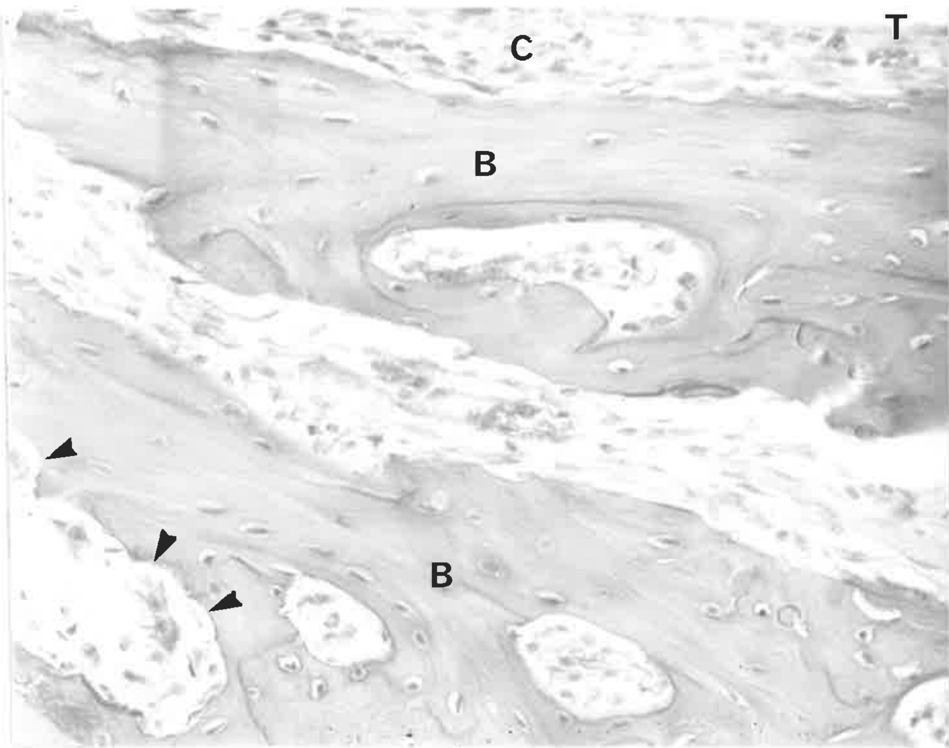

FIGURE 4.18

(v) Twelve Weeks Post-Implantation

(a) *Hydron*

Low power microscopic observations revealed that the outline of the original bony defect was difficult to readily identify. Where it was able to be distinguished, the bony walls of the original defect contained many empty lacunae. All specimens exhibited new deposition on the old acellular bone. The junction between these two bony tissues was readily apparent.

Signs of overt inflammation were not present.

High power observations of the Hydron implant specimens revealed that the triangular defect between the Hydron and the wall of the defect was characterized chiefly by the presence of bone (Fig. 4.19) and maturing fatty marrow (Fig. 4.21). In 6 of the 7 specimens observed in this period post-implantation, there appeared to be absolute contact at the highest resolution possible with the microscope employed in this study, (i.e. x1000 oil) between the bone and the Hydron (Fig. 4.19) for the majority of the interface. The Hydron was separated from either bone or marrow for the remainder of the interface by a fine capsule of mature fibrous connective tissue (Fig. 4.20). In the seventh specimen the Hydron was separated from adjacent bone by a fine connective tissue capsule. There were a range of stromal tissue types from fibrous connective tissue to fatty marrow. Macrophages containing phagocytosed Hydron particles (Fig. 4.21) were evident in the marrow tissue associated with

all Hydron implants. However, these phagocytosing macrophages appeared to be fewer in number compared with earlier post-implantation periods. Macrophages containing phagocytosed Hydron were more numerous close to the implant and were confined overall to the area of this defect. The relative amounts of bone and marrow in the different specimens varied from almost solid bone to fine trabeculae of bone and large quantities of interspersed marrow. There were approximately equal numbers of each.

(b) Controls

Low power microscopic observations of specimens containing implants of both AH26 and Teflon (Fig. 4.22) revealed similar features to those described adjacent to the Hydron implants.

Signs of overt inflammation were not present.

High power microscopic observations of specimens containing implants of both AH26 and Teflon similar features to those observed in specimens containing Hydron implanted for 12 weeks. However, all specimens containing either implanted AH26 or Teflon exhibited a fibrous connective tissue capsule between the implant and the regenerated bone in the defect (Fig. 4.24). The capsule consisted of fibroblasts, collagen, macrophages and a variable number of foreign body giant cells which appeared to be associated with the interface of the particular implant. The tissues in the defect associated with AH26 implants contained a variable number

of macrophages with phagocytosed particles of AH26 within their cytoplasms. However, the numbers of such macrophages appeared to be fewer than those noted in the tissues associated with AH26 implants of shorter post-implantation periods. Two specimens containing implanted AH26 exhibited a significant infiltration of lymphocytes. The capsule associated with control specimens appeared to be thicker than that associated with Hydron implants following the same period post-implantation (compare Fig. 4.22 with Figs. 4.20 and 4.21). Cartilage formation was evident in one specimen (Figs. 4.22 and 4.23). Both membranous ossification (Fig. 4.24) and endochondral ossification (Fig. 4.23) appeared to have been proceeding in adjacent areas in this particular specimen.

FIGURE 4.19: HYDRON, 12 WEEKS POST-IMPLANTATION IN BONE.

Photomicrograph illustrating the junction between the Hydron implant (H) and the bone (B). At this magnification, using oil emersion, there appears to be absolute contact between the bone and the implant.

H. & E. original magnification x1000

FIGURE 4.20: HYDRON, 12 WEEKS POST-IMPLANTATION IN BONE.

Photomicrograph illustrating a piece of Hydron (H) that became detached from the main implant. Several different interfaces between the osseous tissues and the Hydron are shown in this photomicrograph. The interface on the right side shows what appears to be absolute contact between the Hydron and the bone (B), similar to the interface shown in Fig. 4.19. On the left side, of this photomicrograph, a thin fibrous connective tissue capsule (smaller arrows) is interposed between the Hydron and the bone. Between the marrow tissue (M) and the implant there is a fine fibrous connective tissue capsule (larger arrow).

H. & E. original magnification x400

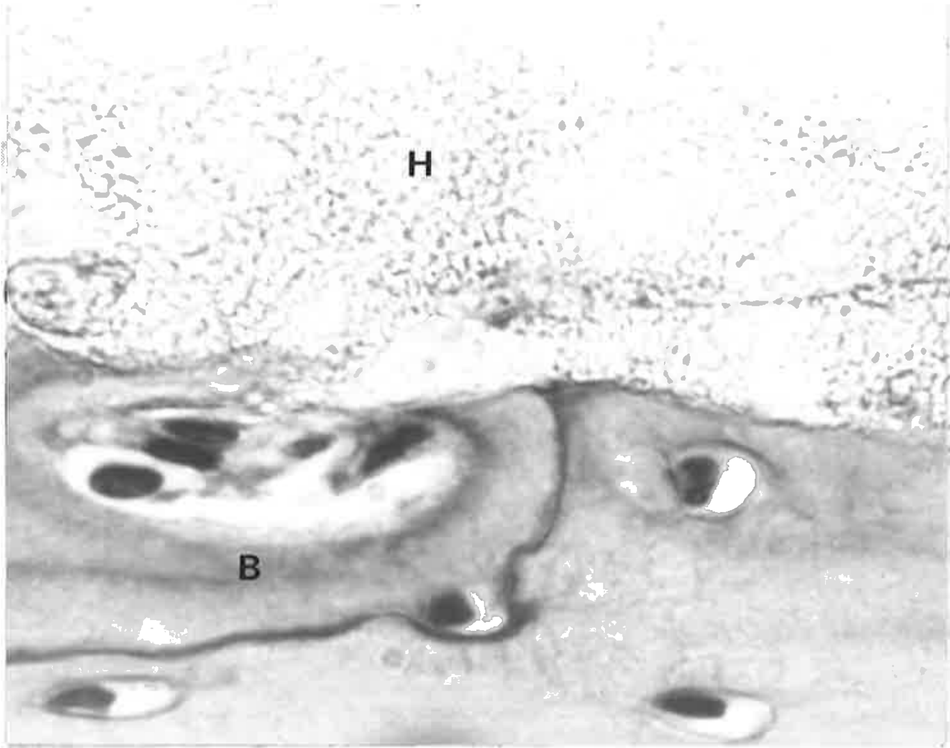

FIGURE 4.19

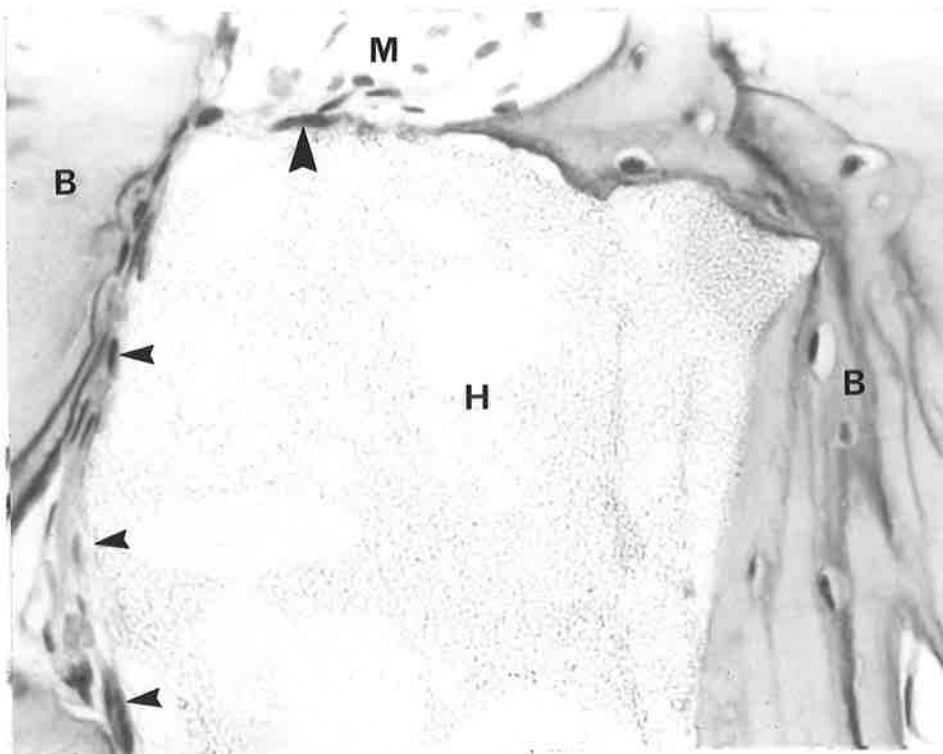

FIGURE 4.20

FIGURE 4.21: HYDRON, 12 WEEKS POST-IMPLANTATION IN BONE.

Photomicrograph illustrating the tissues adjacent to the Hydron implant (H) which has been lost during processing. A fine fibrous connective tissue capsule (arrows) separates the Hydron implant from the maturing fatty marrow. The tissue in the lower left corner (F) is much more fibrous than the majority of the marrow in this photomicrograph. Note the macrophages containing phagocytosed Hydron throughout the marrow. Note also the large thin walled blood vessel (V).

H. & E. original magnification x400

FIGURE 4.22: TEFLON, 12 WEEKS POST-IMPLANTATION IN BONE.

Photomicrograph illustrating the tissues adjacent to the Teflon implant (T). Between this implant and the bone (B) is a fibrous connective tissue capsule (C). Note the cartilage in the top right corner of this photomicrograph. The junction between this cartilage and the bone is indicated with a larger arrow.

H. & E. original magnification x40

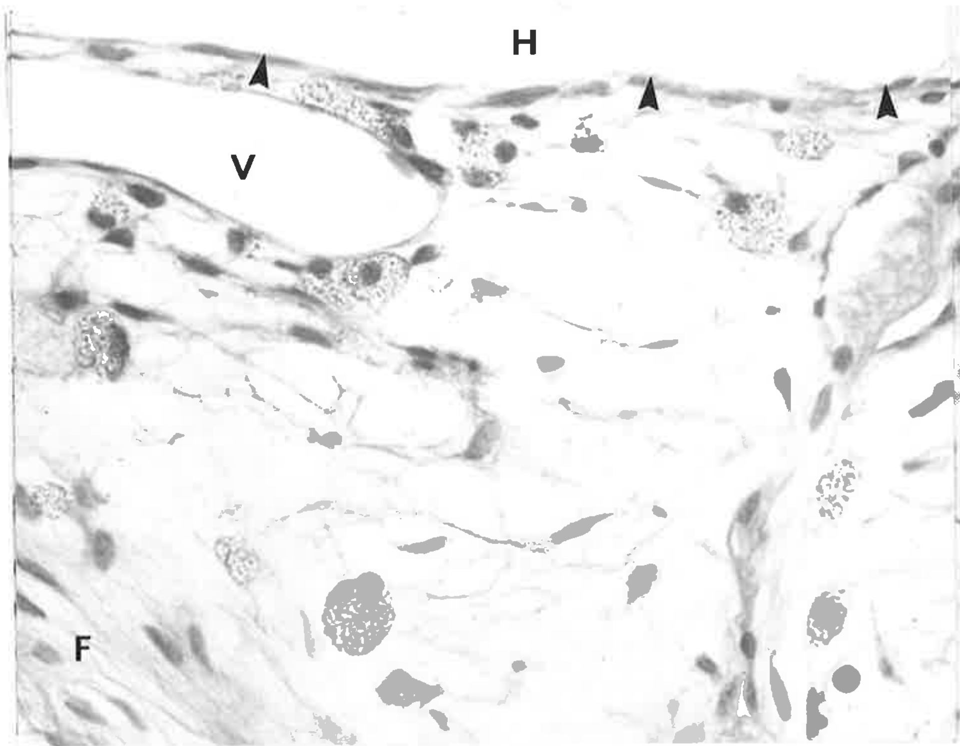

FIGURE 4.21

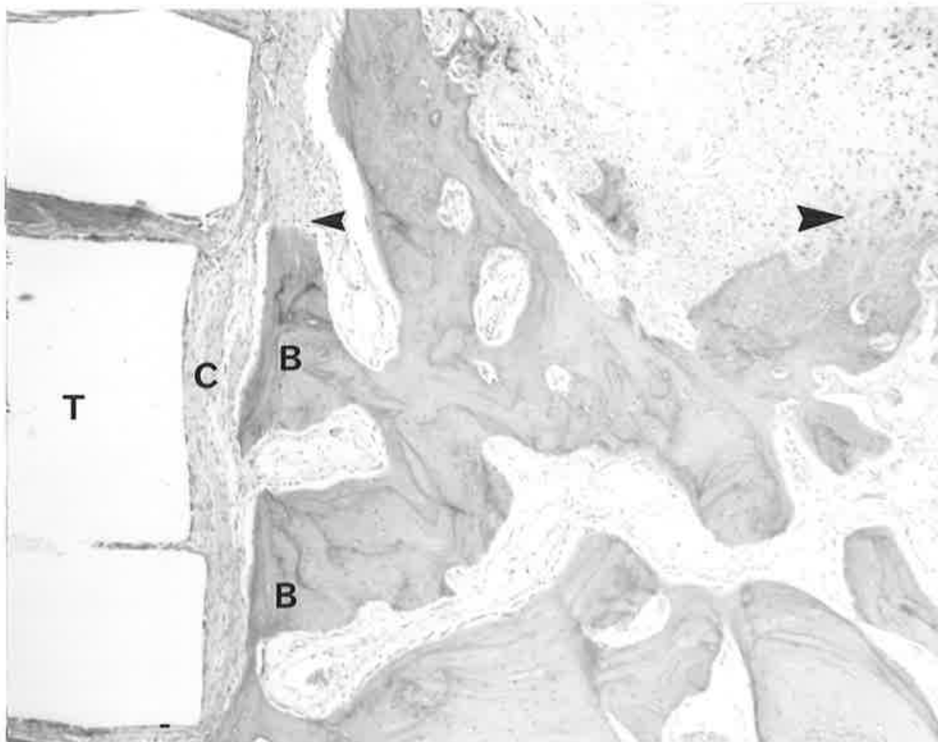

FIGURE 4.22

FIGURE 4.23: TEFLON, 12 WEEKS POST-IMPLANTATION IN BONE.

Photomicrograph illustrating (at higher magnification) the junction between cartilage (C) and bone (B), previously mentioned in Fig. 4.22. The stromal tissues illustrated are immature fibrous connective tissue.

H. & E. original magnification x400

FIGURE 4.24: TEFLON, 12 WEEKS POST-IMPLANTATION IN BONE.

Photomicrograph illustrating (at higher magnification) the area indicated with a smaller arrow in Fig. 4.22. Sharpey fibres (arrows) are continuous between the bone (B) and an area of condensed collagen (X). Both tissues are separated from the Teflon implant (not included in this photomicrograph) by a fibrous connective tissue capsule (C). The large cells at the junction of the bone and the condensed collagen would appear to be immature osteoblasts. The stromal tissue in this photomicrograph is immature fibrous connective tissue. Note the large thin walled blood vessel (V) located within this stromal tissue.

H. & E. original magnification x400

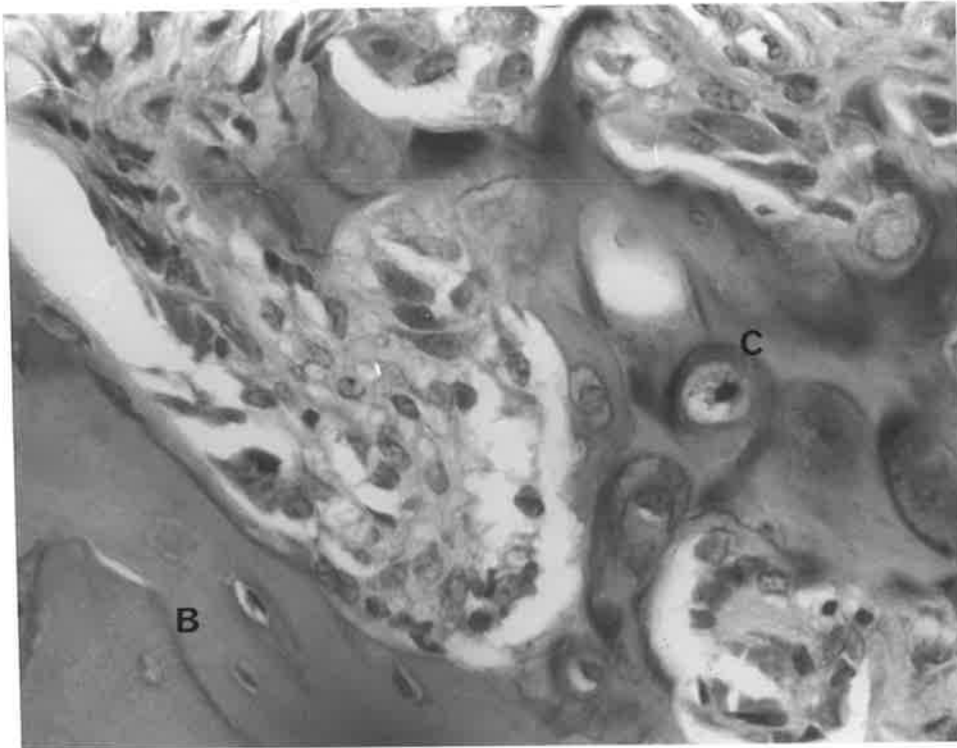

FIGURE 4.23

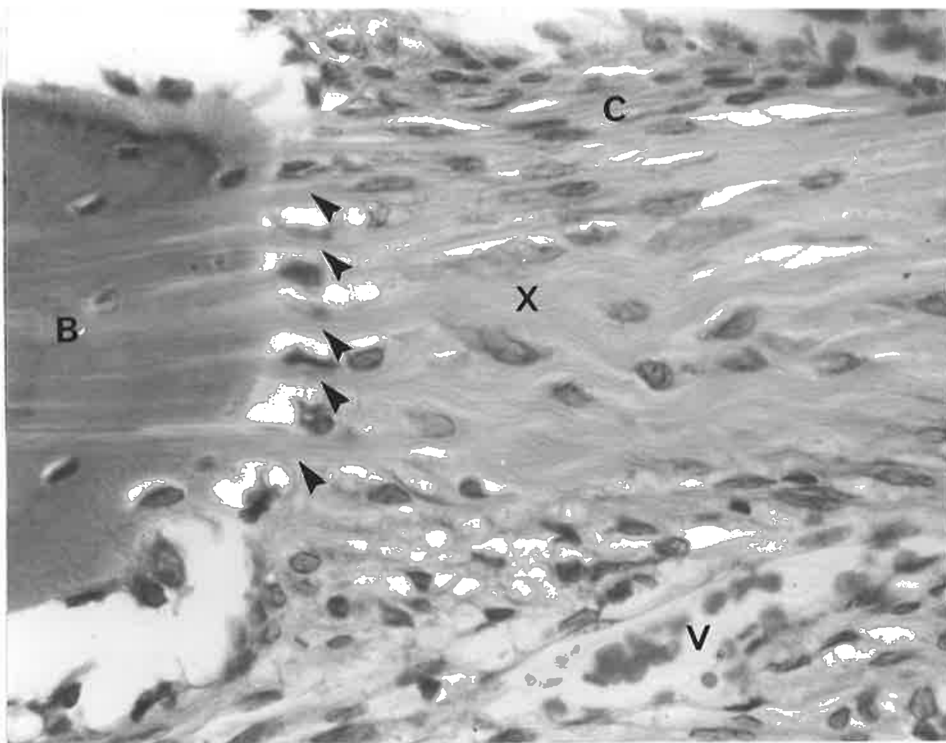

FIGURE 4.24

(vi) Twenty-Six Weeks Post-Implantation

(a) *Hydron*

Low power microscopic observations revealed that in all specimens the outline of the original bony defect was difficult to distinguish in its entirity (Fig. 4.25). This feature could not be distinguished at all in 3 of the 7 specimens examined. In specimens where the bony defect could be distinguished, the older bone adjacent to the defect contained many empty lacunae (Fig. 4.27). Even where the outline of the defect was not distinguishable, some areas of bone devoid of viable osteocytes could be observed. This acellular bone was taken to be the bone of the original defect wall. All 7 specimens exhibited new bone deposition on the acellular bone.

Signs of overt inflammation were not present.

High power microscopic observations revealed that the triangular defect between the Hydron and the bone was characterized chiefly by the presence of bone and mature fatty marrow (Fig. 4.26). The bone in all but one of the specimens observed was in direct contact with the Hydron (Fig. 4.26). The remaining specimen exhibited a mature fibrous connective tissue capsule between the Hydron and the bone. The marrow tissue was fatty marrow (Fig. 4.26). In all cases macrophages containing phagocytosed particles of Hydron were a feature of the fatty marrow. However, the numbers of these macrophages appeared to be fewer than those observed in specimens

from earlier post-implantation periods. There was variation in the degree of fibrosis of the marrow similar to that noted in tissues adjacent to Hydron implants at 12 weeks. In one specimen, the mature fatty marrow appeared to be being replaced by cells from the adjacent haemopoietic marrow (Fig. 4.25).

(b) Controls

Low power microscopic observations of specimens containing implants of either AH26 or Teflon revealed a similar situation to that described adjacent to the Hydron implants.

Signs of overt inflammation were not present.

High power microscopic observations of specimens containing implants of both AH26 and Teflon showed features very similar to those observed in specimens containing Hydron implanted for 26 weeks. However, in specimens containing either implanted AH26 or Teflon, a fibrous connective tissue capsule was always present between the implant and the bone (Figs. 4.28 and 4.29). A small number of foreign body giant cells were present and appeared to be associated with the interface of the particular implant (Fig. 4.29). In the tissues of the original defect associated with AH26 implants, macrophages containing phagocytosed particles of AH26 were noted (Fig. 4.28). However, the number of macrophages appeared to be fewer than those apparent in tissues associated with AH26 implants post-implantation periods. There was a similar variation in the fibrosis of the fatty marrow, to that seen in specimens containing implanted Hydron.

Of special interest was one specimen containing implanted AH26. The area between the implant and the walls of the bony defect was characterized by the presence of fibrin and there was a noticeable paucity of any cells.

FIGURE 4.25: HYDRON, 26 WEEKS POST-IMPLANTATION IN BONE.

Photomicrograph illustrating the tissues adjacent to Hydron implant (H). The junction (arrows) between the newer bone (B) and the older acellular bone (O) is present only for a short distance. The tissues between the Hydron implant and the walls of the original defect are characterized by bone (B) and mature fatty marrow (F.M.). This marrow on the left side of this photomicrograph appears to be replaced by haemopoietic marrow (H.M.). The bone appears to be in absolute contact with the entire surface of the Hydron implant. Note the periodontal ligament (P.L.) and ameloblasts (A) associated with the guinea pig incisor.

H. & E. original magnification x40

FIGURE 4.26: HYDRON, 26 WEEKS POST-IMPLANTATION IN BONE.

Photomicrograph illustrating (at greater magnification) the contact between the bone (B) and the Hydron implant (H) seen in Fig. 4.25. Note the mature fatty marrow (F.M.) and also the prominent reversal lines (arrows) in the bone.

H. & E. original magnification x400

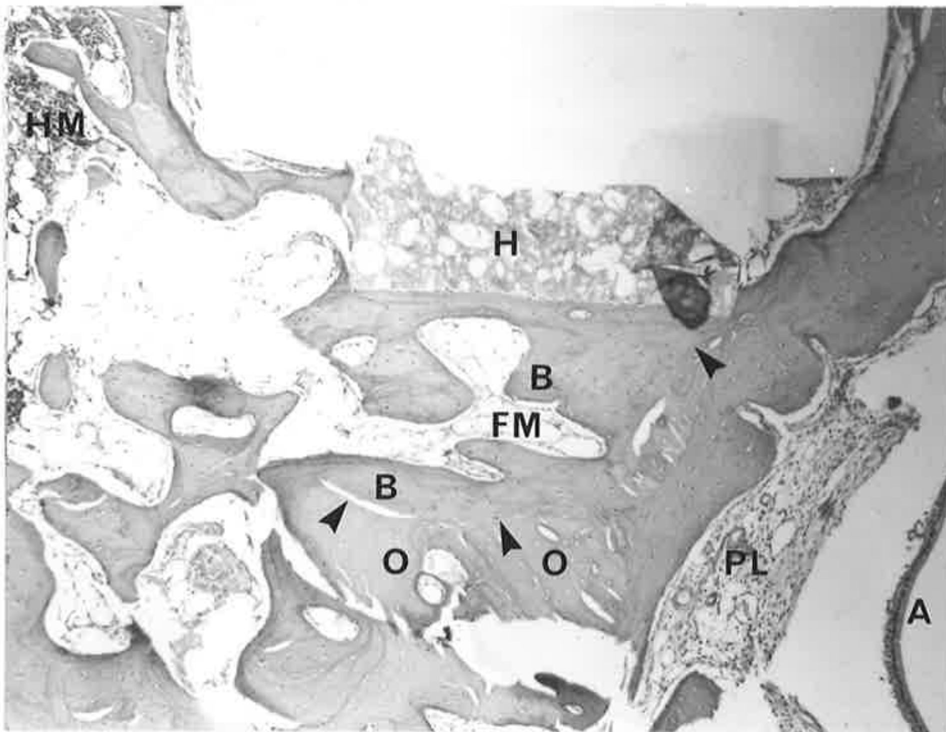

FIGURE 4.25

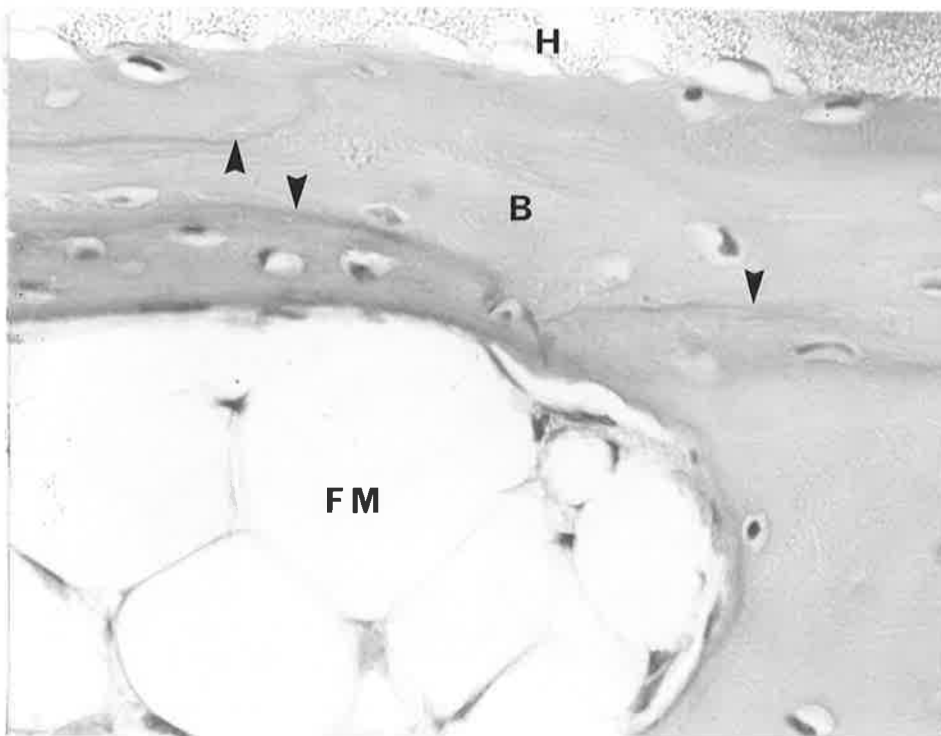

FIGURE 4.26

FIGURE 4.27: HYDRON, 26 WEEKS POST-IMPLANATION IN BONE.

Photomicrograph illustrating the junction between the newer cellular bone (N) and older acellular bone (A) seen in Fig. 4.25.

H. & E. original magnification x400

FIGURE 4.28: AH26, 26 WEEKS POST-IMPLANTATION IN BONE.

Photomicrograph illustrating the capsule between the bone (B) and the AH26 implant (A). Several macrophages (arrows) contain phagocytosed particles of AH26. Note the large thin walled blood vessel (V) within the capsule.

H. & E. original magnification x400

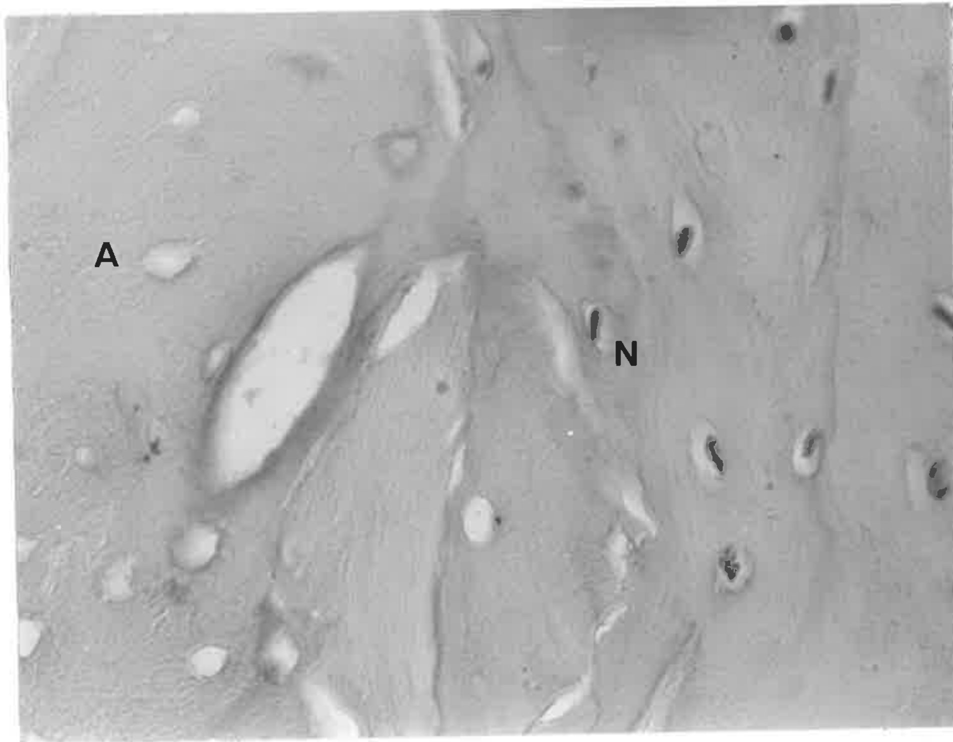

FIGURE 4.27

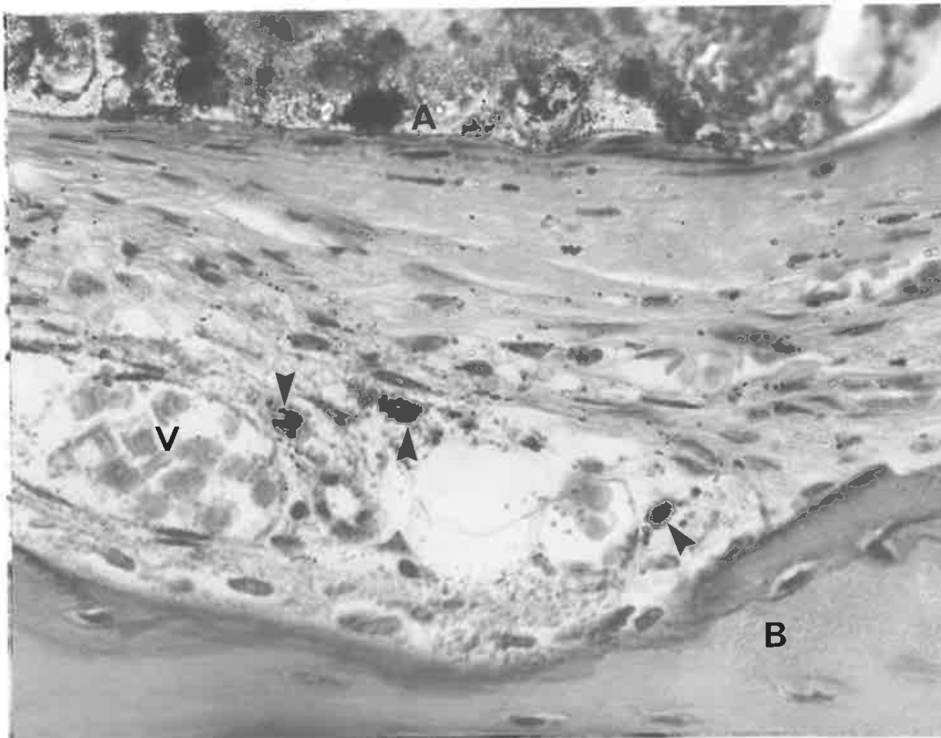

FIGURE 4.28

FIGURE 4.29: TEFLON, 26 WEEKS POST-IMPLANTATION IN BONE.

Photomicrograph illustrating the tissues adjacent to the Teflon implant (T) lost during processing. There are several multinecleated giant cells (arrows) associated with the surface of the implant. The capsule (C) is characterized by the presence of fibroblasts, collagen and macrophages. Note the fatty marrow (F) in contact with this capsule.

H. & E. original magnification x400

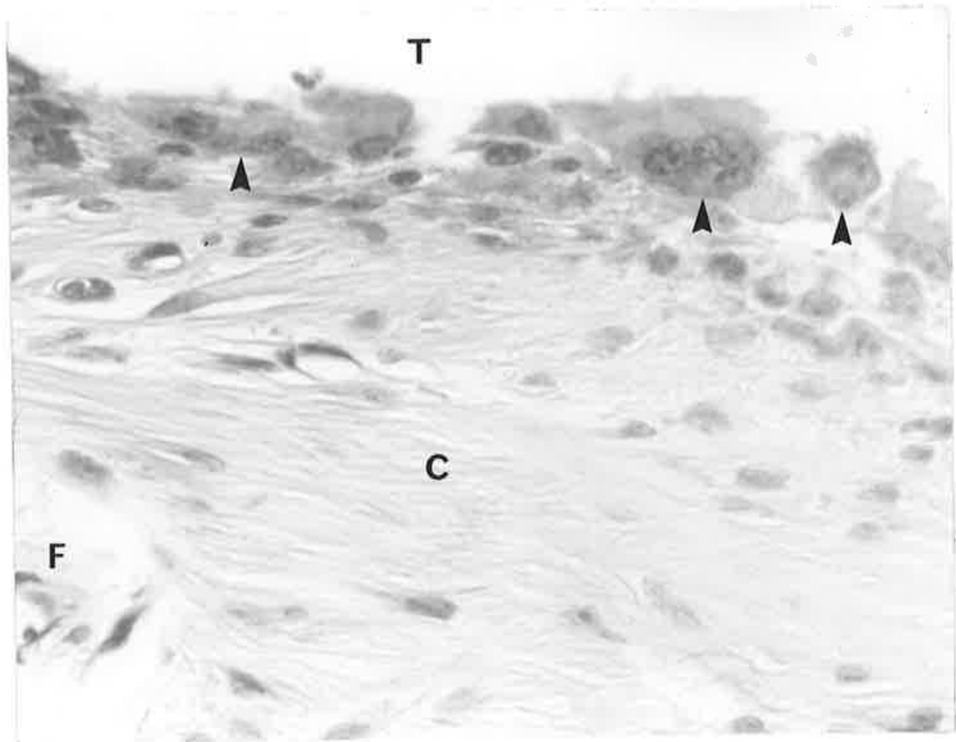

FIGURE 4.29

4.2.2 MUSCLE

CAPSULAR ZONE - THE NATURE OF THE CAPSULAR ZONE

(i) Two Days Post-Implantation

(a) Hydron

Microscopic observations of the tissues adjacent to the implanted Hydron revealed the presence of fibrin, extravasated erythrocytes polymorphonuclear leukocytes and few lymphocytes (Fig. 4.31). Immediately adjacent to the implant the muscle appeared altered as evidenced by decreased nuclei numbers, less striations and altered P.T.A.H. staining. Both the staining properties and features of the peripheral muscle appeared normal.

(b) Controls

Microscopic observations of the tissues adjacent to either the implanted AH26 or the implanted Teflon (Figs. 4.32 and 4.33) revealed features similar to those observed in Hydron specimens. These were the presence of fibrin, extravasated erythrocytes and similar inflammatory cells. The adjacent muscle appeared to be similar to that adjacent to Hydron specimens.

FIGURE 4.30: HYDRON, 2 DAYS POST-IMPLANTATION IN MUSCLE.

Photomicrograph illustrating the tissues adjacent to the Hydron implant (H). There is an area of inflammatory exudate (I) between the Hydron and the muscle (M).

H. & E. original magnification x100

FIGURE 4.31: HYDRON, 2 DAYS POST-IMPLANTATION IN MUSCLE.

Photomicrograph illustrating (at greater magnification) the area adjacent to the Hydron implant (H) as seen in Fig. 4.30. The extravasated erythrocytes are indistinct. There are numerous polymorphonuclear leukocytes in the area illustrated.

H. & E. original magnification x400

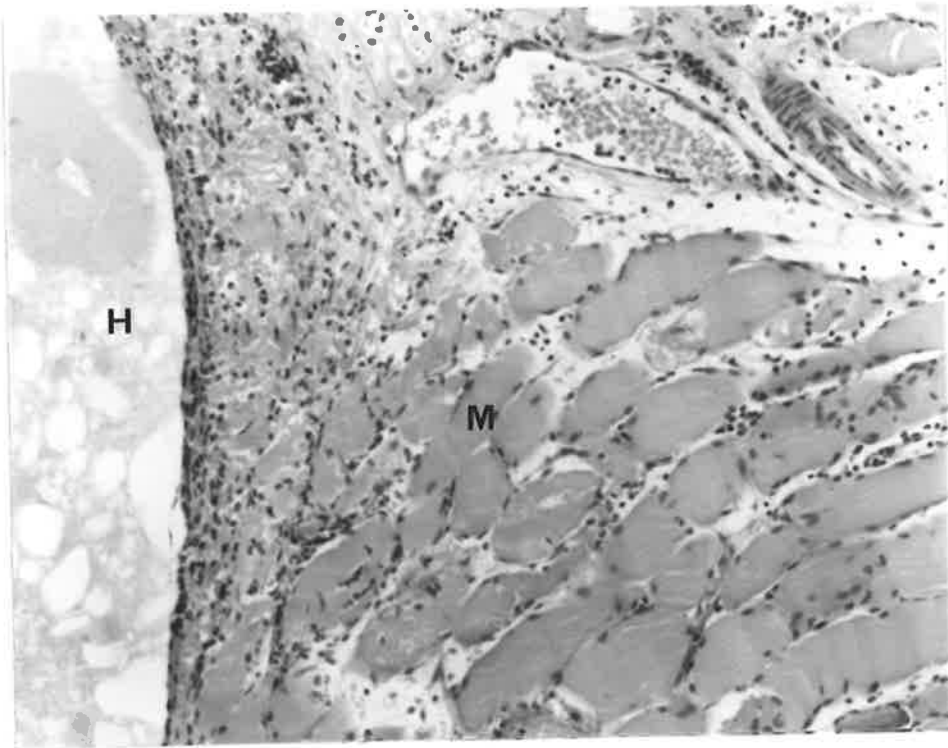

FIGURE 4.30

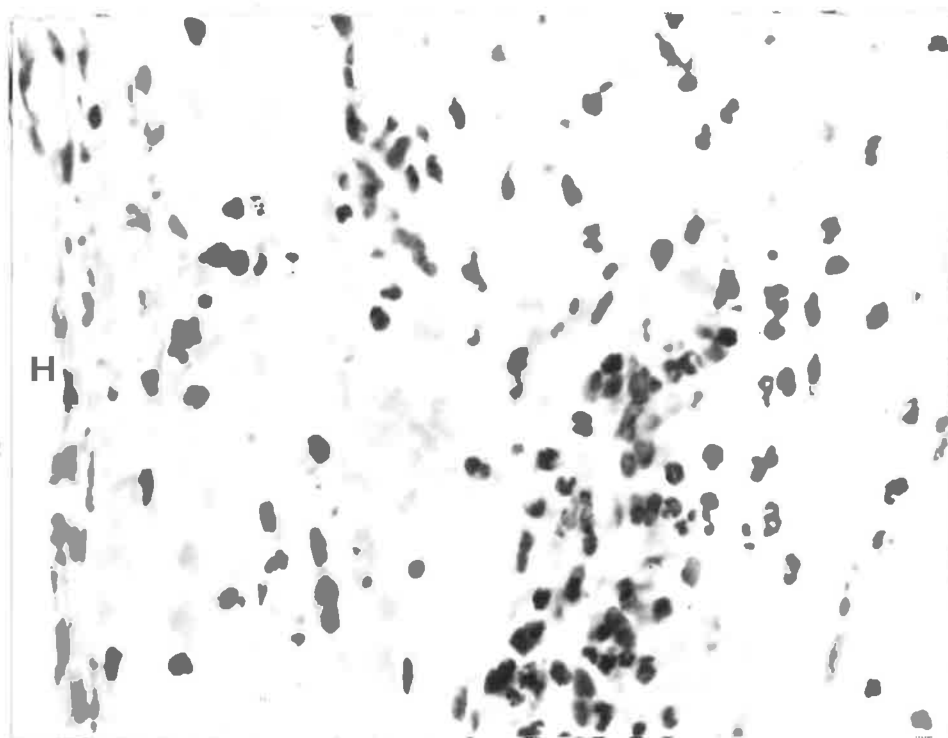

FIGURE 4.31

FIGURE 4.32: AH26, 2 DAYS POST-IMPLANTATION IN MUSCLE.

Photomicrograph illustrating the tissues adjacent to the AH26 implant (A). Areas such as that designated (E) consist mainly of extravasated erythrocytes while other areas such as that designated (F) consist mainly of fibrin. Polymorphonuclear leukocytes appear throughout the area illustrated either scattered or in clumps.

H. & E. original magnification x400

FIGURE 4.33: TEFLON, 2 DAYS POST-IMPLANTATION IN MUSCLE.

Photomicrograph illustrating the tissues adjacent to the Teflon implant (T). The area illustrated consists mainly of fibrin. There are also a number of polymorphonuclear leukocytes and extravasated erythrocytes present.

H. & E. original magnification x400

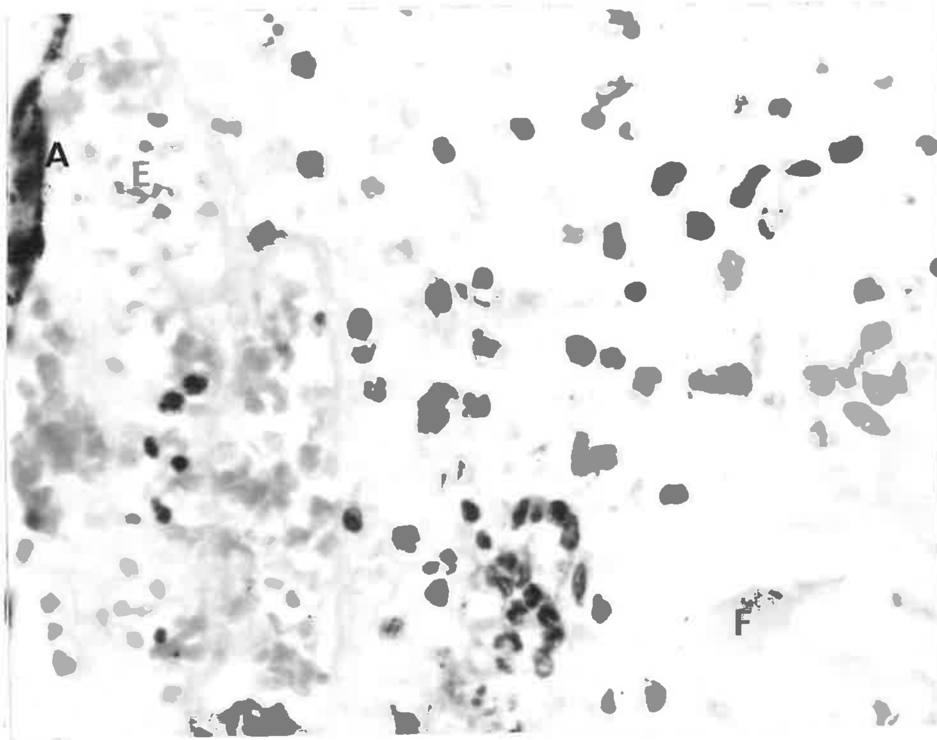

FIGURE 4.32

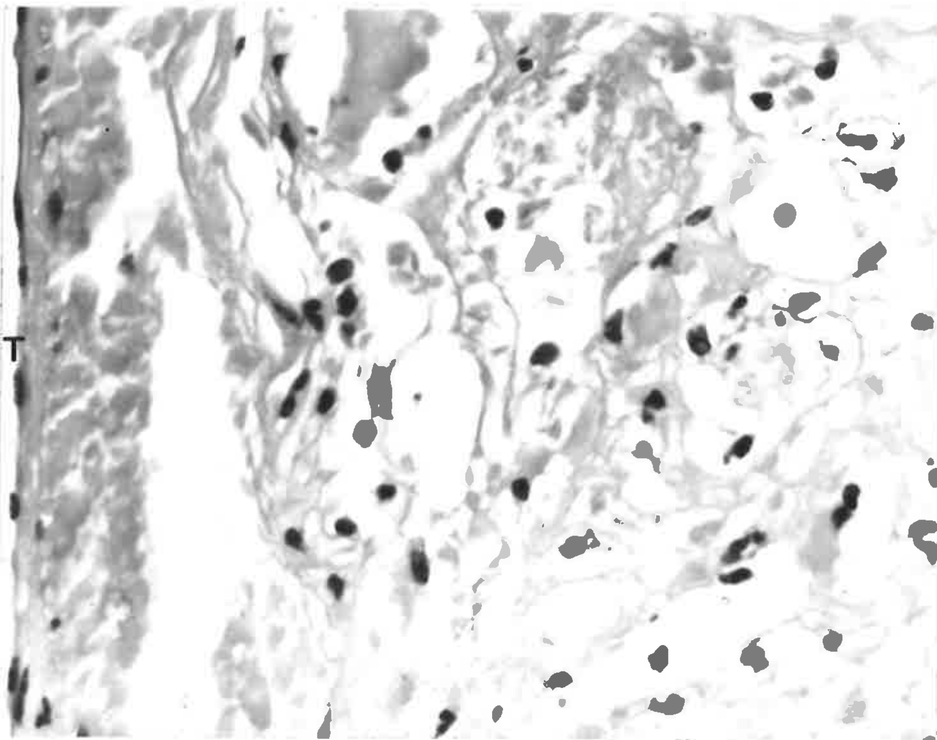

FIGURE 4.33

(ii) One Week Post-Implantation

(a) Hydron

Microscopic observations of the tissues adjacent to the implanted Hydron showed two distinct zones. The zone adjacent to the implant was more densely cellular, with the cells being aligned parallel to the surface of the implant (Fig. 4.34). This zone hereafter will be referred to as the inner zone. Between this inner zone and the main muscle mass was a less cellular and more randomly oriented zone, which will hereafter be referred to as the outer zone. This pattern of an inner and an outer zone occurred adjacent to all materials implanted for 1 week or more.

The inner zone associated with Hydron, 1 week post-implantation, was characterized by the presence of fibrin, macrophages, fibroblasts and small numbers of extravasated erythrocytes (Fig. 4.35). The outer zone was characterized by the presence of immature fibroblasts, macrophages, little collagen and occasional chronic inflammatory cells (Fig. 4.35). Regenerating muscle cells were also noted within this outer zone. When staining with haematoxylin and eosin, the regenerating muscle cells often appeared similar to foreign body giant cells (Fig. 4.34). Muscle was, however, readily distinguished from other cells when adjacent serial sections, stained with P.T.A.H., were compared.

(b) Controls

The tissues adjacent to the control materials revealed features similar to those adjacent to the Hydron implants. The tissues adjacent to one AH26 implant were infiltrated by a number of lymphocytes.

The outer zone associated with both AH26 and Teflon implants, 1 week post-implantation, exhibited features similar to those associated with implanted Hydron.

FIGURE 4.34: HYDRON, 1 WEEK POST-IMPLANTATION IN MUSCLE.

Photomicrograph illustrating the tissues adjacent to implanted Hydron (H). The inner zone (demarkated by smaller arrows) is densely cellular and the cells are aligned parallel to the surface of the Hydron. The outer zone (demarkated by larger arrows) is less cellular and less oriented. Note the cellular response to this implantation extends throughout the adjacent muscle. Several regenerating muscle cells (R) resemble in some aspects foreign body giant cells.

H. & E. original magnification x100

FIGURE 4.35: HYDRON, 1 WEEK POST-IMPLANTATION IN MUSCLE.

Photomicrograph illustrating (at greater magnification) the tissues adjacent to the Hydron implant (H) as seen in Fig. 4.34. The inner zone (extent shown by the smaller arrow) is highly cellular and parallel to the surface of the implant. The outer zone (from the larger arrow and extending beyond the right side of this photomicrograph) is less cellular and less oriented. Note the darkly staining lymphocytes present in this outer zone.

H. & E. original magnification x400

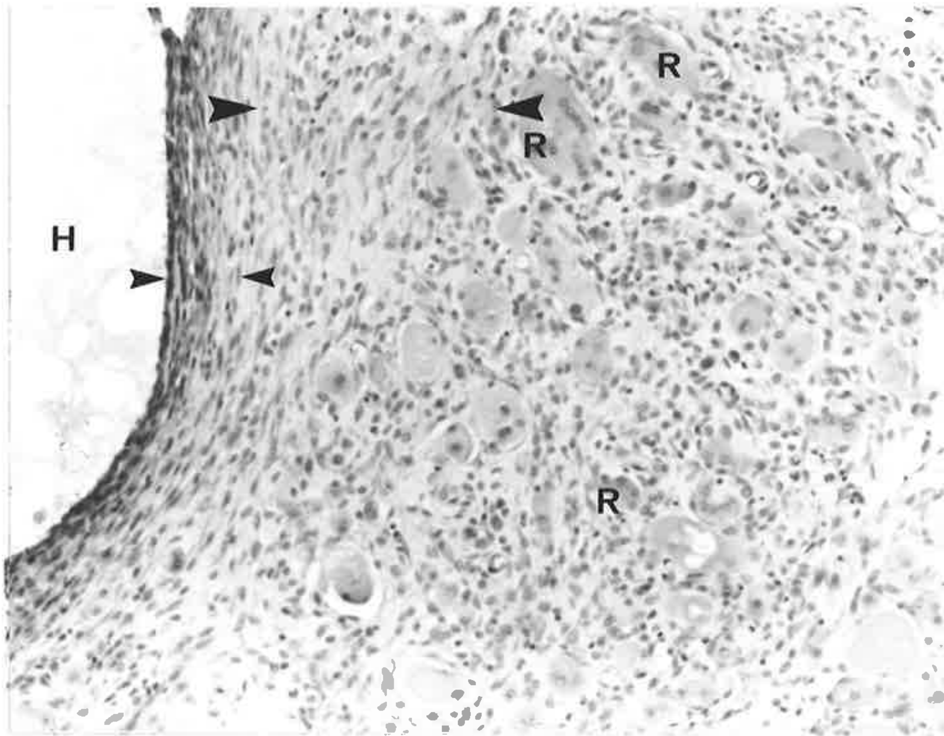

FIGURE 4.34

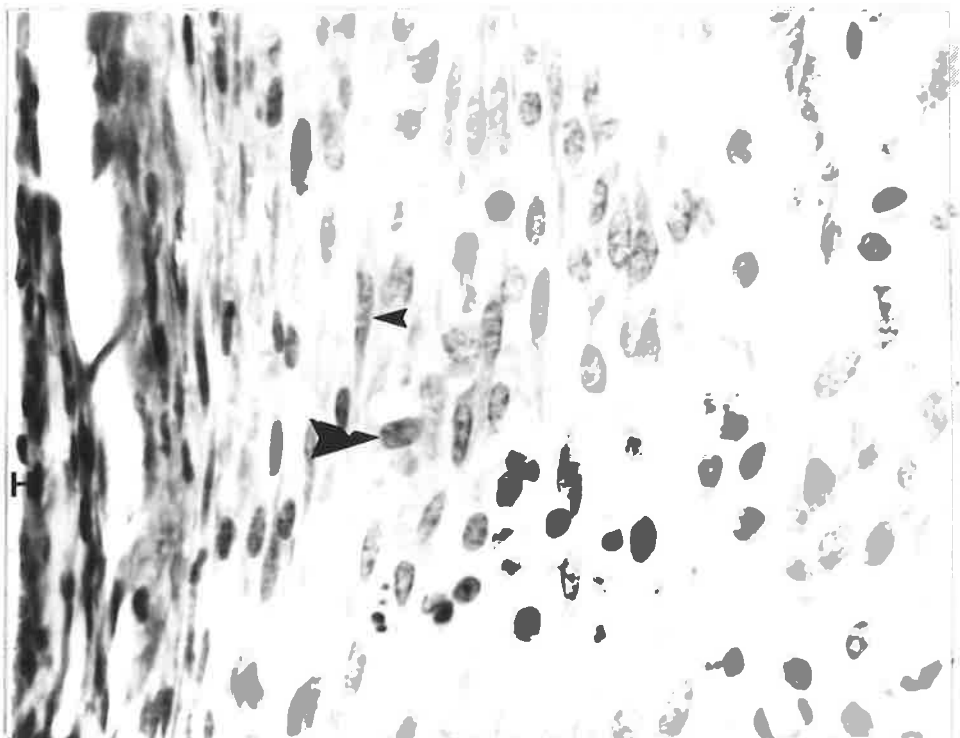

FIGURE 4.35

(iii) Two Weeks Post-Implantation

(a) *Hydron*

Both the inner zone and the outer zone appeared more mature than those observed 1 week post-implantation. The tissues adjacent to the implanted Hydron revealed an inner relatively cellular zone characterized by the presence of fibrin, fibroblasts, collagen and macrophages (Fig. 4.36). There were no extravasated erythrocytes in this zone. The densely cellular area adjacent to the implant was surrounded by a more collagenous zone. This outer zone was characterized by the presence of fibroblasts, collagen, macrophages and regenerating muscle (Fig. 4.37). Occasionally there was a scattered infiltration of lymphocytes, in the outer zone. Fat cells were present in the outer zone of all specimens.

(b) *Controls*

The tissues adjacent to implants of both AH26 and Teflon were basically similar to those adjacent to Hydron, 2 weeks post-implantation. However, extravasated erythocytes were noted in the tissues adjacent to both AH26 and Teflon implants. Fat cells were noted in the outer zone associated with both control implant materials.

FIGURE 4.36: HYDRON, 2 WEEKS POST-IMPLANTATION IN MUSCLE.

Photomicrograph illustrating the tissues adjacent to the Hydron implant (H). The inner and outer zones are still readily discernible. Note the mild infiltration of chronic inflammatory cells in the outer zone of this particular specimen.

H. & E. original magnification x100

FIGURE 4.37: HYDRON, 2 WEEKS POST IMPLANTATION IN MUSCLE.

Photomicrograph illustrating (at greater magnification) the tissues adjacent to the Hydron implant (H) seen in Fig. 4.36. The inner and outer zones can be differentiated, as well as the occasional chronic inflammatory cell in the outer zone.

H. & E. original magnification x400

FIGURE 4.36

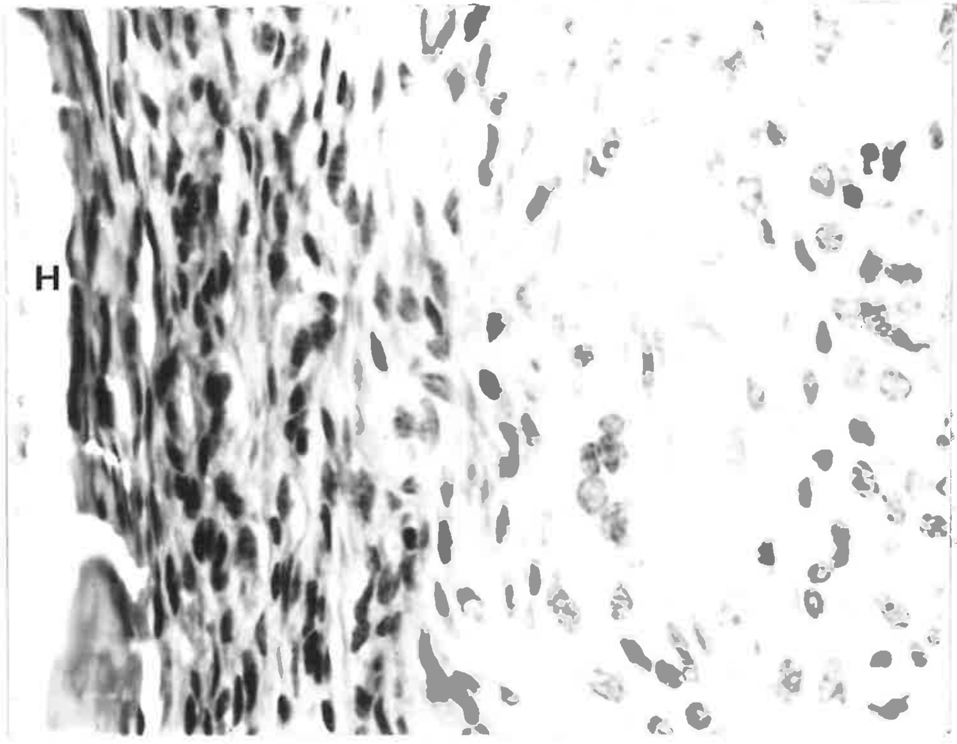

FIGURE 4.37

(iv) Four Weeks Post-Implantation

(a) *Hydron*

The tissues adjacent to the implanted Hydron revealed an inner zone characterized by the presence of fibrin, fibroblasts, collagen and macrophages (Fig. 4.38). The outer zone was characterized by the presence of fibroblasts, collagen, macrophages and regenerating muscle. Observation of sections stained with Van Gieson revealed a large amount of collagen present in both the inner and the outer zones (Fig. 4.39). This was consistent with the impression that the tissues adjacent to the implants, 4 weeks post-implantation, were more fibrosed than tissues associated with Hydron, implanted for shorter time periods.

(b) *Controls*

The tissues adjacent to both AH26 and Teflon implants revealed features similar to those seen adjacent to the Hydron implants, 4 weeks post-implantation. However, as in the case of 2 week specimens, extravasated erythrocytes were present in the inner zone associated with both control materials. Similar maturation of the connective tissue to that observed in the tissues adjacent to Hydron implants were apparent in the tissues adjacent to both AH26 and Teflon implants, 4 weeks post-implantation.

FIGURE 4.38: HYDRON, 4 WEEKS POST-IMPLANTATION IN MUSCLE.

Photomicrograph illustrating the tissues adjacent to the Hydron implant (H). The more densely cellular inner zone, and less cellular outer zone are readily discernible. The muscle (M) in this particular section is quite close to the implant.

H. & E. original magnification x400

FIGURE 4.39: HYDRON, 4 WEEKS POST-IMPLANTATION IN MUSCLE.

Photomicrograph illustrating a similar area to that illustrated in Fig. 4.38. The tissue has been stained with Van Gieson. The amount of collagen present in these tissues is highlighted by this staining method.

Van Gieson original magnification x400

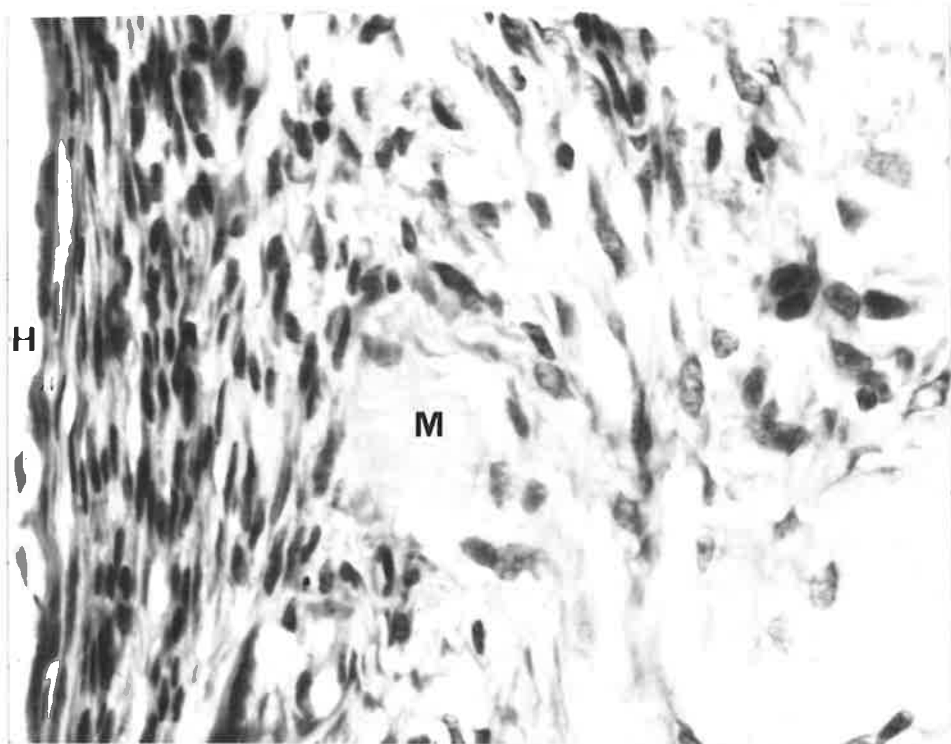

FIGURE 4.38

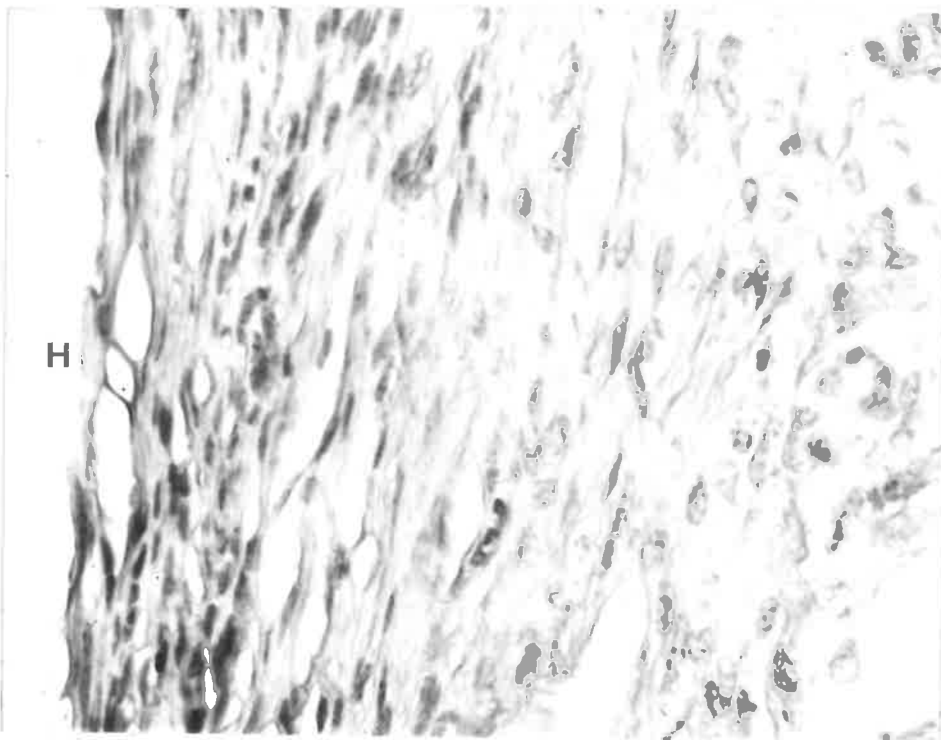

FIGURE 4.39

(v) Twelve Weeks Post-Implantation

(a) Hydron

The persistence of an inner zone and an outer zone was observed between the implant and the adjacent muscle. Both zones comprised relatively mature fibrous connective tissue (Figs. 4.40 and 4.41). Fat cells were noted in the outer zone of all specimens and was a prominent feature in several specimens. Observation of both zones revealed features indicative of further maturation than was evident in the 4 weeks post-implantation specimens. Increased maturation was characterized by increased collagen formation.

(b) Controls

The tissues adjacent to the implants of both AH26 and Teflon were characterized by features similar to those observed adjacent to implanted Hydron, 12 weeks post-implantation. Extravasated erythrocytes were noted adjacent to half the implants of AH26 and adjacent to one Teflon implant.

FIGURE 4.40: HYDRON, 12 WEEKS POST-IMPLANTATION IN MUSCLE.

Photomicrograph illustrating the tissues adjacent to the Hydron implant (H). The cellular inner zone is readily distinguished from the less cellular outer zone. The fibrous connective tissue of this outer zone appears more mature than the tissues of the outer zone of specimens from shorter time periods post-implantation. Several fat cells (arrows) can be seen in the outer zone, as well as a considerable number of them between cells in the main muscle mass. However, fat cells are not a prominent feature in this particular specimen.

H. & E. original magnification x100

FIGURE 4.41: HYDRON, 12 WEEKS POST-IMPLANTATION IN MUSCLE.

Photomicrograph illustrating (at greater magnification) the tissues adjacent to Hydron implant (H) as seen in Fig. 4.41. The cellular inner zone and part of the less cellular outer zone are illustrated in this photomicrograph.

H. & E. original magnification x400

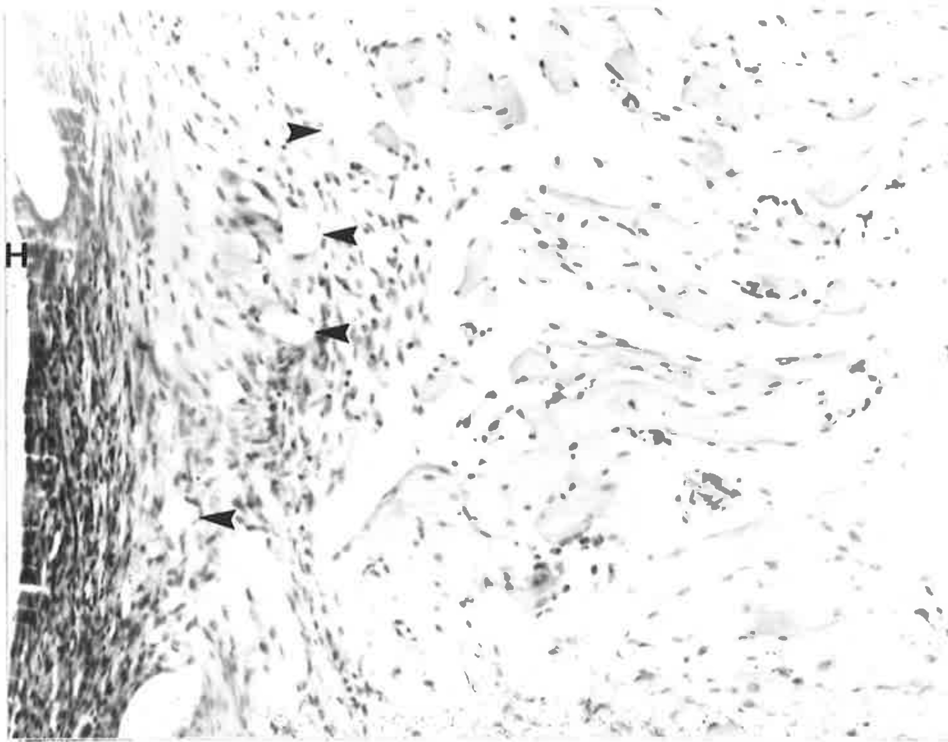

FIGURE 4.40

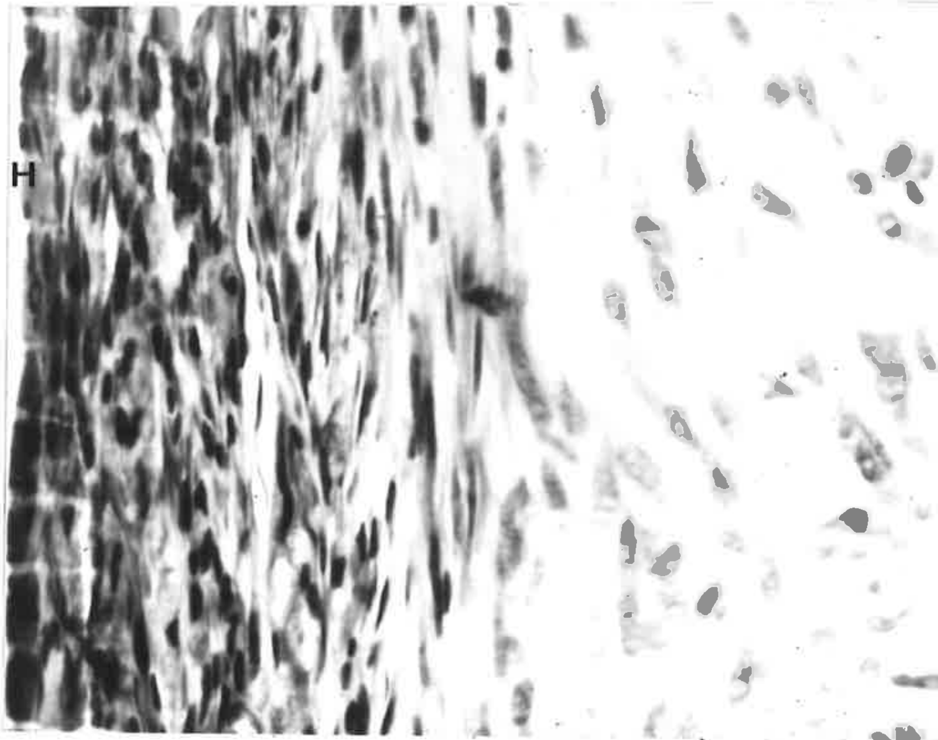

FIGURE 4.41

(vi) Twenty-Six Weeks Post-Implantation

(a) *Hydron*

Adjacent to the implanted Hydron, an inner zone was observed consisting of fibroblasts, collagen and macrophages. It resembled mature fibrous connective tissue. The outer zone was characterized by the presence of both mature areolar tissue and mature fibrous connective tissue. Few macrophages were noted in either tissues of the outer zone.

(b) *Controls*

The tissues adjacent to implants of both AH26 and Teflon were characterized by features similar to those observed adjacent to Hydron specimens. The outer zones adjacent to implants of both AH26 and Teflon were similar to the outer zone adjacent to implanted Hydron. As with the Hydron specimens, fatty tissue was a prominent feature of the tissues adjacent to the control materials.

FIGURE 4.42: HYDRON, 26 WEEKS POST-IMPLANTATION IN MUSCLE.

Photomicrograph illustrating the tissues adjacent to the Hydron implant (H). The thin inner zone (demarcated by arrows) consists of fibroblasts, collagen and macrophages. The outer zone between the inner zone and the adjacent muscle (M) consists of both mature areolar tissue (A) and mature fibrous connective tissue (F). Note the dark area (basophilic) within the Hydron at its junction with the inner zone.

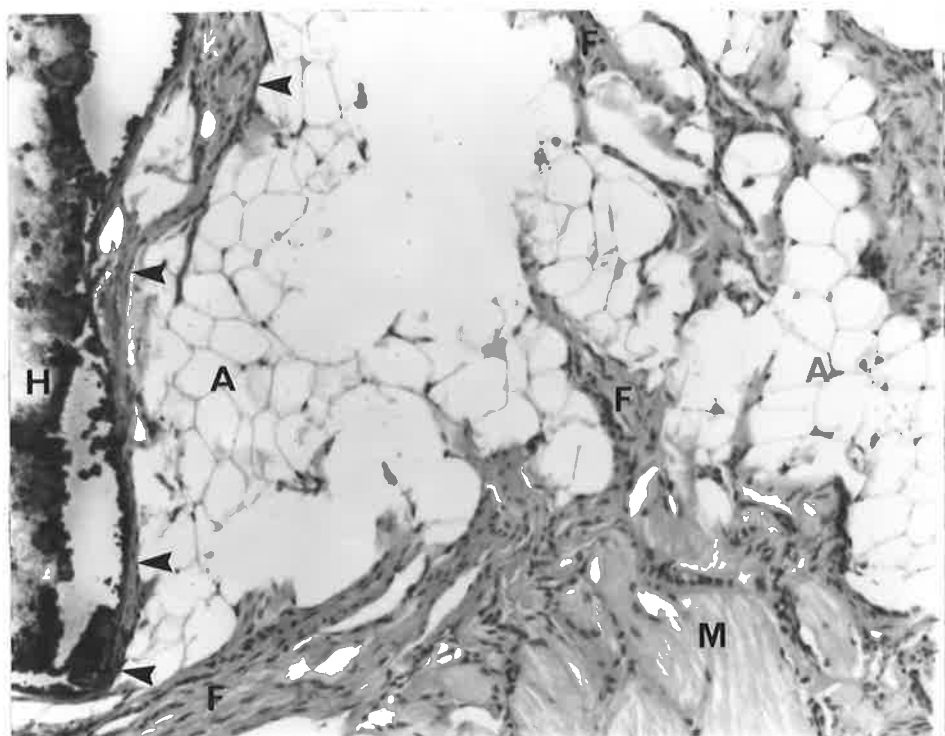

FIGURE 4.42

INFLAMMATORY CELLS ADJACENT TO THE IMPLANTS

The number of inflammatory cells in the designated area (see Section 3.2.13) were counted for each specimen and the mean calculated. Where the mean number of inflammatory cells was greater than 10, a score of 3 was assigned. Where 5-10 inflammatory cells occurred, a score of 2 was assigned and where less than 5 inflammatory cells occurred, a score of 1 was assigned. The results are presented in Table 4.1.

THE EXTENT OF THE CAPSULAR ZONE

The measurement employed to indicate the extent of the capsular zone was the distance from the interface of the implant to the nearest viable muscle cell, using the gradations on the graticule referred to in Section 3.2.13. Measurements were made employing a magnification of x400. Viable muscle was readily distinguishable in sections stained with phosphotungstinic acid and haematoxyline (P.T.A.H.) - (Fig. 4.43).

Means were plotted against time (Fig. 4.44). Hydron was compared with each control material. Student t-tests were computed to register statistically significant differences between these means at the various periods post-implantation. Differences between Hydron and Teflon were significant at 2 weeks ($P = 0.05$) and 4 weeks ($P = 0.01$), while differences between Hydron and AH26 were significant at 2 weeks ($P = 0.05$). At all other time periods the differences were not significant at the 5% level.

MEAN NUMBER OF INFLAMMATORY CELLS ADJACENT TO IMPLANTS

	HYDRON	AH26	TEFLON
2 DAYS	***	***	***
1 WEEK	*	**	*
2 WEEKS	**	**	**
4 WEEKS	*	*	**
12 WEEKS	*	*	*
26 WEEKS	*	*	**

* < 5 cells/x400 field.

** 5-10 cells/x400 field.

*** > 10 cells/x400 field.

TABLE 4.1: The mean number of inflammatory cells per x400 field in the tissues adjacent to the respective implants.

FIGURE 4.43: AH26, 4 WEEKS POST-IMPLANTATION IN MUSCLE.

Photomicrograph illustrating the tissues adjacent to implanted AH26 (A). Using this particular stain, the muscle (M) is readily distinguishable from the adjacent tissues, not only by its colour but also by the characteristic striations. The number of gradations (or part thereof) between the implant and the muscle were measured. Note the macrophages containing phagocytosed particles of AH26.

P.T.A.H. original magnification x400

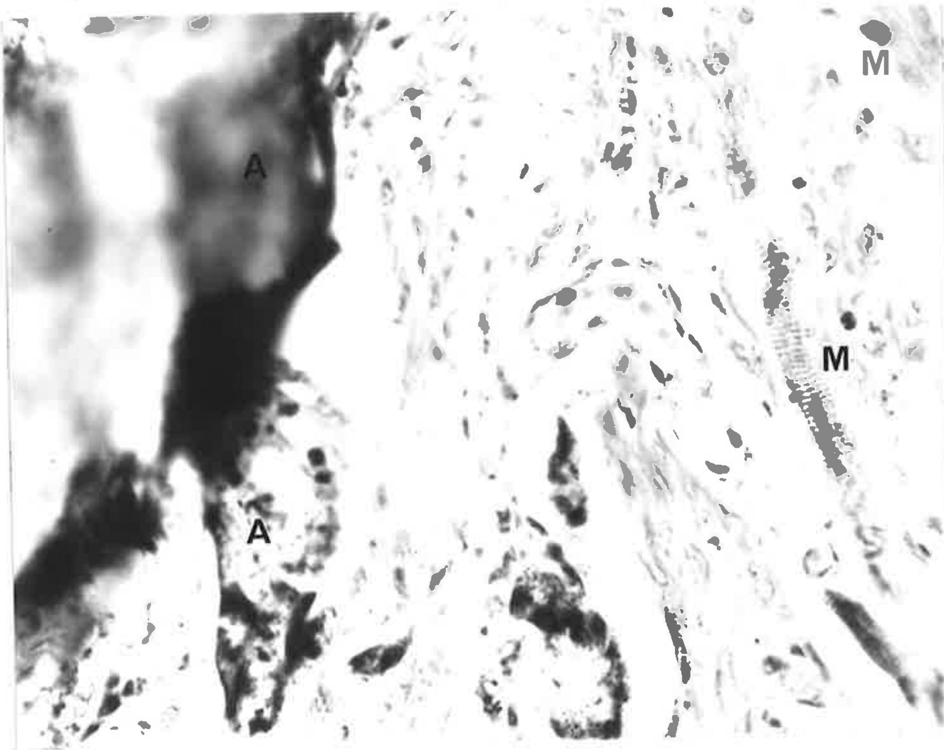

FIGURE 4.43:

TIME	MEAN (HYDRON)	S.E.	MEAN (AH26)	S.E.	MEAN (TEFLON)	S.E.
1 week	7.7	1.1	10.1	2.3	12.9	2.3
2 weeks	5.0	0.8	9.6	1.2*	10.3	1.9*
4 weeks	3.0	0.8	5.2	1.3	8.1	1.4**
12 weeks	3.4	0.8	4.1	1.2	3.5	1.2
26 weeks	3.8	1.0	6.7	1.3	5.6	1.6

* P = 0.05

** P = 0.01

FIGURE 4.44: Graph illustrating the extent of the tissue response adjacent to the various implanted materials as plotted against time post-implantation.

The magnification employed was x400.

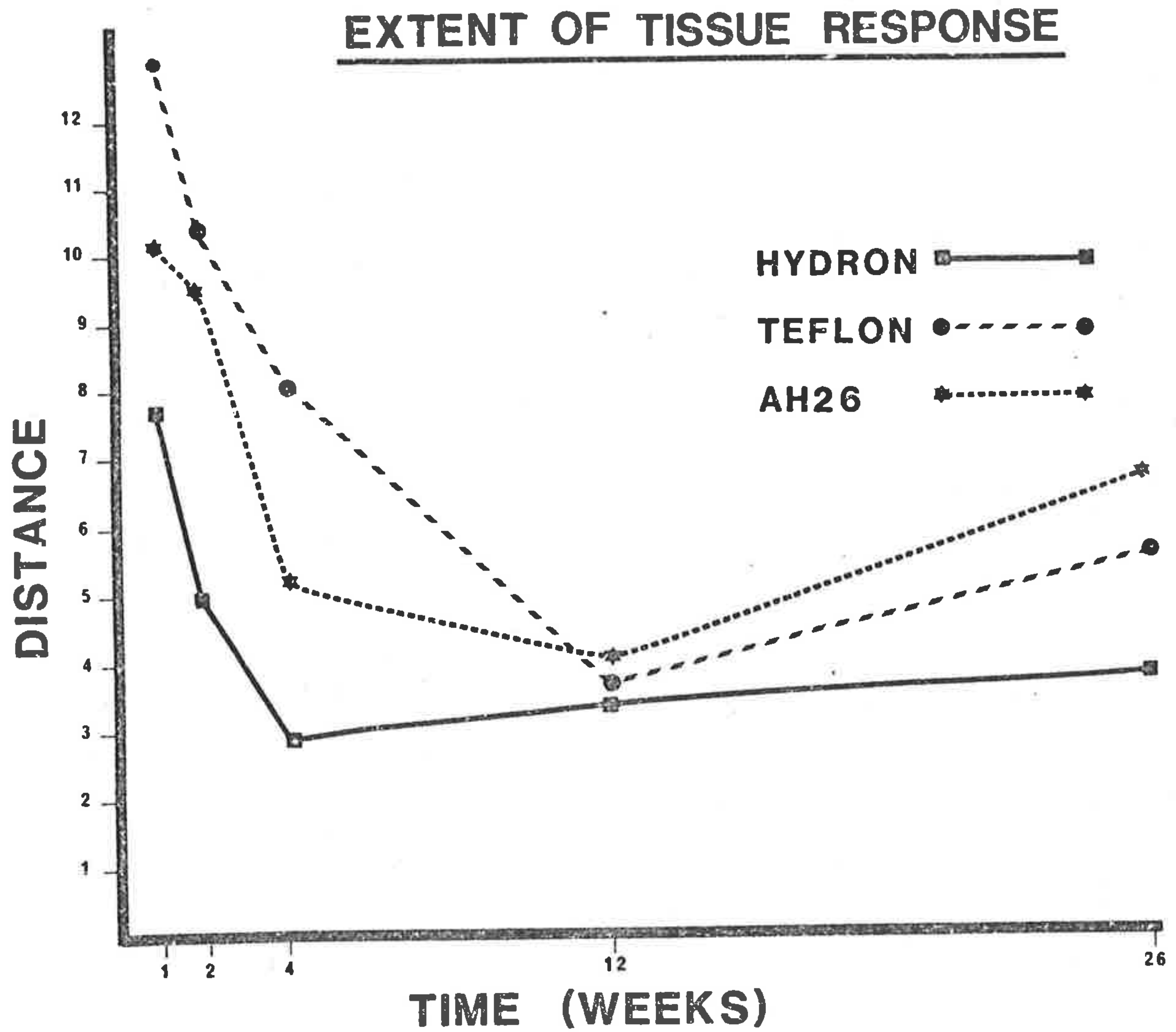

FIGURE 4.44

(a) Foreign Body Giant Cell Response

Foreign body giant cells were noted adjacent to the three implanted materials. However, no foreign body giant cells were observed adjacent to the 2 days post-implantation specimens. They occurred most commonly with implants from longer time periods post-implantation but there appeared to be no other trend. They were rarely observed with implanted Hydron. The occurrence of foreign body giant cells appeared to be associated with surface irregularities. Since the surface of AH26 implants were more irregular than those of the other materials, foreign body giant cells occurred more commonly adjacent to these implants. These cells did not appear to be phagocytosing the implant material.

(b) Macrophage Response

Microscopic observations revealed that mononuclear macrophages occurred in the tissues adjacent to implants of all three materials at all time periods from 1 weeks to 26 weeks post-implantation.

Many macrophages in the tissues adjacent to implanted Teflon contained phagocytosed material that resembled haemosiderin. Selected sections were stained with Prussian Blue (Appendix VII) to confirm that this material was haemosiderin. The phagocytosed material stained positively in all cases (Fig. 4.45).

The tissues adjacent to implants of AH26 contained phagocytosing macrophages that contained either AH26 only, haemosiderin only or a mixture of both. Because of the difficulty encountered distinguishing between AH26 and the haemosiderin contained within the cytoplasm of these macrophages, quantitation of the number of macrophages containing phagocytosed AH26 was not possible.

Phagocytosing macrophages in the tissues adjacent to implanted Hydron contained either Hydron, haemosiderin or both in their cytoplasm. Phagocytosed Hydron could be distinguished from haemosiderin in haemotoxylin and eosin stained sections. In these H. & E. sections haemosiderin particles appeared brown whereas particles of Hydron appeared almost clear. The two materials refracted at different levels. To confirm the identity of the material thought to be haemosiderin, selected sections were stained with Prussian Blue. The abovementioned material stained positively with Prussian Blue (Fig. 4.46).

(i) *Quantitation of macrophages phagocytosing Hydron*

The numbers of macrophages containing phagocytosed Hydron in the tissues adjacent to each Hydron implant were quantitated, according to the method outlined in Section 3.2.13. These results were plotted against time and are shown in Fig. 4.47.

Considerable differences occurred not only between the various implants in each time period, but also between the two ends of the same implant (as shown by the relatively large standard error of the mean, expressed as a positive and negative value at each point on the graph). The greatest number of macrophages occurred at 2 weeks post-implantation and thereafter decreased with time.

FIGURE 4.45: TEFLON, 2 WEEKS POST-IMPLANTATION IN MUSCLE.

Photomicrograph illustrating phagocytosing macrophages in the tissues adjacent to implanted Teflon. These are the only cells that stain positively with Prussian Blue. This is indicative of material containing iron, in this instance haemosiderin.

Prussian Blue original magnification x400

FIGURE 4.46: HYDRON, 4 WEEKS POST-IMPLANTATION IN MUSCLE.

Photomicrograph illustrating phagocytosing macrophages in the tissues adjacent ot the implanted Hydron. Macrophage (M1) contains particles of phagocytosed Hydron only. Macrophage (M2) contains phagocytosed Hydron and phagocytosed haemosiderin. Macrophage (M3) contains phagocytosed haemosiderin only.

Prussian Blue original magnification x400

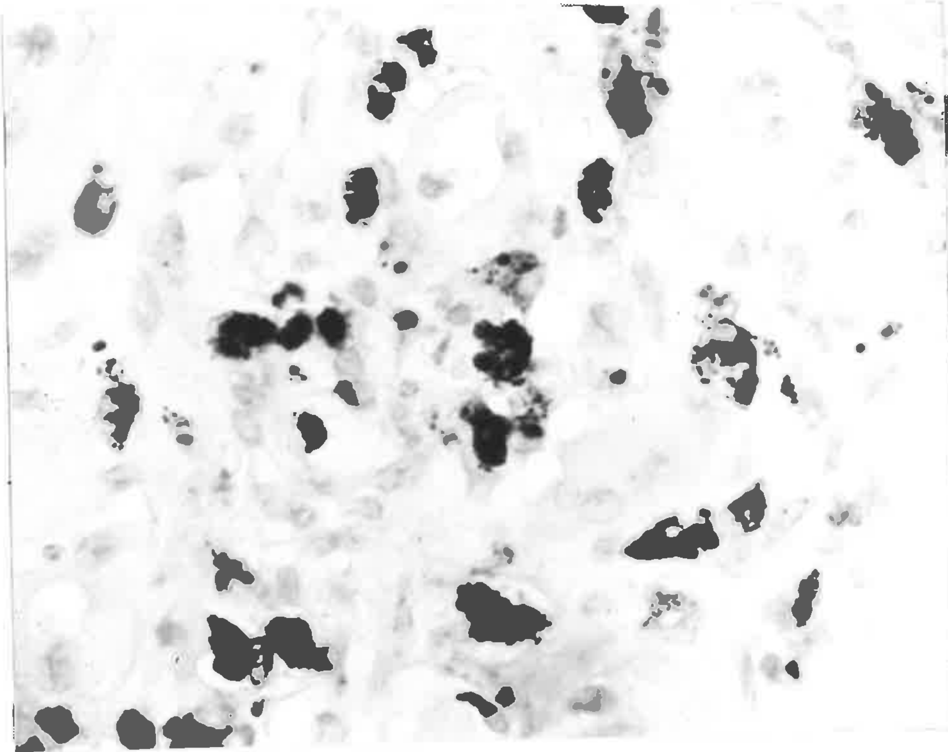

FIGURE 4.45

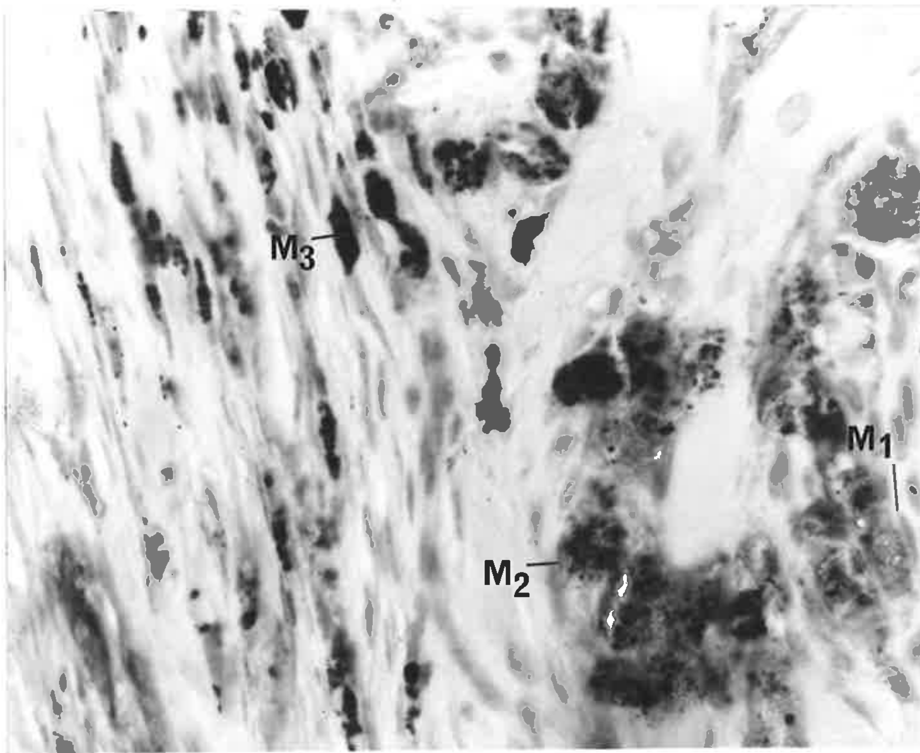

FIGURE 4.46

FIGURE 4.47: Graph illustrating the number of macrophages containing phagocytosed Hydron for the various time periods post-implantation. No attempt was made to quantitate the amount of Hydron in each macrophage.

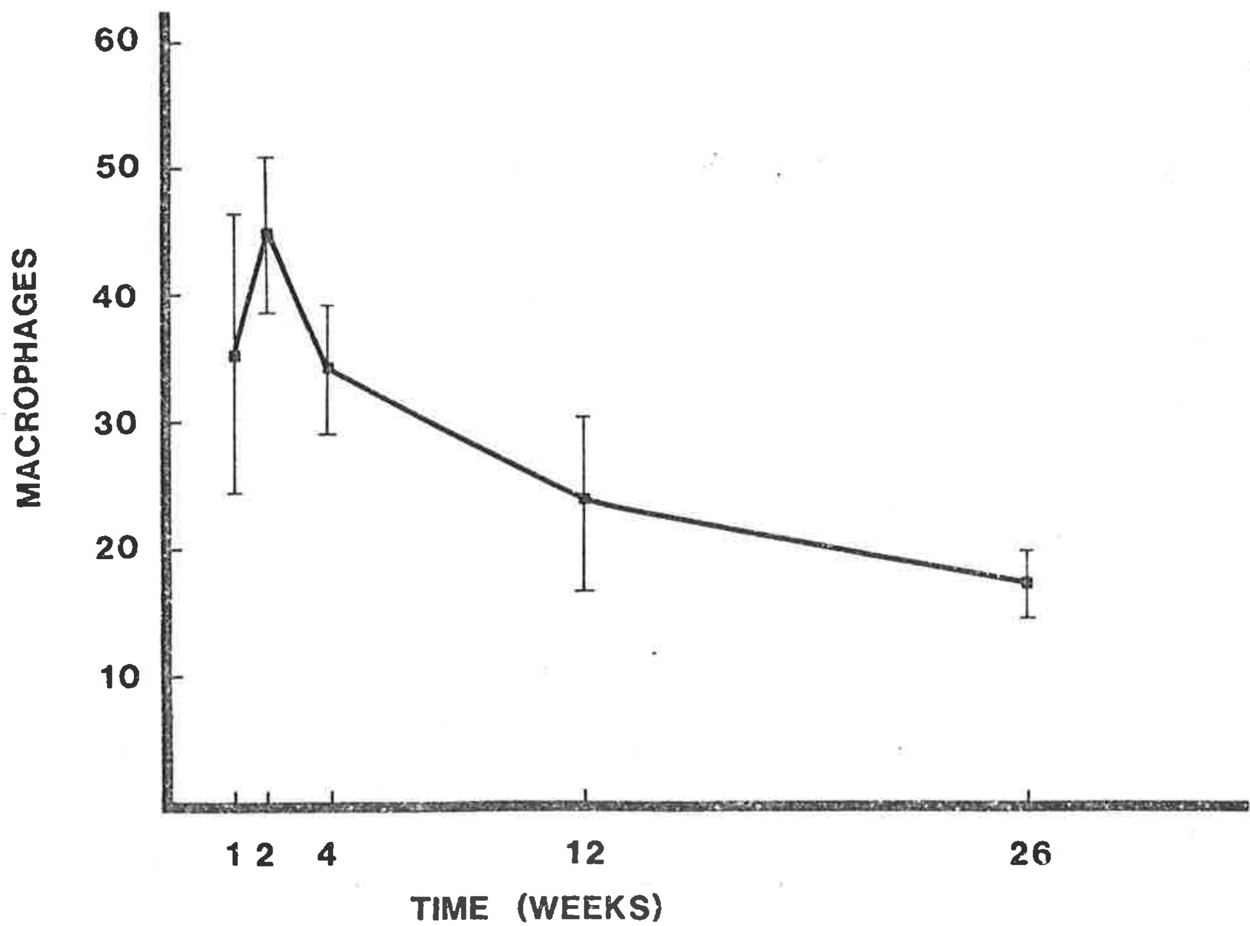

FIGURE 4.47

(*ii*) *Distribution of macrophages phagocytosing Hydron (Poisson Analysis)*

The distribution of macrophages containing phagocytosed Hydron was investigated in the same central area of the implant described in the previous section (Quantitation of macrophages phagocytosing Hydron). Central sections from two implants from each time period post-implantation were used. The magnification employed was x400. The counting grid contained 100 small squares, and the number of phagocytosing macrophages contained in each small square was recorded as in Fig. 4.48. (Each example given below is for implant 85 M/L/H, Hydron implanted for 4 weeks, and is recorded to illustrate the procedure used for all sections assessed.)

From Fig. 4.48 the following table can be compiled.

NUMBER OF MACROPHAGES/SQUARE	OBSERVED OCCURRENCE f
0	51
1	26
2	15
3	17
>3	1

TABLE 4.2

The mean in this case was 0.81 macrophages per square and the variance, was calculated to be 1.00.

FIGURE 4.48: Number of macrophages observed per small square in a counting grid. The area assessed was the tissues adjacent to the central area of a Hydron implant, 4 weeks post-implantation.

Magnification x400

0	0	2	1	0	1	3	1	2	1
2	1	1	0	0	1	1	0	0	0
2	0	3	0	1	0	1	2	0	3
1	1	0	1	0	1	0	1	3	4
1	1	0	0	0	1	2	2	1	2
2	0	0	0	3	0	1	3	3	1
0	0	0	0	1	1	1	0	2	0
0	0	0	1	2	2	0	0	0	0
2	2	0	0	0	0	0	0	0	0
2	1	0	0	0	0	0	0	0	0

FIGURE 4.48

The Coefficient of Dispersion (see Statistical Methods) was determined ($\frac{S^2}{\bar{Y}}$) and was 1.24. This value is very indicative of a Poisson distribution. To determine the goodness of the "fit", a χ^2 test was performed between observed and expected values (Table 4.3).

Y	OBSERVED OCCURRENCES (O)	EXPECTED OCCURRENCES (E)	DEVIATIONS FROM EXPECTED (O-E)	DEVIATIONS SQUARED $(O-E)^2$	$\frac{(O-E)^2}{E}$
0	51	45	6	36	0.80
1	26	36	-10	100	2.78
2	15	14	1	1	0.07
3	7	4	3	9	2.25
>3	1	1	0	0	0.00
	100	100	$\Sigma \frac{(O-E)^2}{E} = \chi^2$	=	5.90

TABLE 4.3

The χ^2 value at the 5% significance level for 4 degrees of freedom is 9.49. Since the χ^2 value obtained from Table 4.3 is less than 9.49, the difference between the observed values expected in a Poisson distribution is not significant, i.e. the distribution is random.

When similar calculations were performed with the other sections, the following table (Table 4.4) was produced. The distribution in each case is random.

DISTRIBUTION OF MACROPHAGES CONTAINING HYDRON

	Coefficient of Dispersion ($\frac{s^2}{\bar{Y}}$)	χ^2	ν	χ^2 at 5% Significance Level	
1 week	0·93	4·37	4	9·49	☆
	1·13	8·03	4	9·49	☆
2 weeks	1·02	5·35	3	7·81	☆
	1·08	1·72	3	7·81	☆
4 weeks	1·19	2·50	2	5·99	☆
	1·24	5·90	4	9·49	☆
12 weeks	0·95	1·86	2	5·99	☆
	1·17	3·76	4	9·49	☆
26 weeks	1·23	4·21	3	7·81	☆
	0·99	0·30	3	7·81	☆

☆ = NOT SIGNIFICANT

TABLE 4.4

(c) *Presence of Fat Cells*

In the capsule adjacent to the Hydron and both controls, initially there was an insignificant amount of fat present. This increased up to 2 weeks post-implantation and then fell at 4 weeks. Thereafter, the amount of adipose tissue associated with the implants of all 3 materials increased.

The quantitative results of this investigation are expressed graphically for the various times post-implantation (Figs. 4.49 and 4.50).

When the difference between the values obtained were compared using Student t-tests, no significant differences were recorded (P = 0.05).

TIME	MEAN (HYDRON)	STANDARD ERROR	MEAN (AH26)	STANDARD ERROR
2 days	0	-	0	-
1 week	3.1	1.68	0	-
2 weeks	9.4	5.19	6.1	1.67
4 weeks	6.4	2.69	1.1	0.68
12 weeks	15.4	5.81	13.0	3.39
26 weeks	37.7	9.80	44.4	7.81

FIGURE 4.49: Graph illustrating the number of squares where fat cells were observed in the tissues adjacent to either Hydron or AH26 implants for each time post-implantation. No attempt was made to quantitate the amount of fat in each square, i.e. the particular square either contained fat cells or no fat cells. Observations were made in a section from the central area of the particular implant and this section had been stained with H. & E.

The magnification at which these observations were made was x100.

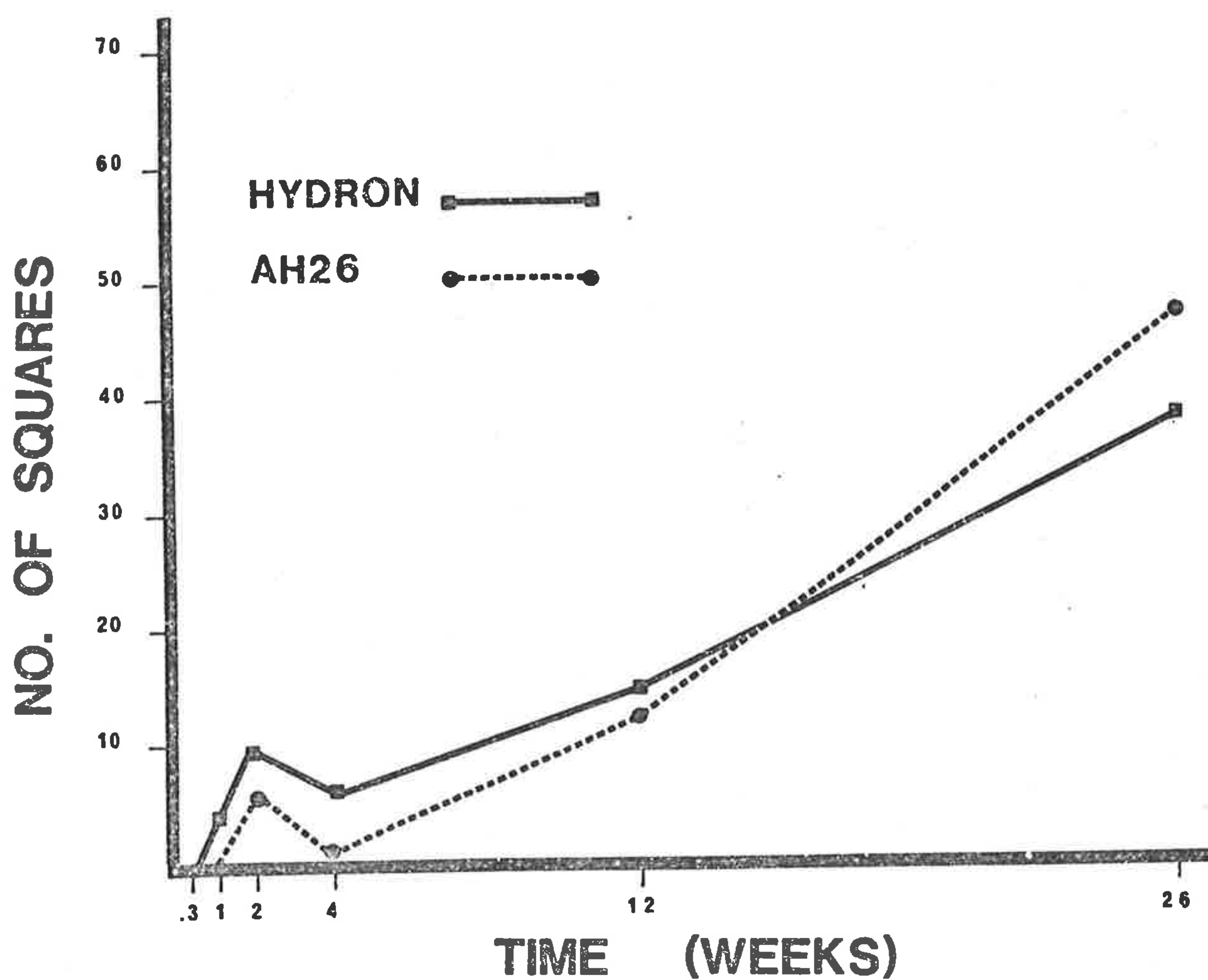

FIGURE 4.49

TIME	MEAN (HYDRON)	STANDARD ERROR	MEAN (TEFLON)	STANDARD ERROR
2 days	0	-	0	-
1 week	3.1	1.68	1.9	1.30
2 weeks	9.4	5.19	9.5	1.61
4 weeks	6.4	2.69	5.1	1.94
12 weeks	15.4	5.81	19.0	10.00
26 weeks	37.7	9.80	70.4	12.33

FIGURE 4.50: Graph illustrating the number of squares where fat cells were observed in the tissues adjacent to either Hydron or Teflon implants for each time post-implantation. No attempt was made to quantitate the amount of fat in each square, i.e. the particular square either contained fat cells or no fat cells. Observations were made in a section from the central area of the particular implant. Sections were stained with H. & E.

The magnification at which these observations were made was x100.

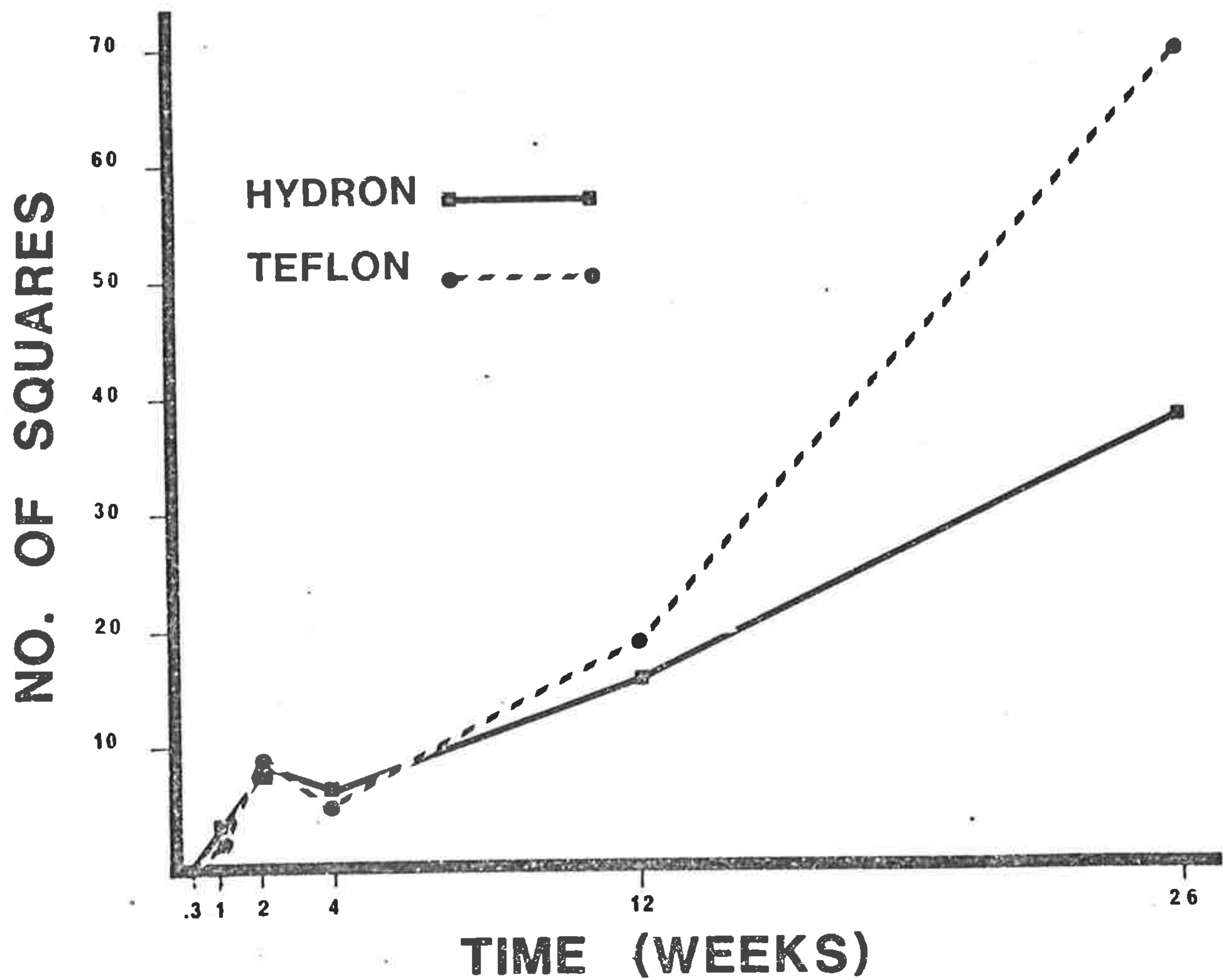

FIGURE 4.50

(d) Mineralization of Hydron

(i) Histochemistry

A central section from each implant of Hydron was stained with a modified Von Kossa stain (Appendix VI).

(i) Two Days Post-Implantation

Microscopic observations of the Hydron implants revealed small, discrete Von Kossa positive bodies situated within the mass of the implant (Fig. 4.51).

(ii) One Week and Two Weeks Post-Implantation

Observations of the Hydron implants revealed the small, discrete Von Kossa positive areas similar to those noted in Hydron at 2 days post-implantation. As well as these areas, a number of larger more densely staining areas were observed within the Hydron adjacent to the interface between it and the tissues (Fig. 4.52). These positively staining areas occurred in the majority of sections from these periods post-implantation but not in every section. When present these areas were seen to be of variable size and discrete.

(iii) Four Weeks Post-Implantation

Microscopic observations of the Hydron implants revealed that the postively staining areas of the interface of the implant formed an almost continuous band. This area was however quite thin. The small discrete areas observed in the 2 day post-implantation specimens were present in the remainder of the implant (Fig. 4.53).

(iv) Twelve Weeks Post-Implantation

Observations of the Hydron implants revealed a thick continuous band of calcium containing material at the interface of the implanted Hydron and the tissues. The small areas of positive staining (initially observed at 2 days) within the main body of the implant, were also observed in the specimens from this period post-implantation.

(v) Twenty-Six Weeks Post-Implantation

A thick continuous band of calcium containing material was present at the interface of the implant and the tissues. The small positively staining areas adjacent to this band appeared more numerous than those observed at shorter periods post-implantation and appeared to coalesce in some areas. Small positively staining areas within the main body of the implant resembling those observed at all other times post-implantation were noted (Fig. 4.55).

FIGURE 4.51: HYDRON, 2 DAYS POST-IMPLANTATION IN MUSCLE.

Photomicrograph illustrating the interface between the Hydron implant (H) and the tissues (T). Throughout the Hydron there are a number of small, discrete Von Kossa positive bodies.

Modified Von Kossa original magnification x250

FIGURE 4.52: HYDRON, 1 WEEK POST-IMPLANTATION IN MUSCLE.

Photomicrograph illustrating the interface between the Hydron implant (H) and the tissues (T). At the interface, the positively staining areas are larger and more numerous. Note the small positively staining areas throughout the body of the implant.

Modified Von Kossa original magnification x250

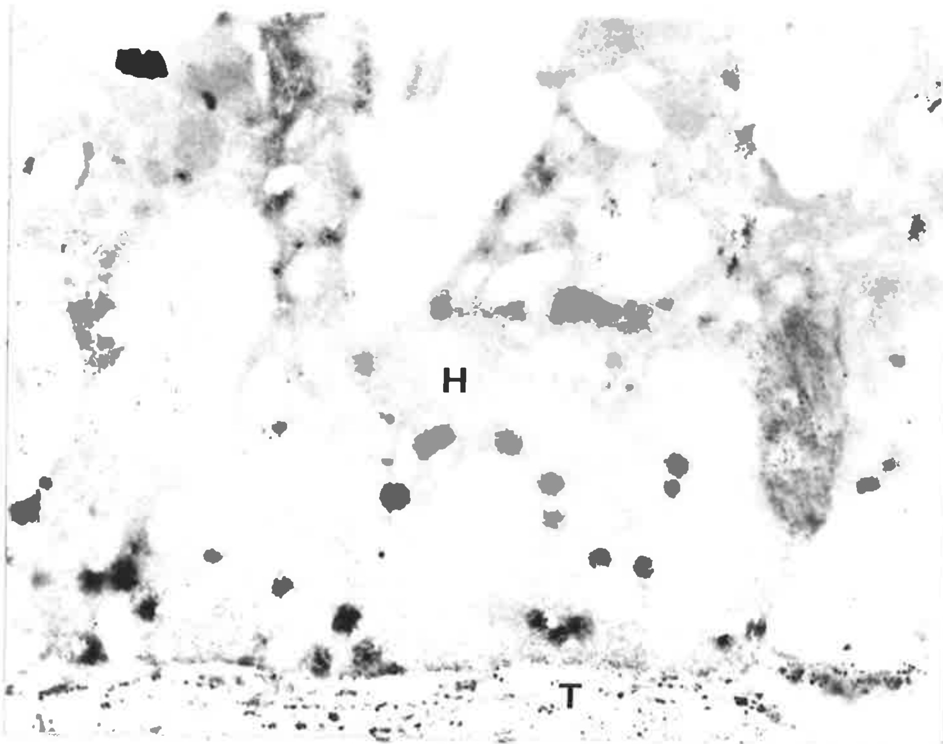

FIGURE 4.51

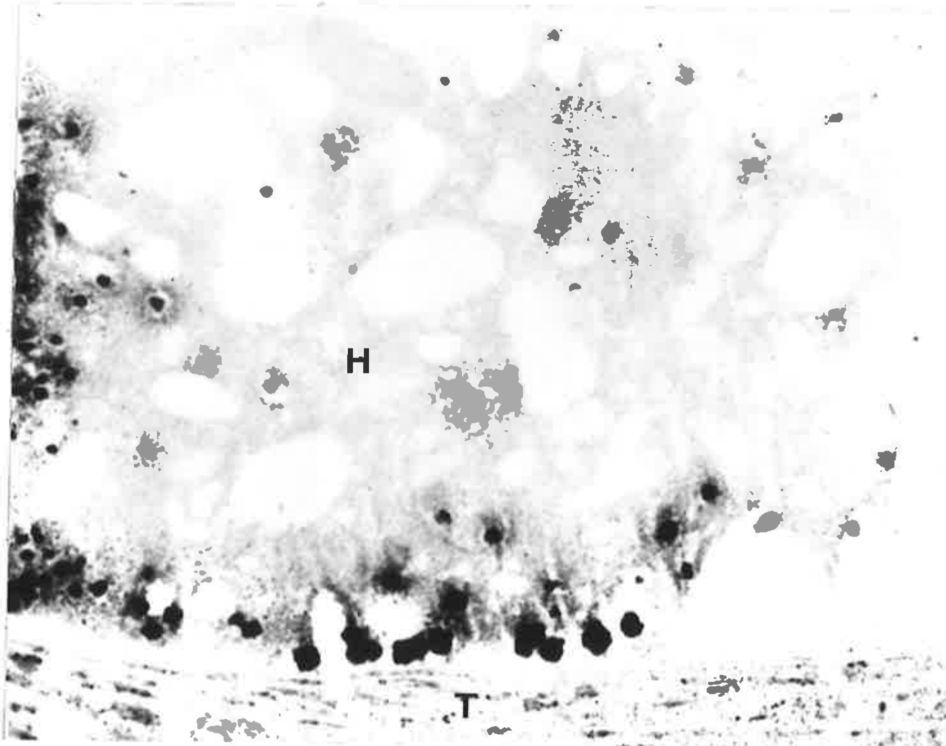

FIGURE 4.52

FIGURE 4.53: HYDRON, 4 WEEKS POST-IMPLANTATION IN MUSCLE.

Photomicrograph illustrating the interface between the Hydron implant (H) and the tissues (T). The positively staining areas are continuous along part of the interface. Adjacent to the interface the positively staining areas are larger and more numerous than those scattered throughout the remainder of the implant.

Modified Von Kossa original magnification x250

FIGURE 4.54: HYDRON, 12 WEEKS POST-IMPLANTATION IN MUSCLE.

Photomicrograph illustrating the interface between the Hydron implant (H) and the tissues (T). There is a relatively thick, continuous Von Kossa positive band along the interface of the Hydron with the tissues. The small positively staining areas are more numerous adjacent to the stained band at the interface than throughout the remainder of the implant.

Modified Von Kossa original magnification x250

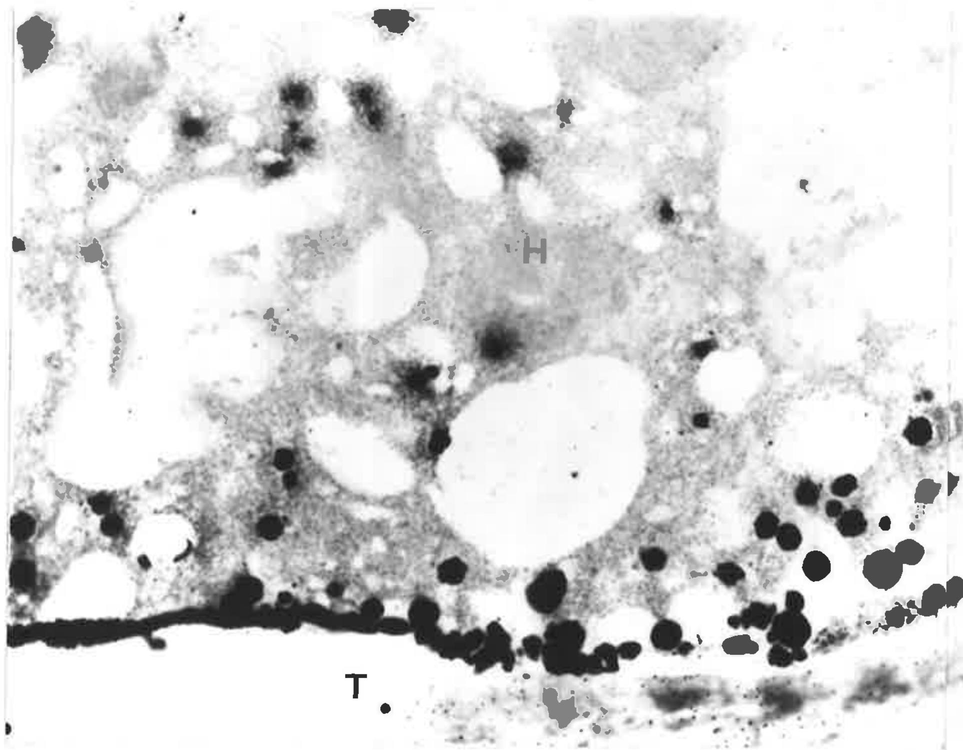

FIGURE 4.53

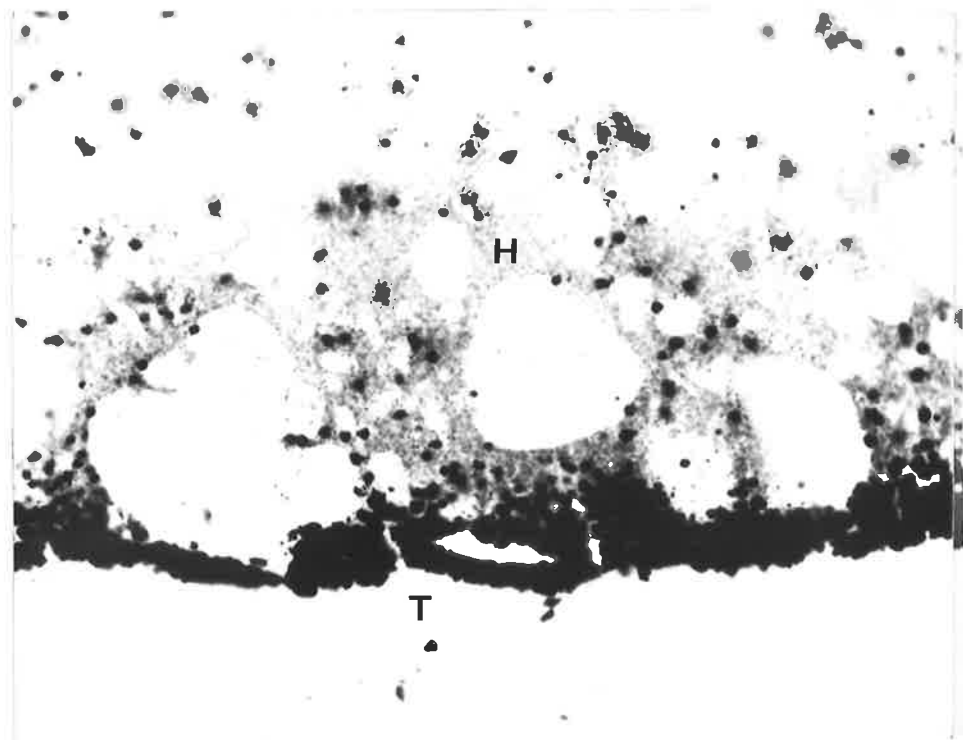

FIGURE 4.54

FIGURE 4.55: HYDRON, 26 WEEKS POST-IMPLANTATION IN MUSCLE.

Photomicrograph illustrating the interface between the Hydron implant (H) and the tissues (T). There is a relatively thick, continuous Von Kossa positive band at the interface of the Hydron with the tissues. Within the implant but adjacent to the interface there are many small positively staining areas some of which appear to have coalesced. Toward the top of this photomicrograph (within the implant) the small positively staining areas appear similar to those of the other periods post-implantation.

Modified Von Kossa original magnification x250

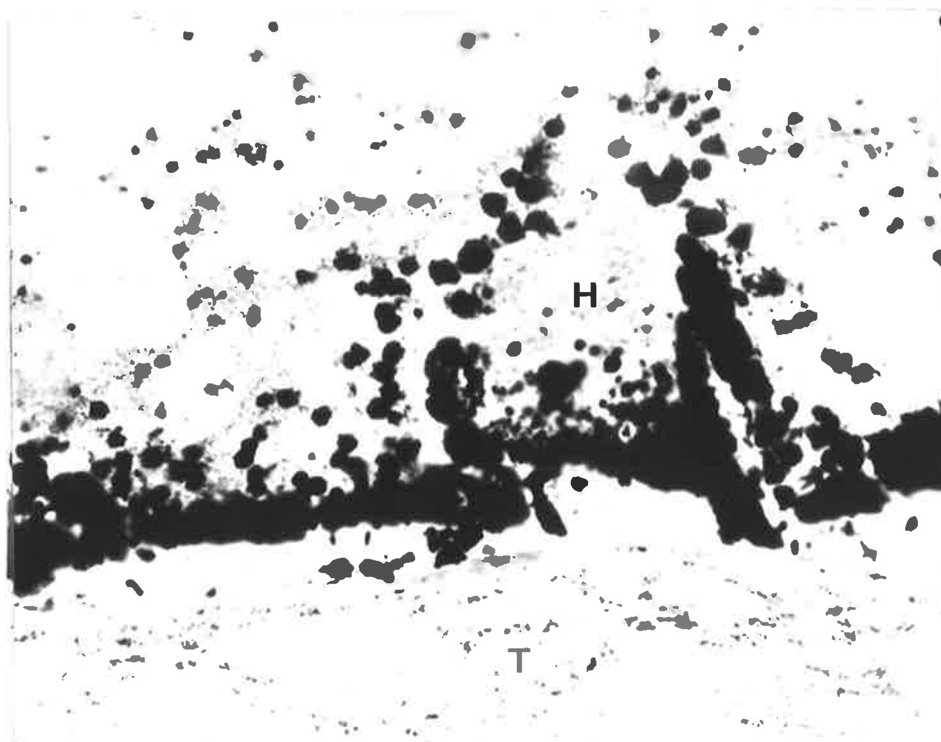

FIGURE 4.55

(ii) *Scanning electron microscopy*

The interface zone between the implanted Hydron and the tissues were analysed using a scanning electron probe and an energy dispersive analysis system. Using the scanning electron probe the main elements detected at the interface were calcium and phosphorous. Minimal amounts of barium and sulphur were detected (Fig. 4.56). Within the implant barium and sulphur were the only elements detected (Fig. 4.57). Using the energy dispersive analysis system, a high concentration of elemental calcium was observed at the interface of the implant with the tissues (Figs. 4.58 and 4.60). Also using the same method, barium was found mainly within the Hydron implant (Fig. 4.62).

(e) *High Pressure Liquid Chromatographic Analyses*

The results of the high pressure liquid chromatographic analyses of Hydron paste, Hydron powder and distilled HEMA (liquid) are shown (Figs. 4.63 and 4.65). Analysis of these tracings was carried out by the chief research chemist at Sola Optical Australia Pty. Ltd. (who provided the tracings). They are said to show the presence of at least one as yet unidentified compound, not mentioned in previous research papers, in both the Hydron paste and the Hydron powder (Sothman, 1980).

FIGURE 4.56: Photograph of the screen of the JOEL Superprobe during analysis of Hydron section.

The area being analysed is the interface of the Hydron implant with the tissues. The red peak represents elemental phosphorous. The blue area represents elemental calcium, being composed of the tall Kα peak and the smaller Kβ peak. The green and brown peaks represent respectively sulphur and the Kα and smaller Kβ peaks of barium.

FIGURE 4.56a: Photograph of the screen of the JOEL Superprobe during analysis of Hydron section.

The area being analysed is within the Hydron implant. The colours represent the same elements as those in Fig. 4.56. The yellow peaks at the right of the Kβ peak of barium represent the silicon of which the glass microscope slide is composed.

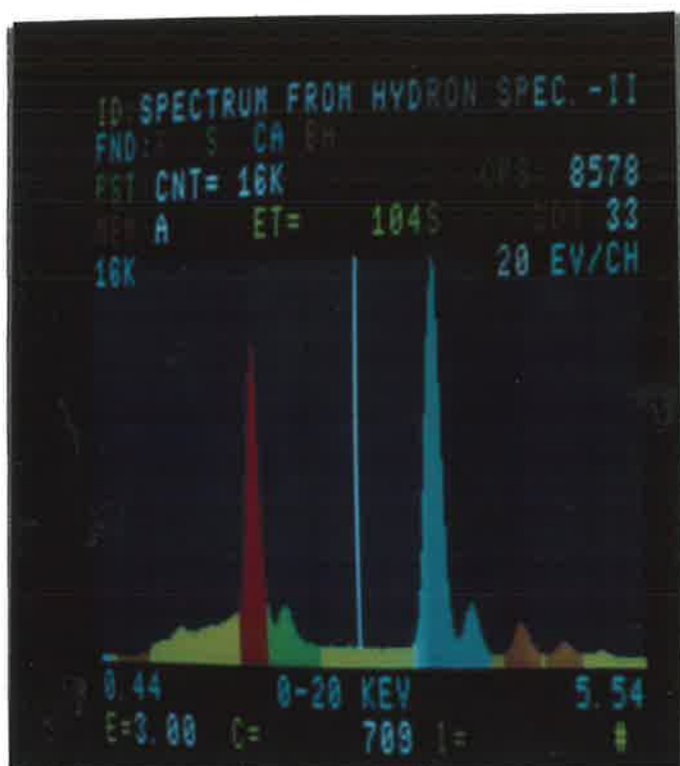

FIGURE 4.56

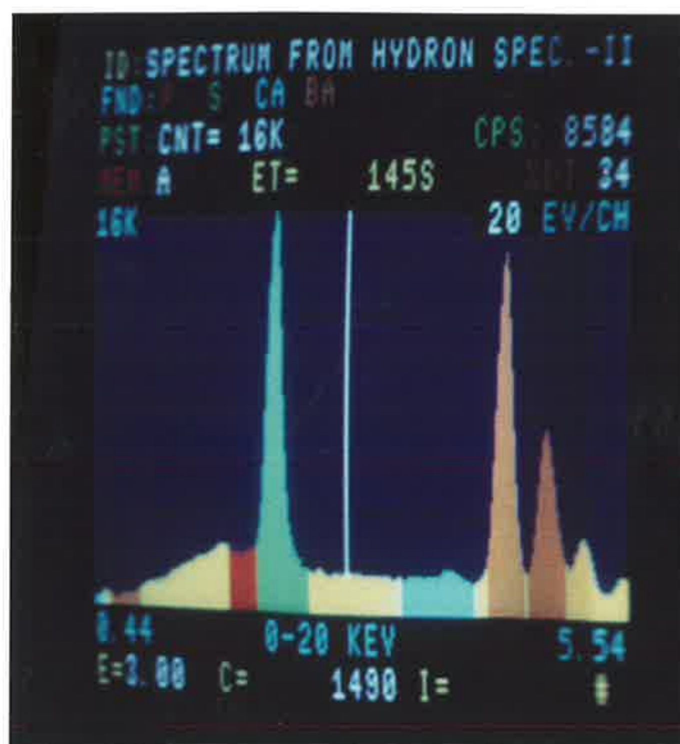

FIGURE 4.56a

FIGURE 4.57: Scanning electron micrograph illustrating the zone of mineralization (Z) between the implanted Hydron (H) and the tissues (T).

Original magnification x600

FIGURE 4.58: Scanning electron micrograph illustrating the distribution of elemental calcium in the same area as illustrated in Fig. 4.57. The highest concentration of calcium (greatest density of white dots) occurs at the zone of mineralizations (Z) between the implanted Hydron (H) and the tissues (T). There are small areas within the Hydron where there appears to be concentrations of elemental calcium.

Original magnification x600

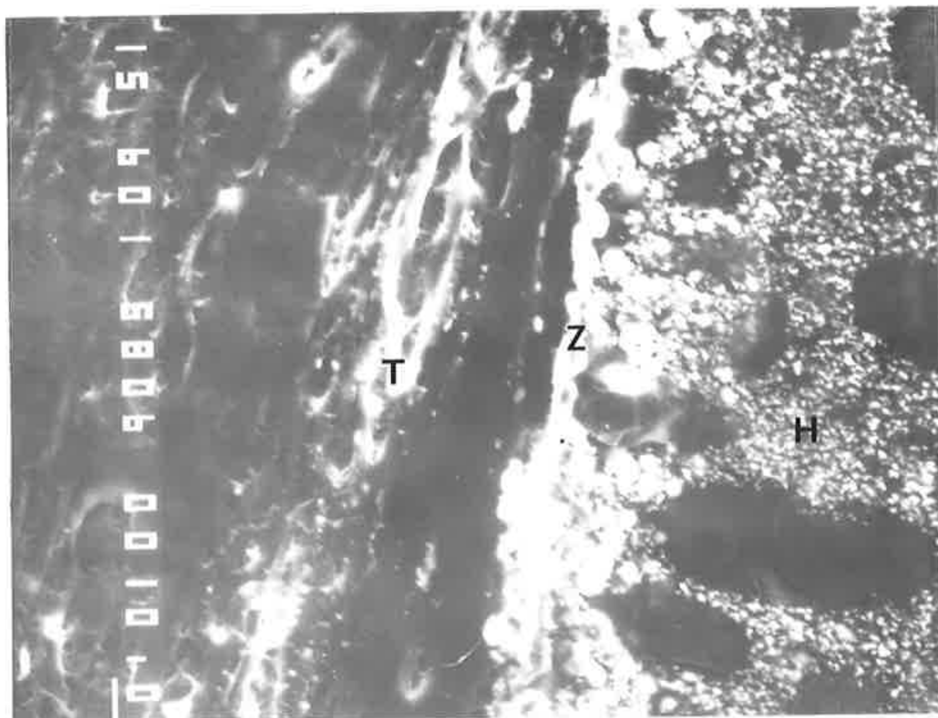

FIGURE 4.57

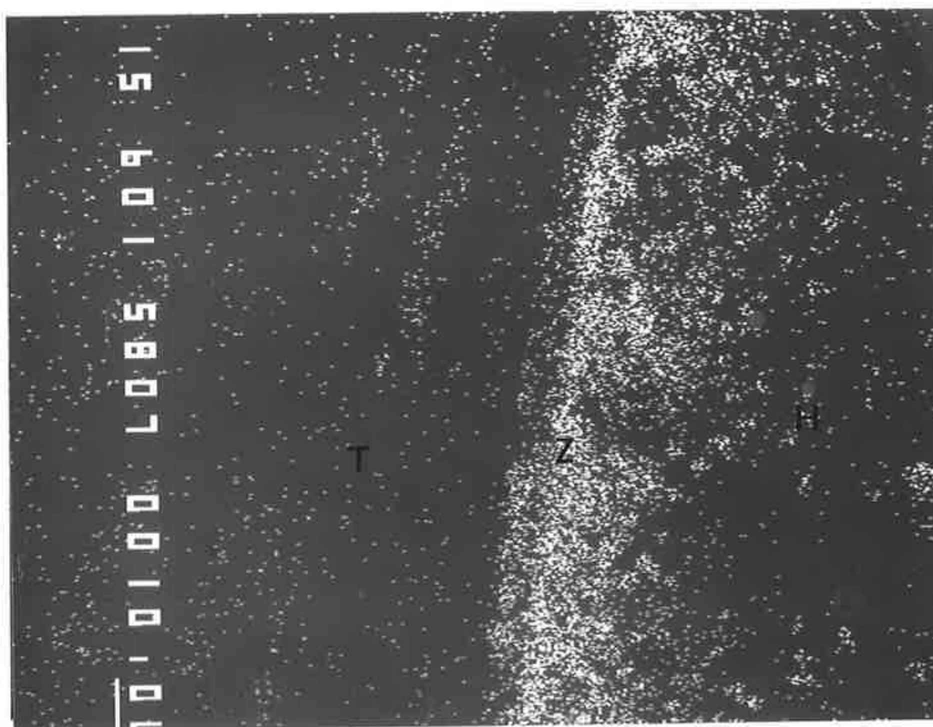

FIGURE 4.58

FIGURE 4.59: Scanning electron micrograph illustrating (at greater magnification) the zone of mineralization (Z) between the implanted Hydron (H) and the tissues (T).

Original magnification x6000

FIGURE 4.60: Scanning electron micrograph illustrating the distribution of elemental calcium in the same area as illustrated in Fig. 4.59. The greatest concentration of calcium occurs at the zone of mineralization (Z) between the implanted Hydron (H) and the tissue (T). The contrast between the zone of mineralization and the Hydron is not as distinct as in Fig. 4.58. There appears to be a high concentration of elemental calcium in the Hydron adjacent to the zone of mineralization.

Original magnification x6000

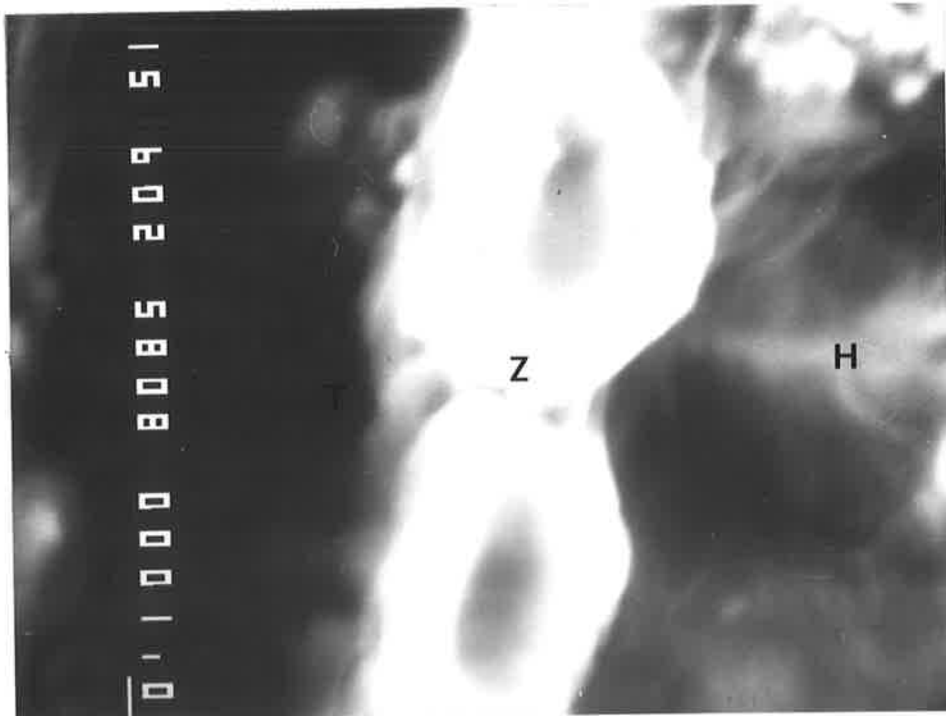

FIGURE 4.59

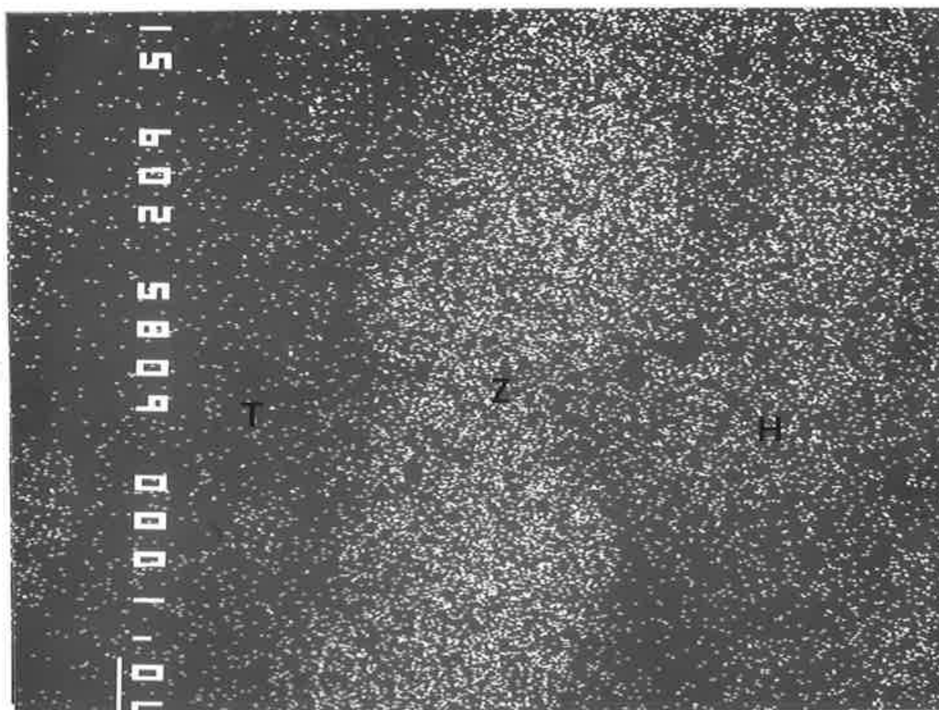

FIGURE 4.60

FIGURE 4.61: Scanning electron micrograph illustrating the zone of mineralization (Z) between the implanted Hydron (H) and the tissues (T), using a scanning electron microscope.

Original magnification x600

FIGURE 4.62: Scanning electron micrograph illustrating the distribution of elemental barium in the same area as illustrated in Fig. 4.61. There is very little barium in the zone of mineralization (Z) and the tissues (T) while there is a high concentration within the implanted Hydron (H).

Original magnification x600

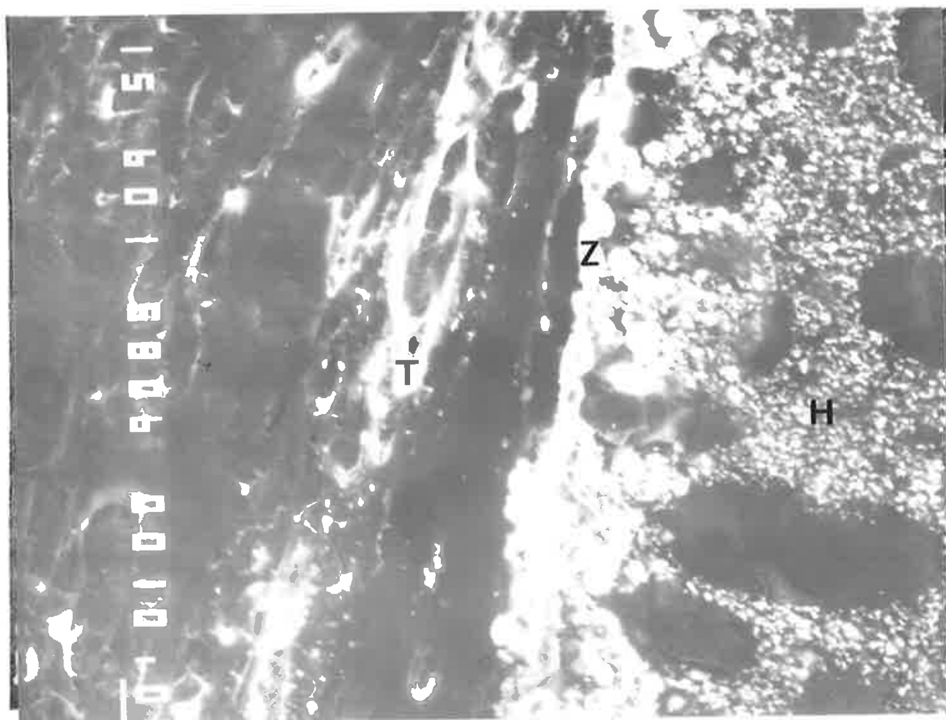

FIGURE 4.61

FIGURE 4.62

FIGURE 4.63: High pressure liquid chromatographic tracing of distilled HEMA.

This liquid used by Sola Optical to manufacture soft contact lenses is said to be more than 90% pure.

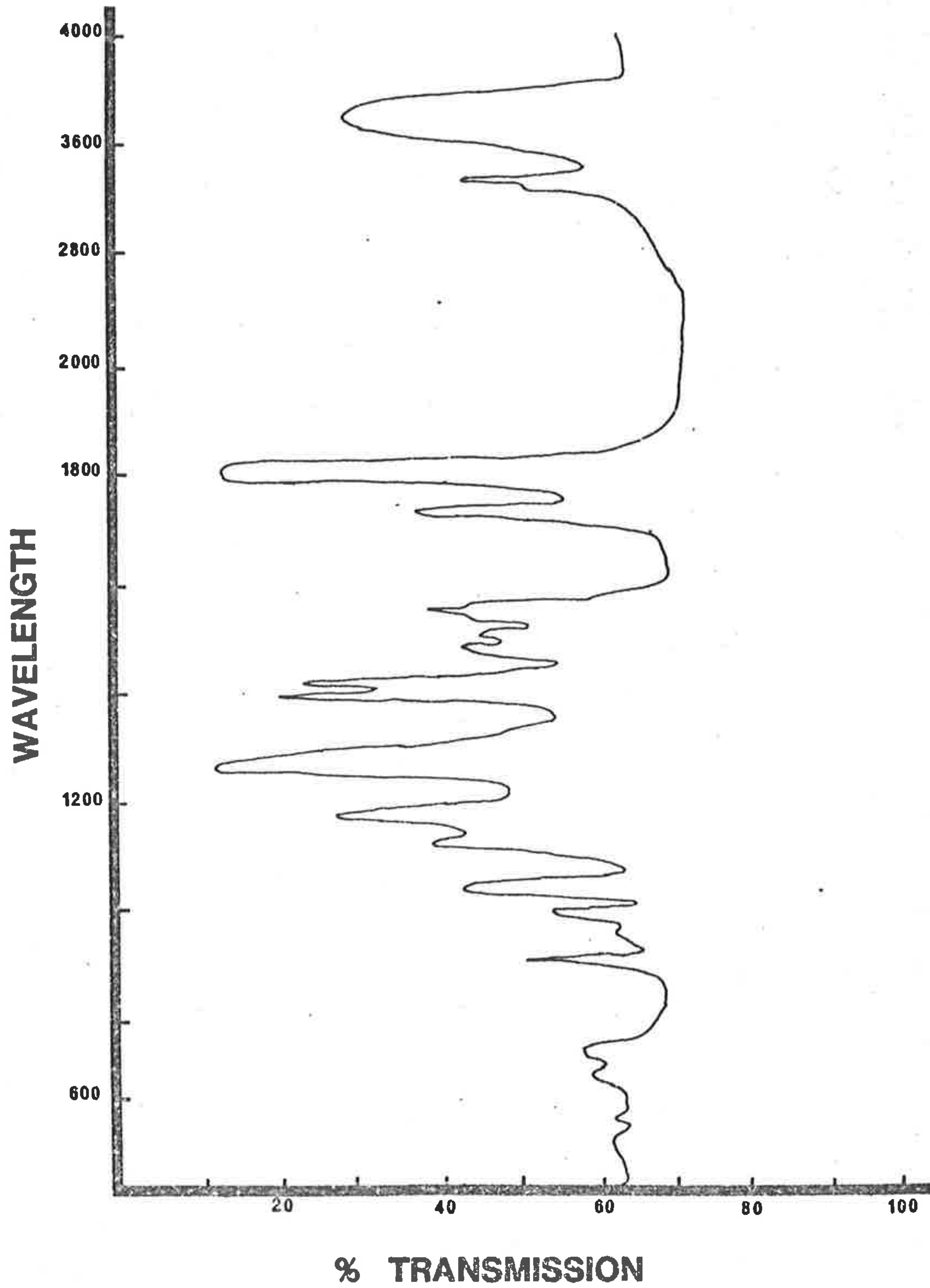

FIGURE 4.63

FIGURE 4.64: High pressure liquid chromatographic tracting of Hydron paste.

The arrow shows a peak that does not correspond to any of the peaks of the tracing of distilled HEMA (Fig. 4.63). This peak indicates a compound that is not present in the distilled HEMA.

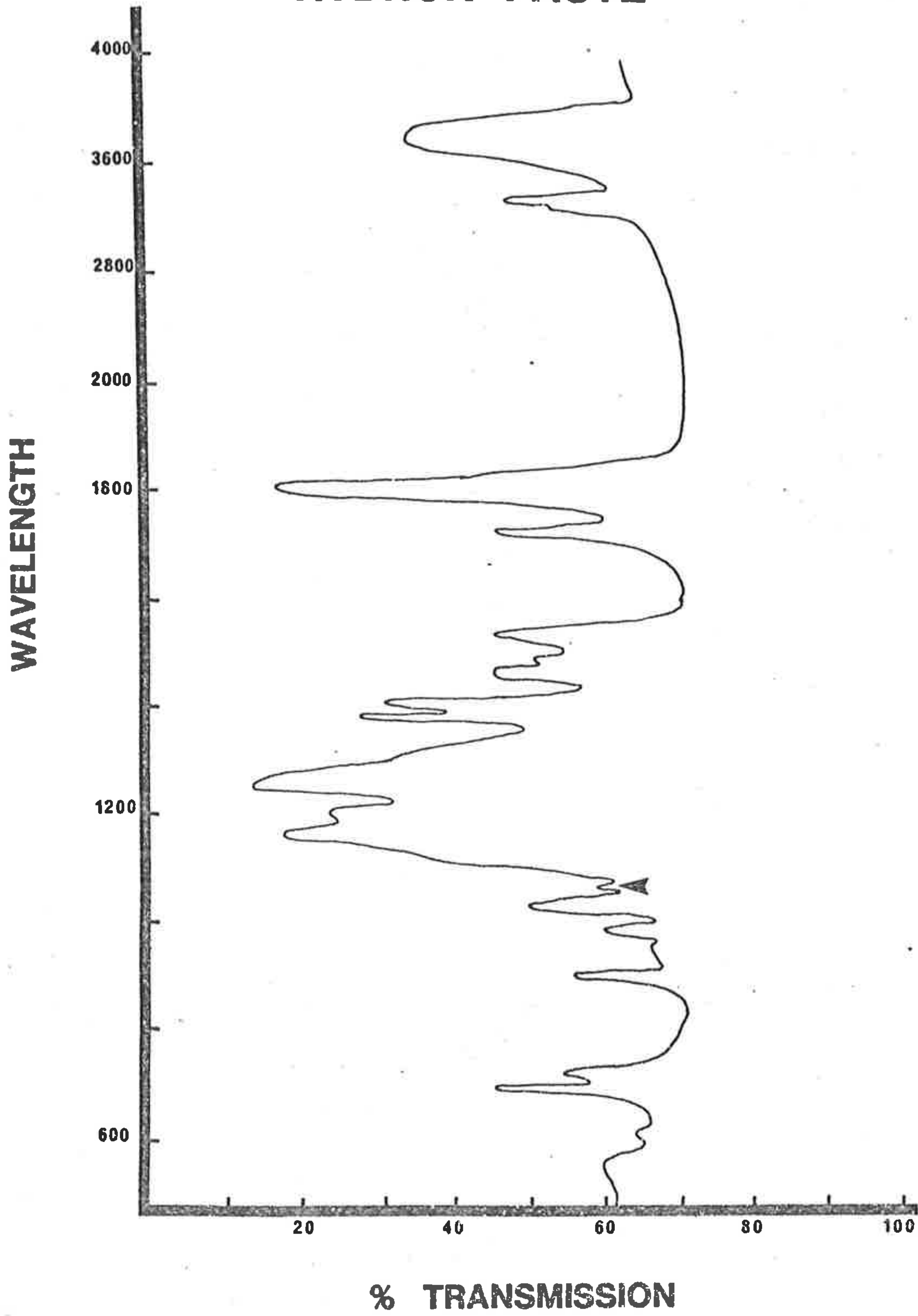

FIGURE 4.64

FIGURE 4.65: High pressure liquid chromatographic tracing of Hydron powder.

The peaks labelled LP indicate the liquid paraffin medium in which the powder was introduced into the apparatus. The peak labelled C indicates the catalyst benzoyl peroxide. The peaks indicated with arrows are an unidentified compound or compounds.

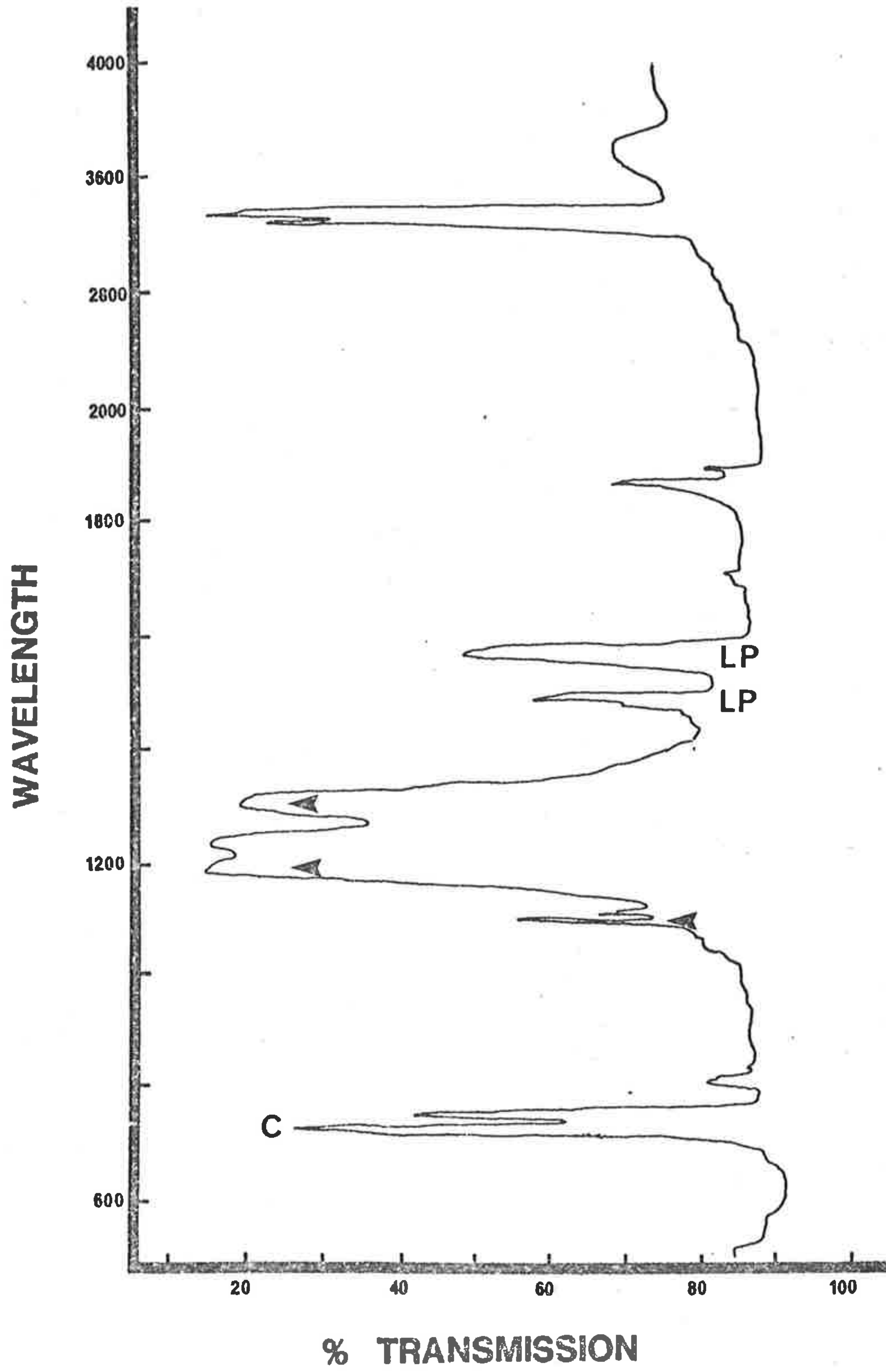

FIGURE 4.65

CHAPTER 5.

DISCUSSION.

5.1 INTRODUCTION

In recent years there has been an increased awareness of the importance of the biological aspects of dental materials. The biological characteristics of many dental materials has been extensively reviewed (Torneck, 1961; Spångberg, 1969a; Paffenbarger, 1972; Klötzer and Langeland, 1973; Dahl, 1974). More recently, researchers have attempted to establish standardized programmes to evaluate the biological characteristics of dental materials (Powell et al, 1970; Langeland, 1973; Autian, 1974; Tronstad et al, 1978). This and other research has led to the publication of two extensive guidelines for the biological testing of dental materials (American Dental Association, 1979; Federation Dentaire Internationale, 1979).

The biological effects of a material are of fundamental concern in endodontics where root filling materials have a direct influence on living tissue for prolonged periods.

In the promotional literature distributed by the manufacturer, it is claimed that Hydron is "inert and non-irritating to tissue". A review of the published research referred to as supporting this claim (Dreifus, 1962; Barvic, 1962; Kocvara et al, 1967; Singh, 1969; Tollar et al, 1969) revealed that in all instances the material had been pre-polymerized, sterilized and cleansed of impurities. In the case of dental Hydron, polymerization occurs *in vivo*. Any impurities present remain in the set material.

Ideally, a biological test should simulate the clinical conditions in which a biomaterial is to be used. Since Hydron root canal filling material is introduced into the canal freshly mixed, the Hydron implanted experimentally should be unset. While it was the intention in this study to use freshly mixed, unpolymerized material, the pilot study (Section 3.1.3 and 3.1.4) showed that in both implant sites, the material could not be satisfactorily contained within the Teflon tubes, thus making standardization impossible. As a result, the original aim of this study (namely to investigate pre-polymerized material) was changed to an examination of the biological properties of freshly mixed but polymerized Hydron root canal filling material (as supplied by the manufacturer).

The effect a root canal filling material might have on bone is accepted as a most important consideration when investigating its biocompatibility. In particular, the effect a material might have on osseous repair is of paramount importance. Previous experimental models which employed compact bone as the target tissue did not investigate osseous repair. The assessment methods used in these models, for example Haugen and Mjör (1979) and Hoover et al (1980) were based on an assessment of the effect the test material had on the relatively sparse population of osteocytes in bone. Spångberg (1969d) attempted to investigate osseous repair. However, the methodology employed by Spångberg assessed changes in a blood clot, the dimensions of which varied considerably.

The effects of potential biomaterial might have on bone marrow has been investigated by researchers such as Taylor et al (1971) and Weneger et al (1979). The bone medulla tissues have advantages from the point of view of ease of histological assessment of bone responses, especially osseous repair. However, in the present study the relatively dense bone, found in the guinea pig mandible, was considered to be a more suitable implant site (especially with the added modification of the triangular defect which allowed the observation of all stages of wound repair through from blood clot formation to bone reproduction and remodelling)than for example long bone marrow.

To investigate aspects of bone repair following the implantation of Hydron in bone, it seemed desirable that the surgically created site into which the implant was placed should be larger than the implant. In these circumstances, the progress of osseous repair (if any occurred) could be ascertained relative to a standardized defect. A methodology (not previously described) to achieve the above aim was devised for this study. This methodology has been detailed in Section 3.1.3(ii). In the context of an endodontic material, the defect between the implant and the walls of the bony lesion in the present study can, in some respects, be likened to the deficiency in mineralized bone created by a periapical pathology. Obviously it is not a truly parallel situation since the implant material in the present study initially contacted an organizing blood clot rather than chronic inflammatory tissue.

In the present study, Hydron was also implanted in soft tissue. This was done in an attempt to gain an insight into the biocompatibility of the material in general.

Unlike most biomaterials which have their whole surface area in contact with reactive tissue, endodontic materials only have contact at or through the apical foramen of a root filled tooth. As the whole canal is generally filled, the volume of the material relative to the exposed surface area is large. While the diffusion of toxins from a root filled tooth is relatively slow, diffusion could take place for a longer period. In the present study, the implant materials were encased in a container of "inert" material (Teflon), thus simulating a root canal and apical foramen, although the latter was of far greater dimensions than that normally encountered in clinical practice. Introducing the test material in an "inert" container has been the technique most commonly employed by other researchers (Torneck, 1966; Browne and Friend, 1968; Friend and Browne, 1969; Langeland et al, 1969; Spångberg, 1969d; Haugen and Mjör, 1978; Wenger et al, 1978; Deemer and Tsaknis, 1979; Marion et al, 1980; Langeland et al, 1980; Olsson et al, 1981). A Teflon container had been previously employed by such investigators as Langeland et al (1969); Spångberg (1969d); Langeland et al (1981) and Olsson et al (1981).

5.2 THE GUINEA PIG AS AN EXPERIMENTAL ANIMAL

The guinea pig was a demanding experimental animal. Developing a suitable anaesthetic regime was a major problem (refer Appendix I). The regime developed in this study proved to be satisfactory. Little appears to be known about the metabolism of the guinea pig (Hoar, 1969).

Socially the guinea pig is an aggressive animal despite its docile appearance (Hoar, 1969). There appeared to be a definite "pecking order" that was encountered even with the small number of animals kept in each cage. The aggression shown by the larger, stronger animals may have been responsible for the small number of deaths encountered in the initial stages of this study. However, this is retrospective supposition only.

The chief advantage of the guinea pig, in the context of the present study, was that a suitable area of sufficiently thick bone for implantation existed in a readily accessible site. The area of bone in the anterior mandible into which the implants were placed, was of such proportions that usually the experimental cavity could be cut without impinging on the tissues of either the periodontal membrane or the symphysis. This finding supports similar observations regarding this technique made by Spångberg (1969d) and Langeland et al (1981).

A feature noticed in the present study was that subjectively, the mandibular bone, as observed in histological sections, appeared to be very labile as evidenced by the large number of resting and reversal lines.

5.3 INTRAOSSEOUS IMPLANTS

The most striking histologic feature following the implantation of Hydron, AH26 and Teflon into bone was the repair that occurred adjacent to the Hydron implants. The majority of Hydron implants at 2 weeks post-implantation or longer, showed bone in what appeared to be absolute contact with the Hydron. This phenomenon was also noted by Murray and Dow (1975) following the implantation of a modified Hydron in rabbit femurs. In contrast, soft tissue capsule separated the regenerated bone from implants of AH26 and Teflon. When soft tissue was present between the Hydron implant and bone, it was thinner than that observed between bone and the control materials. According to Williams (1976), the thickness of a fibrous capsule surrounding an implant is indicative of the biocompatibility of a material. It is exceedingly rare for any tissue to repair in absolute contact with an implant. While this appeared to be the case in respect to Hydron (as observed at maximum resolution using light microscopy), assessment employing transmission electron microscopy would be necessary to substantiate this observation. Murray and Dow (1975) using transmission electron microscopy, in fact observed a thin layer of collagen between their implants of modified Hydron and regenerated bone.

Calcifications in Hydron implanted in bone were noted by Benkel et al (1976), Kronman et al (1977) and Tanzilli et al (1981) in monkey implant experiments. However, how these authors were able to detect calcifications in decalcified sections was not clear. On the basis of observations made in thc present study it could be suggested that

calcification of the Hydron adjacent to the interface with the tissue took place in the experiments devised by these investigators. Calcifications were observed in the surface zone of intramuscular Hydron implants in the present investigation. This finding was confirmed by special histochemical staining procedures and by electron probe analysis (see Section 4.2.2). In this study, the bone implant specimens were decalcified prior to histological processing. Whether a similar pattern of dystrophic calcification occurred within the Hydron implanted in bone is thus uncertain. Undecalcified sections would need to be cut to verify this assumption. Whether presumed dystrophic calcification adjacent to the surface of the intraosseous Hydron implants could influence ossification in the adjacent tissues is highly speculative. However, it is possible that a high concentration of calcium ions adjacent to the surface of the implant could be one factor influencing the bone regeneration observed adjacent to the Hydron.

The first specimens to exhibit evidence of osseous repair in the defect produced in the bone were Teflon implants which had been implanted for one week. Osseous repair was not evident in the defect adjacent to either AH26 or Hydron implants one week post-implantation. Thereafter, the Hydron specimens exhibited, subjectively assessed, greater bone formation than either of the other implanted materials. This observation contrasted with the soft tissue results where no significant differences could be detected between the experimental and control materials.

Macrophages containing phagocytosed Hydron were numerous in reparative soft tissues 2 weeks post-implantation, in both intraosseous and intramuscular implant specimens. Since the Hydron was implanted preset, it can only be assumed that the phagocytosed Hydron originated from the implant and probably from its surface. Macrophages appeared to be "transporting" the Hydron from the area adjacent to the implant, as evidenced by the fact that these cells were less numerous in the tissues adjacent to Hydron in specimens from longer time periods. As pointed out by Tanzelli et al (1981) it is uncertain whether such macrophages represent a low turnover lesion or a high turnover granuloma. The eventual fate of the intracellular Hydron was not further investigated in the present study due to time constraints. Further research with regard to this particular parameter would be of interest. It is of interest that Hydron could not be demonstrated within the bone which ultimately replaced the soft tissue adjacent to intraosseous implants thus indicating that the Hydron had been completely removed before bone regeneration occurred.

5.4 INTRAMUSCULAR IMPLANTS

5.4.1 CAPSULAR ZONE

In general the tissue responses immediately adjacent to the implant face were similar in Hydron, AH26 and Teflon specimens. There were some slight differences in the prominence of certain features, but overall the tissue responses occurring adjacent to all three implant materials were assessed as being essentially similar.

There did not appear to be any real differences between the extent of the capsules adjacent to the three implanted materials. While statistically significant differences were observed between Hydron and control materials in some of the shorter time periods post-implantation (refer Figure 4.44) there did not appear to be any significant trends.

Inflammation was not a prominent feature in the tissues adjacent to all three implanted materials at periods greater than one week. This observation is in accord with those of Benkel et al (1976). More lymphocytes were observed adjacent to implanted Teflon than the other materials in the 12 week and 26 week observation periods. The significance of this observation is not certain.

5.4.2 FOREIGN BODY GIANT CELL RESPONSE

Browne and Friend (1968) postulated that a foreign body giant cell response is associated with surface irregularities and therefore is a response to mechanical irritation rather than chemical irritation. Foreign body giant cells were not a prominent feature in any group examined in the present study. Foreign body giant cells were however most commonly associated with implanted AH26 which appeared to have the most irregular surface. All foreign body giant cells were in close proximity to the surface of the implant.

5.4.3 MACROPHAGE RESPONSE

After 4 weeks, there appeared to be a decrease in the rate at which macrophages containing phagocytosed Hydron were cleared from the tissues adjacent to Hydron implants. If the initial rate of clearance observed between 2 and 4 weeks had been maintained, no macrophages containing Hydron would have been observed at 12 and 26 weeks. Whether the macrophages observed at these longer time periods represented macrophages that had recently engulfed Hydron or were cells which had earlier phagocytosed the material and had not yet left the area, is uncertain. Future ultra-structural study of these cells might provide some insight into this question. The observations pertaining to phagocytosing macrophages in the tissues adjacent to intramuscular implants were similar to intraosseous implant specimens. Certainly the fibrosis evident in the tissues surrounding the implants in the longer post-implantation specimens could make the clearance of cells such as macrophages difficult.

The random distribution of macrophages, established employing a Poisson evaluation (Section 3.2.14), in the tissues adjacent to the implanted Hydron would indicate that the macrophages were neither attracted to or repulsed by the Hydron.

5.4.4 FAT CELLS

Kaminski et al (1977) indicated that fat cell presence in tissues adjacent to an implant might be a useful indicator of biocompatibility. By employing a method in the present study similar to that outlined by Kaminski et al (1977), no significant differences could be demonstrated between the biocompatibility of Hydron and that of the controls (refer Figs. 4.49 and 4.50).

5.4.5 MINERALIZATION OF HYDRON

The calcification observed in intramuscular Hydron implants from the longer time periods appeared similar to that illustrated by Sprinel et al (1973) in their investigation of macroporous HEMA gel. Similar results were obtained by Winter and Simpson (1969) and Winter (1970) in experiments in pigs. Sprinel et al (1973) described their results as follows: "deposition of compounds giving a positive reaction to calcium in the margin of the implant is massive - the central part is uncalcified". The stimulus for this deposition of calcium remains conjectural. Cerny et al (1970) suggested that the HEMA acted as an ion exchanger. However, this seems an unlikely explanation since Sprinel et al (1973) has shown that modification of the polymer backbone by the introduction of ionogenic groups does not affect the process of deposition calcium salts in the implant. The sequence of events suggested by Sprinel et al (1973) was initial cell penetration, necrosis and subsequent calcification of these structures. Support for this theory could not be found in the present study.

Examination of the calcifications at the interface area of a Hydron specimen, employing scanning electron microscopy, generally supported observations made with the light microscope with respect to the distribution and composition of these calcifications.

5.5 COMPARISON WITH OTHER RESEARCH

A study employing similar materials and techniques to those in the present study was reported by Langeland et al (1981). Langeland et al (1981) implanted Hydron, contained in Teflon containers, into the subcutaneous tissue of rats and the mandibles of guinea pigs. Freshly mixed Hydron was used. Langeland et al (1981) did not report the difficulty of containment of the fluid-like freshly mixed material within the Teflon containers as was encountered in the pilot study in the present investigation. Certainly the Teflon cups employed by Langeland et al (1981) would be less likely to distort than Teflon tubing. The Teflon tubes employed for the intramuscular implants were similar to those employed in the present study.

The results reported by Langeland et al (1981) would seem to contrast markedly with those of the present study. When Hydron was implanted in soft tissue, the present author found little or no inflammation present after one week, while Langeland et al (1981) reported acute and chronic inflammation for all periods up to and greater than one year. They also reported a foreign body giant cell response. When implanted intraosseously, Langeland et al (1981) observed inflammation and foreign body giant cells adjacent to the Hydron up to

6 months post-implantation. Such features were not evident in the present investigation. Langeland et al (1981) also noted the transportation of the Hydron in "vessels" (the type of vessel was not described) and "biological transportation". A common finding between the two studies was the presence of phagocytosed Hydron particles in macrophages.

Another section of the study reported by Langeland et al (1981) investigated the histological responses in periapical tissues adjacent to canals obturated with Hydron. The responses in any remaining pulp tissue and in the adjacent periapical tissue was similar to that adjacent to implanted Hydron.

Tanzelli et al (1981) observed severe inflammatory responses to excess Hydron similar to those observed by Langeland et al (1981). Whether the observed response was a low-turnover lesion or a high turnover granuloma appeared to be uncertain (Tanzelli et al, 1981). Particles of phagocytosed Hydron were observed by Tanzelli et al (1981) within the cytoplasm of macrophages as was the case in the study of Langeland et al (1981) and in the present study. Kronman et al (1977) expressed the view that phagocytosed particles consisted of dissociated barium sulphate. This opinion was not supported by Langeland et al (1981) and Tanzelli et al (1981). Detailed analysis of the phagocytosed material within macrophages was not undertaken in the present study. However, based on the optical microscopic similarity between intact Hydron and phagocytosed material, the findings of the present study tend to support the views of Langeland et al (1981) and Tanzelli et al (1981).

The prime difference between the study of Langeland et al (1981) and the present study relates to the polymerization of the Hydron. In the former study, polymerization took place *in vivo*, while polymerization was effected immediately prior to implantation in the present investigation. Since the composition of the material prior to setting was identical in both instances, it would seem that the setting reaction must differ in the different environments. One possible explanation for the differences in the results is that tissue fluid interferes with the polymerization reaction causing a significant proportion of the HEMA to remain as monomer. Monomers are invariably more reactive than polymers by their nature and the leaching of monomer from the final produce could theoretically cause the irritation observed by Langeland et al (1981). However, it would seem unlikely that monomer would still be leaching from a small length of implant 480 days after its implantation.

The heat of the polymerization reaction could be another factor which could influence the adjacent tissues when the Hydron was implanted unset. Langeland et al (1981) found that temperatures of 80°C occurred in large volumes of bench cured material. However, it is unlikely that this temperature increase would occur in the volume of Hydron present in a small tube or in a root canal. In addition any heat produced would tend to be absorbed by the relatively large bulk of surrounding tissue. Accordingly, it would seem unlikely that the heat caused by the setting reaction would have more than a transitory effect on the adjacent tissue.

Further research would seem necessary to clarify what effect the presence of tissue fluids has on the setting reaction of Hydron. This should include a study of the nature and relative quantity of any leachable materials that remain following polymerization under clinical conditions. Considering the excellent biocompatibility of preset Hydron and pre-polymerized, decontamination HEMA, ways in which this preset material could be adequately introduced to obturate the root canal should be investigated.

SUMMARY

The aim of the study reported was to evaluate aspects of the biocompatibility of Hydron, a proprietary root filling material.

An *in vivo* experimental model involving implantation of pre-polymerized Hydron and control materials into the mandibles and thigh muscles of guinea pigs was designed. Implant materials were contained within short lengths of Teflon tubing which served as a carrier. The use of Teflon carrier tubes allowed standardized assessment techniques to be employed during histological assessment of specimens at various post-implantation periods.

During the preliminary stages of this investigation a number of pilot studies were conducted to design and evaluate the mandibular implant sites with respect to their suitability for post-implantation assessment of tissue reactions. An intrabony implant site design was developed which allowed standardized post-implantation observations.

Optical microscopic assessment of muscle and bone implants and adjacent tissues was carried out at 2 days, 1, 2, 4, 12 and 26 weeks post-implantation. A single 4 week post-implantation muscle specimen was examined, using scanning electron microscopy and x-ray analysis for the purpose of investigating the nature of mineralized material which had been observed in the implanted Hydron.

Assessment of the biocompatibility of Hydron and the control materials, AH26 and solid Teflon, was based on analysis of a number of parameters scored or observed histologically. Results and conclusions obtained of the study were as follows:

1. The experimental *in vivo* implant model employed in this investigation allowed for standardized microscopic assessment of a number of "tissue response" parameters which were used to assess biocompatibility. The design of the intrabony implant sites is considered to provide a potentially useful model system to evaluate *in vivo* a variety of dental materials.

2. Hydron once polymerized is at least as biocompatible as AH26 and Teflon. None of the products tested elicited signs of overt or significant tissue damage. In the context of the design of present study it is thus concluded that polymerized Hydron is a biocompatible material. Clearly however, assessment of the "biocompatibility" of Hydron using other *in vitro* and *in vivo* techniques would be desirable in order to substantiate this claim.

3. The degree of inflammation, an indicator of tissue injury, observed in Hydron specimens was minimal. This finding is in accord with the findings of one group of researchers (Goldman et al, 1978) who have previously investigated the biocompatibility of Hydron. These results however conflict with those of Langeland et al (1981) and Tanzelli et al (1981). It is suggested that the differences observed are due, in part at least, to variation in experimental design.

4. Bone formation in Hydron bone implant specimens occurred in very close apposition to the polymerized Hydron. This observation tends to support the view that polymerized Hydron is a relatively biocompatible material.

5. Hydron is phagocytosed, particularly in earlier post-implantation time periods, by macrophages. No foreign body giant cell response to implanted Hydron was observed. Macrophages containing phagocytosed Hydron remain in the implant area for at least 6 months. Further studies aimed at elucidating the fate of phagocytosed Hydron would be of interest.

6. Mineralization of Hydron along the implant tissue interface was observed in muscle specimens. Whether similar mineralization occurred in bone implant specimens could not be determined. The occurrence of this phenomenon could have implications in clinical endodontics and is an area of study which should be further investigated.

APPENDICES.

APPENDIX I

DEVELOPMENT OF A SUITABLE ANAESTHETIC PROTOCOL

The methods of anaesthesia experimented with or reviewed constitute this Appendix. Atropine sulphate (2.5 mgm) was injected subcutaneously prior to all methods of anaesthesia in order to reduce mucous secretions (Croft, 1960).

(a) Barbiturates

The anaesthetic drug most commonly used for small laboratory animals in this Institution is one of the barbiturate, namely Sagatal* (pentobarbital sodium 100 mgm/ml). The stock solution is diluted: 1 part Sagatal to 9 parts sterile 0.9% sodium chloride, and administered intraperitoneally according to the formula:

$$\text{Dose} = \frac{\text{Wt. (grams)} \times 9}{2240} \text{ ml.}$$

While this regime has been successfully used with rats, mice, etc., in pilot studies conducted by the author, one in 3 guinea pigs failed to regain consciousness following anaesthesia. A reduction in the dose did increase the recovery rate, but the depth of anaesthesia was unsatisfactory and losses were still experienced.

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* May & Barker (Australia) Pty. Ltd.

The intraperitoneal route is more dangerous in the guinea pig than in most other experimental animals. The large bladder, gall bladder and liver make the area where it is safe to inject quite small (Hoar, 1969). In practice, the problems of intraperitoneal injections were not insurmountable. However, because of the mortality rate using intraperitoneal barbiturate it was decided to look for other methods of anaesthesia.

(b) *Urethane*

The intraperitoneal injection of Urethane (ethyl carbamate) at the recommended dose of 1500 mgm/kg (Hoar, 1969) achieved very good anaesthesia. However, the animals remained unconscious for an exceedingly long period (usually 2 to 3 days). Again, one in 3 animals failed to regain consciousness. The long recovery time and the high loss rate made this anaesthetic unsuitable. Also, Urethane is a carcinogen (Barnes and Eltherington, 1966).

(c) *Narcotics*

Either morphine or pethidine could have been used as the anaesthetic agent. However, because of the unpredictable nature of the animal, it was felt that it was essential to be able to deliver an antagonist rapidly. The only predictable way to achieve this was to have a vein permanently cannulated during the course of the operation. All attempts to enter the great saphenous vein (that would be exposed during an intramuscular implantation in the thigh) were unsuccessful. A separate operation would thus be necessary to uncover a major vein near the heart. There would be a high risk of mortality

should this wound become infected, especially considering guinea pigs' tolerance of systemic antibiotics (Gardner, 1980). For these reasons it was decided not to proceed with this anaesthetic regime.

(d) *Ketalar*

Ketamine Hydrochloride* at the recommended dose (MIMS ANNUAL, 1979a) of 6.5 - 13.0 mgm/kg was given intramuscularly in this investigation. Suggested human anaesthetic protocols (MIMS ANNUAL, 1979a) recommended that in addition to the Ketalar, a muscle relaxant also be given. Consequently, Valium** at the recommended dose (MIMS ANNUAL, 1979b) of 0.25 mgm/kg was concurrently administered intraperitoneally.

At this dosage, the anaesthesia was ineffective - in fact, the animal did not lose consciousness. The dose was increased progressively to 50 mgm/kg Ketalar which achieved reasonable anaesthesia. Complete analgesia was not attained at this dosage, but was achieved when supplemented by the local infiltration of Xylocaine*** (2% lignocaine with 1:80,000 adrenaline) in the surgical field. Additionally, the vasoconstrictor in the local anaesthetic helped to control haemostasis. At this dosage good muscle relaxation was achieved, by Ketalar alone and so the injection of valium was discontinued.

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* Parke-Davis and Co.
** Roche Products Pty. Ltd.
*** Astra Chemicals Pty. Ltd.

(e) *Results*

The average weight of the sixty-four guinea pigs of the main study and the experimental models was 688 gms. The average duration of the surgery required for the implantations of the main experiment was 56 minutes and the average duration of the anaesthesia was 192 minutes.

The anaesthetic dose was calculated according to the animal's weight (see preceding section). To test the validity of this method of dosage calculation, the hypothesis: *that the time of anaesthesia depended on the dosage,* was tested. A correlation of variance was computed (refer Correlation of Variance, Section 3.2.14). In this instance r was 0.234. The critical value of r when N = 41 and ν = 40 was 0.3044. Therefore r was not significantly different from zero at the 5% level of significance, i.e. dosage according to weight is a valid method fo anaesthetic dosage calculation.

APPENDIX II

10% NEUTRAL BUFFERED FORMALIN (ph 7.0)

Formalin	500 ml
Tap H_2O	4500 ml
Acid sodium phosphate monohydrate ($NaH_2PO_4.H_2O$)	20 gm
Anhydrous disodium phosphate (Na_2 HPO_4)	32 gm

APPENDIX III

DOUBLE EMBEDDING TECHNIQUE

Method

Blocks of trimmed, fixed tissue to 70% alcohol overnight

To absolute alcohol for 12-24 hours (depending on the size of the blocks)

To a mixture of methyl salicylate 50%, absolute alcohol 50% overnight

To a solution of methyl salicylate containing 1% celloidin until cleared (up to 3 days)

Wax infiltration in a 60°C oven, 1 hour each of:

50% paraffin embedding wax, 50% methyl salicylate;
75% paraffin embedding wax, 25% methyl salicylate;
100% paraffin embedding wax;
100% paraffin embedding wax;
100% paraffin embedding wax;
100% paraffin embedding wax;

vaccum infiltration in fresh pure paraffin embedding wax for at least 1 hour (and until bubbling stops) embedded in fresh pure paraffin wax.

APPENDIX IV

HAEMATOXYLIN AND EOSIN

Method

Dewaxed sections to tap water

Erlicks haematoxylin (10 minutes)

Tap water wash (5 minutes)

Differentiate in acid alcohol (1% HCl in 70% alcohol)

Running tap water (10 minutes)

Eosin (30 seconds)

Differentiate in 70% alcohol

Dehydrate, clear and mount

ERLICKS HAEMATOXYLIN

Haematoxylin	2 gm
Absolute alcohol	100 ml
Glycerin	100 ml
Distilled water	100 ml
Glacial acetic	10 ml

Potash alum $[K_2SO_4\ Al_2(SOE_4)_3\ 24\ H_2O]$ in excess (10-14 gm)

Dissolve the haematoxylin in alcohol before adding the other ingredients. The stain is ripened naturally by standing in a large flask, loosely stoppered, in a warm place, and exposed to sunlight.

EOSIN

95% alcohol	390 ml
1% eosin	50 ml
Glacial acetic acid	2 ml
1% pholoxine	5 ml

APPENDIX V

VAN GIESON'S STAIN FOR COLLAGEN FIBRES

Method

Dewaxed sections to distilled water

Weigerts haematoxylin (10 minutes)

Wash in running tap water (2 minutes)

Wash in distilled water

Van Gieson's solution (1-3 minutes)

Dehydrate: 95% alcohol, absolute alcohol, xylene

Mount

WEIGERTS IRON HAEMATOXYLIN

Solution A:	Haematoxylin crystals	1 gm
	95% alcohol	100 ml
Solution B:	Ferric chloride (20% aqueous)	4 ml
	Distilled water	95 ml
	Concentrated HCl	1 ml

Working Solution: Equal parts of Solution A and Solution B - made immediately before uśe.

VAN GIESONS'S SOLUTION

Acid fuchsin (1% aqueous)	10 ml
Picric acid (saturated)	90 ml

APPENDIX VI

MALLORY'S PHOSPHOTUNGSTIC ACID HAEMATOXYLIN TECHNIQUE

Method

Sections to distilled water

Mordant overnight in dichromate solution

Rinse in distilled water, treat with Lugols iodine for 5 minutes

Decolourise in 5% hypo or 95% alcohol

Wash in distilled water

Oxidise in 0.25% potassium permanganate for 3 minutes

Rinse in distilled water

Decolourise in 5% oxalic acid for 5 minutes

Wash in distilled water, then tap water for 10 minutes

Stain in a closed coplin jar of P.T.A.H.

Differentiate if necessary in 95% alcohol, dehydrate and clear in xylene and mount

REAGENTS

Phosphotungstinic Acid and Haematoxylin (P.T.A.H.):

Haematein	1 mg
Phosphotungstic acid	20 mg
Distilled water	1 litre

Dissolve the haematein in the distilled water, bring solution to boil, cool and add the phosphotungstic acid.

REAGENTS (cont.)

Dichromatemordant:

2.5% potassium dichromate in 5% acetic acid

This solution is unstable and must be prepared fresh from stock solutions.

APPENDIX VII

MODIFIED VON KOSSA STAIN

Method

Dewaxed section to distilled water

Wash in several changes of distilled water

Place in a jar of 1.5% silver nitrate in the dark (10-20 minutes)

Wash in at least 10 changes of distilled water

Reduce in freshly prepared 0.5% hydroquinone (5 minutes)

Rinse in distilled water

Treat with 2.5% sodium thiosulphate (5 minutes)

Wash well in tap water

Counterstain as desired, e.g. neutral fast red (3-5 minutes)

Dehydrate: 95% alcohol, absolute alcohol, xylene

Mount

APPENDIX VIII

PERL'S PRUSSIAN BLUE

Method

Dewaxed sections to tap water

Rinse in distilled water

Transfer to a mixture of equal parts of 2% potassium ferrocyanide and 2% hydrochloric acid in distilled water (20-30 minutes)

Wash in distilled water

Counterstain, e.g. neutral red (10 minutes)

Dehydrate: 95% alcohol, absolute alcohol, xylene

Mount

APPENDIX IX

LENDRUM'S MARTIUS-SCARLET-BLUE METHOD FOR FIBRIN

Method

Sections to water

Stain nuclei with celestin blue for 10 minutes; rinse in water, then stain Mayer's haemalum for 10 minutes

Rinse in tap water

Differentiate nuclei in 0.25% HCl in 70% alcohol, if necessary

Wash well in tap water

Stain with 0.5% martius yellow containing 2% phosphotungstic acid for 2 minutes

Rinse in distilled water; check the staining microscopically

Stain in 1% ponceau 6 R in 2.5% acetic acid for 10 minutes

Rinse in distilled water and stain with 1% phosphotungstic acid to fix and differentiate the red stain for up to 30 seconds

Rinse with distilled water and stain with 0.5% soluble blue in 1% acetic acid for up to 15 minutes

Rinse in 1% acetic acid; blot

Dehydrate, clear and mount

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