THE ABSORPTION OF A LOCAL HAEMOSTATIC AGENT: SURGICEL^{R}

An *in vivo* biochemical and histologic investigation in the rat.

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This thesis is dedicated to my parents

for their unfailing love and support.

PRECIS

Surgicel[R], an absorbable haemostatic gauze, is claimed to consist of oxidized regenerated cellulose. It is a polyanion, the functional unit of which is termed polyanhydroglucuronic acid. The ability of tissues to absorb Surgicel, the tissue responses it evokes, and its inherent haemostatic properties have been extensively studied by a number of investigators. However, the mechanisms of absorption of Surgicel are unknown.

The objectives of the present investigation were:

(1) To determine the time period required for absorption of Surgicel in rat muscle.
(2) To establish techniques for positive identification of the material in sections obtained for optical microscopy, using Alcian Blue.
(3) To identify the ultrastructural morphology of Surgicel using transmission electron microscopy.
(4) To investigate the mechanisms of resorption/absorption of Surgicel in rat muscle.

Young, female Sprague-Dawley Albino rats were used as the experimental animals. Teflon tubes, containing the test material, were implanted into muscles of the anterior chest wall and animals were sacrificed at time periods ranging from 6 hours to 7 days post-implantation. Recovered tubes were:

(1) Biochemically assayed for the presence of uronic acid.

[R] Johnson and Johnson, New Brunswick, U.S.A.

(2) Processed for optical microscopy and either stained conventionally for histologic examination or stained with Alcian Blue at different electrolyte concentrations.

(3) Processed for transmission electron microscopy.

Results of this investigation indicated that:

(1) Surgicel consists of at least two active components. These are (a) a soluble uronic acid component which is absorbed after 6 hours post-implantation and (b) a fibrous component which persists. The latter material resembles Surgicel in the electron microscope and is still evident at the implantation site 48 hours post-implantation. Moreover, Surgicel can be characterized in vitro into at least two components according to its solubility under dissociative salt conditions (4M guanidinium chloride). A residual fibrous material could be hydrolyzed with 0.3N sodium hydroxide.

(2) Surgicel, an analogue of tissue polyanions such as hyaluronic acid and chondroitin sulphate, can be selectively stained using Alcian Blue 8GX at a magnesium chloride concentration of 0.15M.

(3) It appears that absorption of the salt-soluble uronate in vivo is effected by early extracellular degradation and/or systemic clearance whilst removal of the fibrous material requires macrophage phagocytosis.

DECLARATION

This thesis is submitted in partial fulfilment of the requirements for the Degree of Master of Dental Surgery in The University of Adelaide. Candidature for the degree was satisfied by completion of a qualifying examination in 1982.

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

Permission is granted by the author for loan or photocopying of this thesis.

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ABBREVIATIONS

COOH	carboxyl
OH	hydroxyl
mg	milligram
g	gram
µg	microgram
ml	millilitre
µm	micrometre
mm	millimetre
cm	centimetre
S-tubes	Surgicel-filled Teflon tubes
C-tubes	Empty Teflon control tubes
H-tubes	Healon-filled Teflon tubes
4M Gu.HCl	4M guanidinium (hydro)chloride
r.p.m.	revolutions per minute
T	post-implantation recovery time
Hanks' B.S.S.	Hanks' Balanced Salt Solution
CEC	Critical Electrolyte Concentration
PMNs	polymorphonuclear leukocytes
GAGS	glycosaminoglycans
PGS	proteoglycan subunits
PGA	proteoglycan aggregate

CHAPTER ONE

INTRODUCTION

INTRODUCTION

Throughout the world, there is widespread use of local haemostatic agents in the treatment of excessive or uncontrolled haemorrhage following surgery or trauma. In such situations, careful haematological evaluation may reveal the presence of an underlying bleeding diathesis and, although systemic therapy may ensue, the use of local measures forms an essential adjunct in treatment.

Surgicel (oxidized regenerated cellulose) is a local haemostatic agent, popular amongst surgeons not only for its efficacy in producing haemostasis, but also because it is absorbed by body tissues, such a property obviating the need to re-enter a wound site for pack removal.

The clinical applications, haemostatic properties, biological effects and absorbability of Surgicel have been extensively investigated in a variety of sites in a variety of experimental animals. However, results regarding the time required for absorption of the material post-implantation seem somewhat inconsistent and it appears that such a time period may vary quite dramatically between implantation sites. Furthermore, previous inconsistencies may have resulted from difficulties associated with positive microscopic identification of the material.

There are few therapeutic materials which are absorbed by the body and, although extensive literature exists concerning mechanisms of absorption of certain suture materials, there is a paucity of data regarding the absorption mechanisms of Surgicel.

The objectives of the present study were to establish the time required for absorption of Surgicel to occur in rat muscle and to elucidate the mechanisms responsible for its removal, attention being focused on the role of the macrophage in such. The problem of microscopic identification of the material was addressed as an integral part of these investigations, as was chemical characterization of the material.

CHAPTER TWO

REVIEW OF THE LITERATURE

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REVIEW OF THE LITERATURE

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2.1 Description of Surgicel and History of its Development

Surgicel is a local haemostatic agent used in many areas of surgery including skin grafting and dermatologic surgery (Blau, Kanof and Simonson, 1960; Miller, Ginsberg, McElfatrick and Johnson, 1961b; Shea, 1962; Degenshein, Hurwitz and Ribacoff, 1963), breast surgery (Jantet and Rob, 1960; Degenshein et al., 1963), head and neck surgery (Hurwitt, Henderson, Lord, Gitlitz and Lebendiger, 1960; Georgiade, Mitchell, Lemler and Heid, 1961; Degenshein et al., 1963; Lucas, 1966; Skoog, 1967; Huggins, 1969), cardiovascular and pulmonary surgery (Jantet and Rob, 1960; Hurwitt et al., 1960; Miller et al., 1961b; Degenshein et al., 1963), surgery of the gastrointestinal tract (Jantet and Rob, 1960; Hurwitt et al., 1960; Miller et al., 1961b; Degenshein et al., 1963), and in gynaecological surgery, orthopaedic surgery, neurosurgery and in the treatment of certain blood dyscrasias (Degenshein et al., 1963). Surgicel is presented as a knitted gauze which is composed of oxidized regenerated cellulose fibres.

In 1879, William Halsted (Surgical Papers, Baltimore, The Johns Hopkins Press, 1924) observed that penghawar djambi was used quite frequently in the clinic of Billroth, particularly for checking haemorrhage in papillary cysts of the thyroid. This material consisted of the root hairs of the East Indian bullock tree and was said to be styptic. The material was cellulose.

Frantz (1946) has summarized the development of oxidized cellulose as a haemostatic agent. Haemostasis was a preoccupation in various research laboratories during the combat years of World War II. Preliminary experimental work and clinical trials were performed by

military surgeons, both British and American. As Frantz wrote, "Fortunately the carnage ceased earlier than some of us had dared hope, and before complete comparative studies of the different agents could be made in combat zones".

The impetus for the development of an absorbable haemostatic packing for lacerated vessels, or for application to surface ooze, arose partly as a consequence of the development and clinical usage of thrombin (Tidrick, Seegers and Warner, 1943). Cotton patties, used at that time for haemostasis, were found to be more effective when soaked in thrombin. However, bleeding often began again after their removal. The need for an absorbable patty was thus established.

The first resorbable haemostatic product developed was human fibrin foam. It was used with thrombin for haemostasis in neurosurgical procedures with excellent results (Ingraham and Bailey, 1944).

Another product developed at this time, by Correl and Wise (1945), was Gelfoam^{R}, a gelatin sponge which was found to be equally as effective as fibrin, and was also used as a carrier for thrombin.

Oxidized cellulose was originally prepared at Eastman Kodak Laboratories by Kenyon and his colleagues (Yackel and Kenyon, 1942; Unruh and Kenyon, 1942). It was soluble in dilute aqueous alkalis because of extensive carboxyl (COOH) group formation during oxidation. It was hypothesized that the product would also be capable of gradual dissolution in tissue fluids in areas of relatively undamaged tissue.

R : Upjohn, U.S.A.

The material was released to Columbia University College of Physicians and Surgeons who were looking for a non-irritating absorbable membrane for use in plastic repair of structures such as tendons in a sheath.

The product developed by Kenyon and colleagues consisted of cellulose, in the form of cotton, gauze and paper, which had been subjected to oxidation by nitrogen dioxide. Preliminary animal experimental work on these cellulosics was reported by Frantz (1943). Small amounts of the cotton, paper and gauze were implanted "into almost every tissue in the body" of dogs and cats. No adverse tissue effects were observed (except in bone) and the material was shown to be absorbable. Frantz (1943) reported that, microscopically, the fibres of oxidized cotton and gauze appeared as homogeneous, eosinophilic bands, difficult to distinguish from coagulated protein.

In 1944, Frantz, Clarke and Lattes reported on the results of a further animal study. They looked at the control of freely bleeding lacerations of the liver, kidney and spleen, in dogs and cats. The gauze contained approximately 13.5 - 15.8% carboxyl groups and was submitted, doubly packaged, in glassine envelopes after formaldehyde sterilisation. At this stage, they observed a phenomenon hitherto unnoticed: the oxidized gauze had a specific haemostatic action greater than controls of ordinary gauze. Thus, not only was the oxidized gauze an absorbable packing, but it did not require the use of thrombin as an adjuvant. In fact, the authors suggested that thrombin probably rendered the material less effective.

Frantz and co-workers noted that the gauze turned black within a few seconds of blood welling up into it. They confirmed this observation

by demonstrating the affinity of the material in vitro for haemoglobin in "dilute laked" blood. If ordinary gauze was used, there was no change. Oxidized gauze so placed immediately began to draw out all colour from the solution and appeared, after some hours, as a brown fibrillar mass in a clear, colourless fluid (Frantz and Lattes, 1945). An actual chemical combination was thought to occur and a sticky gelatinous mass was formed. It filled the wound space and was not a blood clot. It could, furthermore, be removed without causing secondary haemorrhage.

Comparison of the haemostatic action of the dry, oxidized gauze with the thrombin-soaked absorbable materials showed the cellulose to be at least equally as effective. Moreover, it was easier to handle than any of the fibrin foams (Frantz, 1946).

Development of oxidized regenerated cellulose (Surgicel) proceeded in the late 1950's at the laboratories of Johnson and Johnson. Regeneration of the cellulose before oxidation gave a product composed of fibres of uniform diameter which ensured the completion of a uniform and reproducible oxidation reaction. This contrasted with the oxidation of natural cotton (cellulose) which does not have a uniform and reproducible molecular size. Thus, the extreme variation in absorbability, tissue reactivity and haemostatic efficacy of the older product was obviated (Hurwitt et al., 1960). Furthermore, Miller and Thomas (1961) suggested that a lack of complete absorption of the older material from the site of implantation prevented wide acceptance of this substance in clinical practice.

The preparation of Surgicel is described in the U.S. patent #3,364,200. It is derived from regenerated cellulose which is prepared by the viscose or Bemberg process. In the viscose process, a solution formed from the treatment of cotton with alkali and carbon disulphide is forced through spinerettes of suitable diameters into an acid bath where the cellulose is regenerated into the pure chemical compound in the form of a fibre (Lebendiger, Gitlitz, Hurwitt, Lord and Henderson, 1959). The regenerated cellulose fibres are of uniform diameter and have a denier of about 1-9, but preferably 1-3. The structure of cellulose is shown in Figure 1. It is an unbranched chain of anhydroglucose units polymerized through carbon atoms 1-4 by a beta-glucosidic bond (White, Handler, Smith, Hill and Lehman, 1978a). The regenerated cellulose is then oxidized, at room temperature, using a solution containing approximately 20% by weight nitrogen dioxide in Freon 113, an inert non-aqueous solvent ($CCl_2F - CClF_2$). Oxidation involves conversion of the hydroxyl (-OH) group on the number 6 carbon atom of the anhydroglucose units to a carboxyl group.

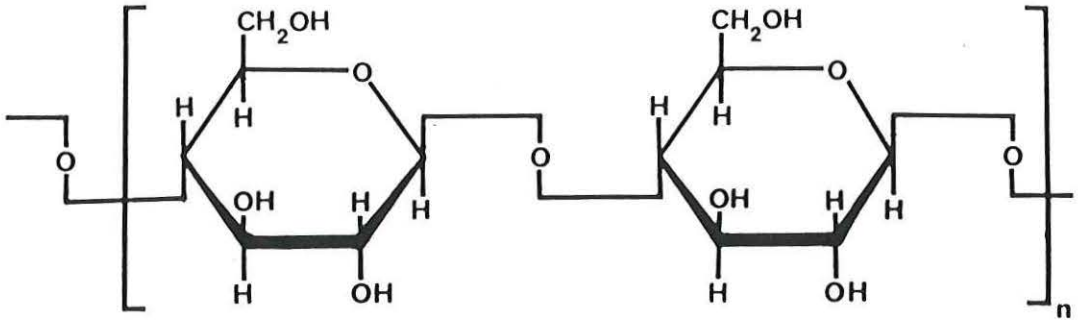
Theoretically, oxidation of the hydroxyl group on the number 6 carbon atom of each anhydroglucose unit in the cellulose polymer would produce oxidized cellulose with a carboxyl content of 25.6% by weight. Such complete oxidation is not desirable for surgical purposes, since optimum haemostasis, tissue absorbability and storage stability are obtained at lower levels of oxidation (U.S. Patent #3,364,200). The carboxyl content of Surgicel is approximately 18-22% by weight, inferring that 72-84% of residuals are oxidized.

The resulting Surgicel polymer (Figure 2) formed from the oxidation of alpha and beta glucose units, is composed of polyanhydro-

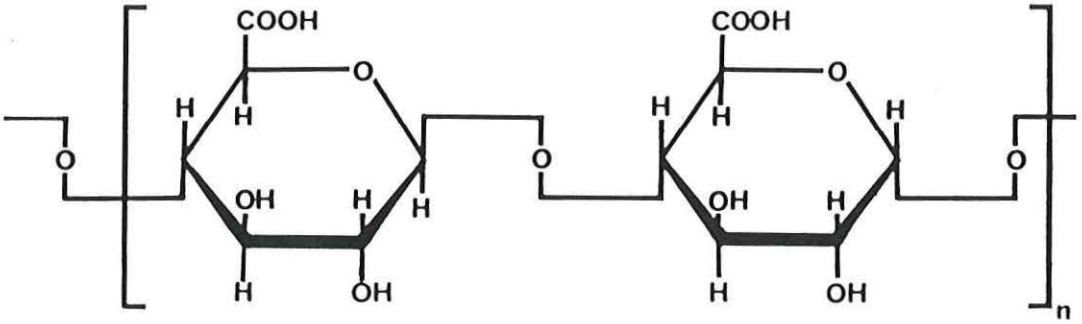
FIGURE 1: Chemical structure of cellulose.

FIGURE 2: Chemical structure of Surgicel.

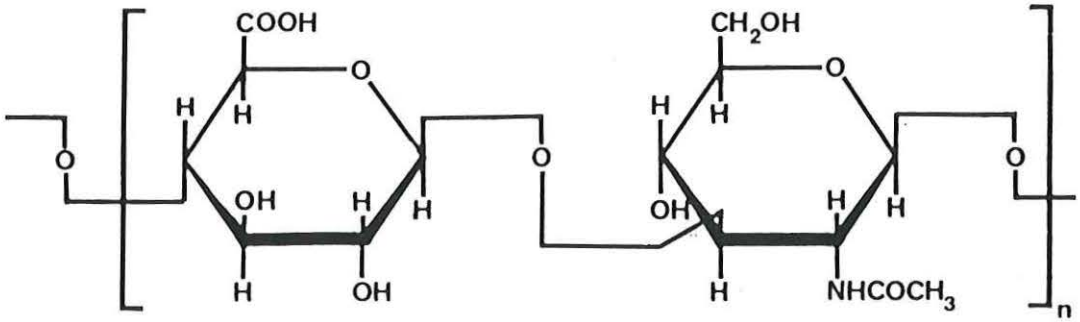
FIGURE 3: Chemical structure of hyaluronic acid.



CELLULOSE



SURGICEL®



HYALURONIC ACID

glucuronic acid units, linked by a C1-C4 beta-glucosidic bond (Miller, Jackson and Collier, 1961a). The similarity of this structure to the naturally occurring glycosaminoglycan, hyaluronic acid, may be observed in Figure 3.

It is essential that all traces of toxic or non-absorbable substances be removed from the final product. The U.S. patent (#3,364,200) describes the washing procedure used to remove impurities, oxidizing agent and by-products of the oxidation reaction from the oxidized cellulose. This procedure employs three different solvents: a non-toxic, inert, non-aqueous solvent, such as Freon 113, to remove the oxidizing agent; a non-toxic aqueous alcohol solution to remove acidic by-products of the oxidation reaction, such as nitric acid; and an inert, non-toxic, non-aqueous solvent having an affinity for water, such as 99% isopropyl alcohol, to remove traces of water from the product.

Currently, formaldehyde sterilisation is used. However, the manufacturers are in the process of substituting irradiation for this purpose (Personal communication, Johnson and Johnson, 1982).

The U.S. patent #3,364,200 gives further details of the chemical composition of Surgicel: chemical analysis of a sample of the material resulted in a total heavy metal content of 7.5 p.p.m. and the following additional constituents:

	% by weight
CH_2O	0.36
COOH	19.1 - 20.3
N_2	0.24
Ash	0.145

Additional information about the absorbability characteristics of the material are also stated in the patent:

> "Materials which will be satisfactorily absorbable in animal tissue within a short enough time to obviate the possibility of causing serious tissue reaction are generally those which are soluble in a 1.0% aqueous solution of sodium hydroxide within 10 minutes".

2.2 Haemostatic Action of Surgicel

Several investigators have proposed the means by which Surgicel effects haemostasis:

Blau et al. (1960) suggested that, although the precise mechanisms were not known, fibres of the gauze rapidly swelled on introduction to the body fluids and the scaffolding so produced promoted local haemostasis and simultaneously formed a soft gelatinous mass that moulded itself to the contours of the wound. They proposed that the polyanhydroglucuronic acid played a significant role in promoting local clot formation.

Hurwitt et al. (1960) reported that the material, which has a pH of 3.5 - 4.5, turned dark brown or black when exposed to blood, due to the formation of acid haematin. They proposed that the acid nature of the material itself probably produced some haemostatic effect due to styptic action. In addition, it was suggested that haemostasis depended, in part, upon the marked affinity of polyanhydroglucuronic acid for haemoglobin and other blood proteins. The large hydrated aggregate thus

formed controlled bleeding by simply acting as a cork in the mouth of the vessels and, secondarily, supplied a scaffolding for clot formation. Thus, they proposed that the haemostatic activity of Surgicel did not depend upon the physiologic clotting mechanism per se, but on the formation of what might be termed an "artificial clot".

Georgiade et al. (1961) also suggested that the polyanhydro-glucuronic acid had an affinity for haemoglobin, resulting in the formation of a gel with blood in the pH range 3.5 to 4.5.

Miller et al. (1961a) demonstrated excellent clot formation when oxidized regenerated cellulose was added to normal blood or blood to which heparin had been added. They found that the basic factor necessary for the fibres to hydrate and form a gel was a slightly alkaline fluid. Furthermore, these investigators suggested that haemoglobin freed from red blood cells by the acidity of the gauze united chemically with the fibres of the haemostatic agent to increase the amount of gelation. Formation of a salt of cellulose by its union with calcium was also proposed as enhancing the degree of gelation. Miller et al. (1961a) postulated that red blood cells, platelets and fibrinogen were not necessary for the gauze to form a gel.

Degenshein et al. (1963) concurred with Hurwitt et al. (1960) in proposing that Surgicel controlled bleeding by virtue of its acidic nature which caused local vessel contraction and initiated local fibrin deposition.

Lucas (1966) suggested three possible mechanisms responsible for the haemostatic action of Surgicel:

1. The formation of a mechanical fibrin-like mesh or framework for the formation of a clot;

2. The provision of a large (non-migratable) negative ionic charge in the surface of the material, attracting calcium and thus accelerating the activation of thrombin;

3. The absorption of blood into the material with swelling to form an artificial coagulum or plug.

Evans (1977) reported that the effectiveness of Surgicel as a haemostatic agent was due to an extrinsic, physical effect and was, therefore, not involved in normal clotting mechanisms. Thus, the formation of a gel when immersed in blood resulted in the mechanical block, artificial coagulum or plug suggested by Hurwitt et al. (1960) and Lucas (1966).

2.3 Clinical Trials

Results of early clinical trials using the older oxidized cellulose were published by Frantz et al. (1944) and Frantz (1945). The gauze was used in 115 various clinical situations and found to be most effective and convenient as a haemostatic agent. Two cases which incidentally came to autopsy confirmed that the material was absorbable and non-irritating in human tissues, as had been found in earlier animal experiments.

Lebendiger et al. (1959) evaluated clinical observations in 50 patients in whom oxidized regenerated cellulose (Surgicel) was used. In no instance was wound infection, toxic reaction or death attributed to

the material. Autopsies were performed on 10 patients (who died due to the underlying disease) at intervals of 1-77 days post-operatively. The Surgicel fabric implanted ante-mortem could not be identified macroscopically in the longer-term specimens, but, at 77 days, small shreds of debris were seen microscopically in areas of subsiding moderate foreign body reaction. These were described as "flecks of amorphous material" in a later report on the same investigation by Hurwitt et al. (1960).

Blau et al. (1960) used Surgicel as a post-operative dressing in 47 cases of dermatologic surgery and found it to be most effective in controlling bleeding. They observed that, in the presence of blood, the gauze formed a soft gelatinous mass which moulded itself to the contours of the wound and was absorbed with little or no reaction.

Hurwitt et al. (1960) reported on the further clinical trials of Lebendiger et al. (1959). By this stage, over 300 cases had been treated with Surgicel and observations confirmed the earlier impressions of applicability and freedom from complications of the material.

Jantet and Rob (1960) used Surgicel in 14 different surgical situations and their clinical impressions were similar to those of earlier trials. In one case, a temporary colostomy was closed 3 weeks after the first operation and the site of suture of the liver was inspected: many fine adhesions were present and some fragments of the cellulose gauze could be seen macroscopically but most of the material had been absorbed. The patient died 4 months after discharge from hospital : at autopsy, only a few strands of gauze were found within the carcinomatous mass on the posterior abdominal wall. The authors

concluded that, even in the presence of carcinoma, absorption of the gauze still occurred, albeit slowly.

Miller et al. (1961b) have also summarized their extensive clinical experience with Surgicel. Reporting on a series of 156 patients in a variety of surgical situations, these investigators observed that Surgicel stopped haemorrhage in two minutes and rapidly adhered to the bleeding surface. The material readily disappeared from the site of implantation and did not delay healing. They further stated that the oxidized regenerated cellulose controlled haemorrhage from certain anatomic sites where the use of conventional methods was impossible.

Shea (1962) dressed the donor sites of 47 graft patients with Surgicel and found complete haemostasis to be effected within 4 minutes. Other advantages enumerated by Shea (1962) were:

(1) avoidance of a moist wound
(2) reduction of bacterial proliferation
(3) minimization of after-care
(4) reduction of mobility, allowing more effective re-epithelialisation

Further clinical trials with Surgicel were undertaken by Degenshein et al. (1963) who also found it to be effective in the control of sanguinous ooze in many different situations. The authors reported complete absorption of oxidized regenerated cellulose at autopsy of a patient in whose liver the material had been implanted during a biopsy procedure 6 months earlier. Degenshein and co-workers suggested that the efficacy of Surgicel was increased by its application to a dry surface

under pressure. The material produced less tissue reaction than other haemostatic agents, absorbed completely and had comparable haemostatic properties.

Tibbels (1963) evaluated the effectiveness of Surgicel with respect to management of 250 cases of epistaxis resulting from trauma, vascular disorders, nasal inflammation, blood dyscrasias and/or coagulation defects, or septum deviation. In this study, 87.6% of all cases were successfully handled on initial packing with the gauze.

Tierney (1964) used oxidized regenerated cellulose to control oozing from the prostate bed after a series of 90 prostatectomies. He found that it was without local or systemic adverse effects and was completely absorbed. Less irrigation was necessary and the incidence of bladder spasm was decreased. Cystoscopic examinations were made on 30 patients over a period of 3-6 months. There were no traces of the cellulose fibres.

One of the first trials on the use of Surgicel in oral surgery was reported by Georgiade et al. (1961). The material was used in 1,036 patients who underwent various oral surgical procedures. Bleeding was easily controlled and no untoward effects from the material were noticed post-operatively.

Lucas (1966) packed dental extraction sockets with oxidized regenerated cellulose saturated in thrombin/0.5% $NaHCO_3$ solution and found it effective in controlling local bleeding, even in a large number of haemophiliacs and patients with thrombocytopenia. This observation contrasted with the proposal of Spouge (1964) that applied thrombin had little or no effect in haemophiliacs.

Skoog (1967) used Surgicel in the repair of maxillary clefts in humans and concluded that, by inserting the gauze subperiosteally, it was able to control the quantity and shape of new bone.

2.4 Antibacterial Activity

Dineen (1976) studied the effect of Surgicel, Gelfoam and Thrombin Topical on bacterial growth *in vitro* and *in vivo*. Bacteria tested included Staphylococcus aureus, Streptococcus pyogenes, Staphylococcus epidermidis, Streptococcus faecalis, Klebsiella aerogenes, Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Bacteroides fragilis and Clostridium perfringens. *In vitro* testing revealed a significant antibaterial activity attributable to Surgicel, but not to the other haemostatic agents. *In vivo* investigations using contaminated wounds in guinea pigs showed that Surgicel also had an antibacterial effect *in vivo*, a property not shared by the other materials.

In a further study, Dineen (1977a) monitored the antibacterial effects of Surgicel in model intravascular infections in dogs. After aortotomies and insertion of Teflon patches, Surgicel was wrapped around the wound in the experimental group of 4 animals. Subsequently, the animals were infected with S.aureus or K.aerogenes. Significantly lower bacterial colony counts were encountered in this experimental group than within a comparable control group of animals. Dineen suggested that the mechanism of antibacterial action of oxidized regenerated cellulose was apparently mediated through its pH effect because, *in vitro*, such an effect could be reduced or eliminated by the use of sodium hydroxide. However, he proposed that the acidity of the material did not completely explain its antibacterial effect because it was more effective on

rapidly multiplying organisms. Furthermore, he concluded that the antibacterial activity of oxidized regenerated cellulose was most evident when the material was placed in direct contact with bacterial agents at the time of challenge to the experimental animals.

In a further attempt to delineate some of the antibacterial activity of Surgicel, Dineen (1977b) packed splenotomy sites in 25 mongrel dogs with either Surgicel or Gelfoam and subsequently challenged the animals intravenously with S.aureus. Animals were sacrificed from zero to 20 days and bacterial colony counts made of the splenotomy sites. Colony counts from the Surgicel splenotomy sites were consistently low whereas those from the Gelfoam and untreated sites were particularly high during the first week following surgery but drifted down to lower levels after 20 days. These results confirmed the author's observations reported in earlier experiments.

Scher and Coil (1982) studied the effects of Surgicel and Avitene[R] (microfibrillar collagen) on bacterial growth and infection in S.aureus contaminated subcutaneous rat wounds. Both materials promoted infection, but to a significantly greater extent with Avitene. The response to Surgicel, however, was dose-related, no incidence of infection being observed for 5 and 10 mg samples.

Huggins (1969) used oxidized regenerated cellulose successfully in a series of 56 patients who underwent otolaryngologic surgery. It was found to be effective in the control of mild to moderate bleeding and produced no evidence of delayed healing or tissue irritation, except in two patients who complained of a local burning sensation.

R Avicon Inc., Fort Worth, Texas, U.S.A.

2.5 Theories of absorption

Not only is Surgicel an effective local haemostatic agent, but it is also absorbed by the body, a property which adds to its clinical appeal.

Blau et al. (1960) suggested that absorption of the gauze from the site of implantation was largely due to its hydrolysis, mediated by body fluid enzymes. Hurwitt et al. (1960) proposed that these enzymes were members of the carbohydrase system. According to Stryer (1975), the linkage between adjacent molecules of glucose in cellulose is not susceptible to attack by any known enzyme of the mammal. Miller et al. (1961a) suggested that although this might be the case, some means of removal must be present, and these authors investigated the effects of adding alpha-amylase, beta-amylase, thrombin, beta-glucosidase and beta-glucuronidase to gauze in a phosphate buffer at pH 7.3. Digestion was only seen in the solution of thrombin. Further experimentation revealed that solubilisation of swollen fibres was encouraged by the slightly alkaline medium provided by the blood.

Tibbels (1963) suggested that, through a process of further oxidation, the oxidized regenerated cellulose was degraded by body enzymatic systems which normally hydrolyzed carbohydrates, and that such reactions were responsible for the material's absorbability.

Tierney (1964) acknowledged that the process of cellulose regeneration during the production of Surgicel resulted in a lower and more uniform molecular weight than that of cotton, and suggested that these two factors were important in producing the ready absorbability of Surgicel in the body tissues.

2.6 Animal Investigations

Results of the early work by Frantz on the absorption and tissue reaction to oxidized cellulose (not regenerated) in experimental dogs and cats were somewhat inconclusive (Frantz, 1943; Frantz et al., 1944). Considerable variation in absorption time was noted, accompanied by varying degrees of phagocytic and foreign body reaction.

Results of further experimentation were published by Frantz and Lattes (1945). The material was implanted subcutaneously into rats which were killed post-operatively at 1, 2, 4, 7, 11, 13, 20, 24 and 30 days. Examination of the implantation sites showed a progressive decrease in the size of the implant, but even at 24 days there was still a residual brown mass. At 30 days the implant was not present macroscopically but, microscopically, tiny residua showed a broad zone of phagocytes and a few remnants of cellulose not invaded by fibrous tissue. There was no significant gross or microscopic evidence of inflammation at any time.

With the refinement of the oxidized cellulose product incorporating regeneration of the cellulose (Surgicel), further animal experiments ensued.

Hurwitt et al. (1960) reported on a series of investigations which also incorporated those of Lebendiger et al. (1959). Surgicel gauze was implanted into the subcutaneous tissues of rats in order to achieve the following objectives:

(1) to obtain a measure of the expected rate of absorption in subcutaneous tissues;
(2) to study the tissue responses to the material.

One hundred and sixty samples of the fabric were implanted and rats were sacrificed at post-implantation periods of 1, 3, 5, 7 and 15 days. Tissues recovered from the implantation sites were stained with haematoxylin and eosin, Van Gieson's stain and Texchrome[R]. Results were evaluated qualitatively.

At day one, the implantation site (in the form of a channel in the subcutaneous tissue) contained a considerable amount of translucent viscous fluid intermingled with fragments of gelatinous material. Nothing resembling the original fabric was observed. Tissue sections stained with Texchrome revealed fragments of amorphous lavender-coloured material associated with oedema, but showing little evidence of a cellular response. At three days, the implant could still be recognized macroscopically as a soft gelatinous mass. Microscopically, the cellular response was moderate and consisted mainly of large macrophages. The area was still oedematous. At five days, there was a significant reduction in the size of the mass and overall response. Microscopically, the degree of oedema was lessened and evidence of fibroplasia was apparent.

At seven days, the implant bore no resemblance to the original fabric but consisted of an off-pink to white, soft, gelatinous material, when examined macroscopically. A brown pigment, considered to be haematin, was frequently observed at the site of implantation. In some cases, the implant appeared to be completely absorbed. Microscopically, the tissue reaction in the area of the implant was characterized by active fibroplasia and moderate numbers of macrophages. When stained

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R : Fisher Scientific Co., New Jersey

with haematoxylin-eosin, the "residue" appeared as an amorphous mass invaded by proliferative fibroblasts and large macrophages frequently containing pigment. There was no suggestion of capsule formation. In most instances, there was "some degree of polymorphonuclear and mononuclear infiltration, with capillary proliferation".

At fifteen days, it was impossible to identify the implant grossly, although, subjectively, the "barest suggestion" of a mass was recorded in some instances. It was not possible to identify this mass microscopically. There were fewer macrophages and usually no polymorphs. Fibroblasts predominated in the cellular compartment near the implant.

Hurwitt et al. (1960) also implanted the gauze, pre-moistened in saline, into the liver, kidney, brain, pleura, intestine and pericardium in five dogs, which were sacrificed at post-implantation periods of 30-62 days.

There was no macroscopic evidence of the implants at the hepatic, pleural, intestinal, subdural, pericardial, and one of the renal sites. The second renal site showed evidence of the material after thirty days. This was subjected to microscopic examination which revealed unabsorbed cellulose mixed with haemolyzed blood. The authors stated that this was the only instance in which a foreign body reaction was observed, as evidenced by giant cells at the periphery of the non-resorbed implant mass. The implant showed no interference with renal function in any of the kidneys examined. Furthermore, no obstruction was caused by the material being wrapped around the intestine. No significant rise in body temperature was observed after subdural implantations but the animals were slightly ataxic the day following surgery. These authors suggested

that it was impossible to identify the material microscopically at any post-implantation time.

Jantet and Rob (1960) implanted Surgicel gauze into the following sites of 18 albino rats:

(1) subcutaneous tissues of the abdominal wall;
(2) intraperitoneally in the right iliac fossa;
(3) intramuscularly into the rectus abdominis muscle;
(4) in the liver after resection of a wedge of the organ 1.5 cm long and 1 cm wide which produced copious venous oozing.

At the first two sites, lengths of gauze 7.5 cm x 5 cm were implanted. Samples measuring 5 cm x 5 cm were used at the other sites. A subjective assessment of the clinical haemostatic effect at the time of operation was recorded. Animals were sacrificed from 1-15 days post-operatively and autopsies performed in order to determine the amount of material absorbed and to assess the reaction of tissue to the material, macroscopically and microscopically.

The results are summarized in Table 1.

The subcutaneous, intramuscular and intraperitoneal implants were all absorbed "almost fully" by 48 hours. It is not stated how such quantitation was achieved. The authors suggested that the material was inert, as the local tissue reaction consisted solely of invasion by macrophages in some of which small particles of material could be "distinctly seen". Absorption from the liver was slower and not complete until 10-14 days. The local tissue reaction around liver implants was

more marked than at other implant sites. A capsule containing polymorphonuclear leukocytes and macrophages was formed at the liver implantation sites in several animals. However, by the time complete resorption of the Surgicel had occurred, the capsule had disappeared. This finding concurred with that of Hurwitt et al. (1960) who reported full resorption of the hepatic implant by 30 days.

Days after implantation	No. of Rats	Comments
1	3	All implants still recognizable.
2-4	3	Liver implants still recognizable; all other implants almost fully absorbed.
5-7	3	Capsule around 2 of the liver implants; all other implants fully absorbed.
8-11	4	Capsule around one of the liver implants; one other liver implant not fully absorbed; all other implants fully absorbed.
12-15	5	Abscess around one of the liver implants; all other implants fully absorbed.

Table 1: Summary of experimental results

(Jantet and Rob, 1960)

Jantet and Rob (1960) concluded that Surgicel appeared to be removed by a process of liquefaction followed by absorption and phagocytosis. At all sites, the gauze was found to be an excellent haemostat for capillary and venous bleeding.

Georgiade et al. (1961) performed a series of 50 tooth extractions on rats and covered the sockets with either Surgicel or gelatin sponges. Animals were sacrificed at 3-day periods, but the maximum post-operative sacrifice-time was not stated. Histologic sections through the surgical sites showed gradual absorption of the haemostatic agents. Surgicel appeared to be completely absorbed within a 21-day period with no foreign body reaction being noted throughout the experiment. The authors did not state how the material was identified in histologic sections.

Miller and Thomas (1961) implanted Surgicel into the livers of 6 mongrel dogs and administered 1×10^6 U penicillin G potassium intramuscularly immediately after each operation. These investigators also implanted the material into the spleen of a further 6 dogs, and proximal to the peritoneum in another 2 dogs. Antibiotic administration was not effected in the two latter experiments. Animals were sacrificed at different times post-operatively and results are summarized as follows:

(1) <u>liver implants</u>

After 3 days, a soft gelatinous mass composed of clot and oxidized regenerated cellulose was found to be firmly adherent to the wound. Contraction of the clot had occurred after 10 days, and this process

continued to 17 days when the clot was observed to be positioned firmly in the wound.

Microscopical analysis at 3 days showed a large defect filled with clotted blood and a hyalin and fibrillar material. A small number of polymorphonuclear neutrophils were scattered in the area. A thin layer of connective tissue separating the implant lesion from the liver cells exhibited a moderate infiltrate of polymorphonuclear neutrophils and lymphocytes. Plasma cells and macrophages were also present, but giant cells were not seen.

At 10 days, the defect contained clot and a large mass of fibrillar and eosinophilic material. A zone of fibrous tissue, containing small numbers of polymorphonuclear neutrophils and lymphocytes separated this area from liver cells. Plasma cells and multinucleated foreign body giant cells were seen in some regions.

At 17 days, the defect contained clot which was separated from liver cells by a narrow band of connective tissue, focally infiltrated with plasma cells and lymphocytes. It appears that the connective tissue capsule around hepatic implants persisted for a longer period in this investigation than in the experiment conducted by Jantet and Rob (1960).

(2) spleen implants

Absorption of the gauze was more rapid than in the hepatic implants. At 10 days, the clot had macroscopically disappeared. Microscopically, the wound was filled with clot and hyalin and fibrillar material. A narrow zone of connective tissue containing leukocytes and lymphocytes

separated the implant site from splenic cells. Plasma cells and a few scattered foreign body giant cells were seen. In some regions, numerous macrophages containing haemosiderin were detected.

(3) peritoneal implants

One animal was sacrificed at 46 days and the other at 64 days. Gauze was not seen in the peritoneal cavity and adhesions were not present in either dog. Microscopically, no evidence of the gauze was seen at 64 days, nor was there an inflammatory reaction.

Degenshein et al. (1963) performed an hepatic lobectomy on a mongrel dog and placed 3 layers of Surgicel gauze over the bleeding wound. The dog was sacrificed at 8 days post-operatively and an amorphous mass of gauze, intimately attached to the edge of the wound, was observed. This finding is consistent with that of Miller and Thomas (1961) who also found the coagulum firmly adherent to the hepatic wound site at this period.

Skoog (1967) studied the tissue response to subperiosteal implantation of Surgicel in the fronto-nasal bones of 12 young rabbits. The animals were sacrificed at 8,14 and 28 days post-operatively. After decalcification several transverse sections were made of the operative field and the opposite control area. Specimens were stained with haematoxylin and eosin and van Gieson's stain. Results are summarized as follows:

8 days

Gross examination revealed a marked change in the elevated periosteum and the normally thin, transparent membrane was thickened and greyish-white with an increased vascularity distinctly prominent on the surface. There was significant absorption of the implant and the material was brownish, soft and gelatinous. The tissue at the site of implantation was characterized, microscopically, by a massive proliferation of osteoblasts and fine vessels into the implants where fibrillar structures had formed. This cellular growth originated from both the bone and the osteogenic layer of the elevated periosteum.

14 days

The original fabric had been absorbed and there was evidence of new bone formation and signs of resorption with osteoclasts lying along the edge of the bone.

28 days

No evidence of the fabric remained and newly formed bone trabeculae were seen extending from the underlying bone into the thickened periosteum.

Skoog (1967) concluded that Surgicel could be used safely in subperiosteal implantation.

The U.S. patent #3,364,200 described an investigation in which wounds were inflicted upon the exposed spleens of 4 anaesthetized dogs. It was

observed that the normal blood clotting time in dogs was about 6 minutes but oxidized regenerated cellulose arrested bleeding in approximately 2-5 minutes.

Thilander and Stenström (1969) studied the effects on bone healing after implantation of Surgicel, Ivalon[R] and collagen chips in a surgical defect created in the premaxillomaxillary suture in guinea pigs. 75 animals were used and sacrificed at 1 week, 2 weeks, 1 month, 2 months and 4 months post-implantation. Sections of specimens were obtained for optical microscopy. Results showed only minimal microscopic remnants of the material to be present at 1 week post-operatively.

There was still evidence of Ivalon and collagen chips at the implantation site after 4 months. In contrast, Surgicel had completely disappeared and the defect was filled with new bone. The authors concluded that Surgicel had a beneficial effect on bone healing whereas Ivalon and collagen chips appeared to retard the process.

Although this study incorporated staining of serial sections with haemalun-eosin (Mayer), azan (Heidenhain), resorcin-fuchsin (Weigent) and picrofuchsin (van Gieson), the authors did not state which technique was suitable for identification of Surgicel, nor did they describe its microscopic appearance.

Engdahl (1972) implanted Surgicel subperiosteally at the site of unilaterally resected rabbit maxillae. He concluded that, although bone was formed at the sites of Surgicel implantation, its genesis was slower and quantitatively less than in the controls. These findings were in contrast to those of Skoog (1967) and Thilander and Stenström (1969).

R : Ivano, Inc., U.S.A.

Rosenquist and Finne (1974) implanted the haemostatic agents Surgicel and Hemofibrine[R] into mandibular and tibial bone cavities of 38 adult rabbits, and studied the effects on bone healing after 2 and 4 weeks, using microradiography. After 2 weeks, the healing of control cavities was better than that of experimental cavities in both bones. After 4 weeks, this difference was less marked, especially with the Surgicel implants. The findings of Rosenquist and Finne (1974) concurred with those of Engdahl (1972), but contrasted with those of Skoog (1967) and Thilander and Stenström (1969).

Larsson, Nisell and Granberg (1978) embedded Surgicel into the traumatized caecum of 20 rats. A further 20 rats were also laparotomized and subjected to a standardized caecal trauma, which was left uncovered. All animals were sacrificed at 14 days post-operatively and examined for intraperitoneal adhesions. The authors found that adhesions were significantly less common ($p < 0.001$) in the group treated with oxidized regenerated cellulose.

In a separate experiment, Larsson et al. (1978) used 8 rats for histological examination of the traumatized caecal wall implanted with Surgicel. No macroscopic residuum was observed after 1 or 2 weeks. Histological examination, however, demonstrated small remnants of Surgicel, surrounded by ingrowing fibroblasts and inflammatory cells. Their methods of identification were not stated.

Uddströmer (1978) developed a model to qualitatively and quantitatively study the influence of Surgicel on periosteal bone

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R : Septodont, Paris

formation in rabbits. A Teflon cup was inserted into a pre-prepared tibial or skull defect. The cups were filled with blood, haematopoietic bone marrow or Surgicel, and the periosteum subsequently sutured over the top. Animals were sacrificed at 2-15 weeks post-operatively. Following retrieval, the Teflon cups were ashed in a furnace and the amount of calcium present determined spectrophotometrically. The amount of calcium produced in the Surgicel-filled cups was found to be a third of that produced by the blood or marrow-filled cups. Uddströmer concluded that Surgicel strongly retarded new bone formation, a quantitative result reinforcing the findings of Engdahl (1972) and Rosenquist and Finne (1974) but contrasting with those of Skoog (1967) and Thilander and Stenström (1969).

Olson, Roberts and Osbon (1982) placed Gelfoam, Surgicel and polylactic acid into extraction sockets of 14 mongrel dogs, and sacrificed the animals at post-implantation periods of 2, 3, 7, 14, 30, 42, 60 and 90 days. Specimens were prepared for histology and stained with haematoxylin-eosin. At 3 days, the structural integrity of all implants was still evident and the authors suggested that fibroblastic invasion was prevented by their presence. By 1 week, all sockets, except the control, showed a great influx of inflammatory cells, including .pa polymorphonuclear leukocytes and lymphocytes. Multiple regions of non-vital bone were noted.

At 14 days, the Surgicel socket revealed satisfactory healing, but fewer trabeculae were present in the alveolus compared with the control socket. Furthermore, the socket was not filled with new bone and an encysted entity noted was thought to represent remnants of the Surgicel. Inflammatory cells were also present. By 30 days, the control site had

healed well. One of the Gelfoam sites showed marked delayed healing and a chronic inflammatory infiltrate. The polylactic acid site revealed a minimal leukocytic reaction and a significant number of multinucleated giant cells. The Surgicel site contained an encysted amorphic remnant of the implant.

At 6 weeks, the Surgicel and Gelfoam sites showed healing comparable to that of the control site. However, the polylactic acid was still present, as were multinucleated cells and lymphocytes. By 60 days, the Surgicel site was well healed but contained an encysted remnant of the Surgicel in the submucosal tissue and a minimal inflammatory response. The Gelfoam site demonstrated an intense chronic inflammatory response superficial to the alveolar crest. Bony apposition was delayed in the polylactic acid site. At 90 days, the control, Surgicel and Gelfoam sites were indistinguishable from one another. Examination of the polylactic acid sites revealed a persistence of some of the implant material, and a few giant cells.

Garach (1982) implanted Surgicel-filled Teflon tubes into the anterior chest wall muscles and femurs of 30 rats. Empty tubes were used as controls in a further 18 rats. Animals were sacrificed at post-implantation periods of 2 days and 1, 2, 3, 4, 6, 8, 12 and 26 weeks. Implants and surrounding tissue were removed and prepared for histology. Sections were stained with haematoxylin-eosin, van Gieson's stain and safranin-aniline blue. The latter stain was used in an attempt to positively identify Surgicel. Selected sections were also stained with Mallory's Phosphotungstic Acid Haematoxylin (PTAH) in order to demonstrate skeletal muscle fibres.

The muscle implant histological material was analyzed qualitatively and quantitatively. Femoral implants were analyzed qualitatively. Criteria used were as follows:

(a) Qualitative Assessment of the Tissue Reactions

Histological observations were confined to the limits of the tube lumina. Features assessed were:

(1) The presence or absence of inflammation. If present, the nature of the cellular infiltrate was assessed.
(2) The intensity and nature (viz. acute-chronic) of the inflammatory response.
(3) The presence or absence of macrophages. Particular attention was paid to signs of phagocytic activity of these cells as evidenced by the presence of cytoplasmic vacuolation and/or particulate material.
(4) The presence or absence of multinucleate foreign body giant cells.
(5) The resorption and possible replacement of the test material.
(6) The nature of the tissues replacing the test material.

(b) The Quantitative Measurement of the Tissue Reactins

In an attempt to quantify the rate of tissue in growth into the Surgicel tubes and the control tubes, the following method was used:

Three sections from the centre of the tube of each specimen were projected onto a piece of paper on a wall usng a Leitz Right Angle Prism on a microscope. The outline of the tube and its contents were traced.

The total inner area of the tube and its contents was determined by using a planimeter. The total area of the tube was designated as "A_{tube}" and the area of tissue ingrowth occupying the tube as "$A_{tissue\ ingrowth}$". The percentage ingrowth into the tube was calculated as follows:

$$\frac{A_{tissue\ ingrowth}}{A_{tube}} \times 100$$

The student's t-test was used to determine whether there was a statistically significant difference between experimental and control tubes.

Microscopic observations of all the intramuscular Surgicel implants revealed that Surgicel was not clearly identifiable in a morphologic form similar to its original knitted appearance, or as discrete fibres. However, in the intrafemoral implants, the fibrous nature of the implant material was clearly identifiable at 2 and 7 days post-implantation.

Histologic examination of all Surgicel specimens at the various post-implantation time periods revealed the following:

Intramuscular Implants

2 days

The tube lumen contained an eosinophilic fibrillar coagulum containing polymorphonuclear leukocytes and macrophages, some of which exhibited a foamy appearance. Many of the polymorphs had pyknotic

nuclei. The eosinophilic coagulum was interpreted as being altered Surgicel.

7 days

Granulation tissue had invaded both ends of the tube, the remainder of which was still occupied by the fibrillar eosinophilic coagulum. The granulation tissue was characterized by an abundance of erythrocytes, macrophages and fibroblasts. Many of the macrophages had a foamy appearance. A few polymorphs were evident, as were small numbers of plasma cells and lymphocytes.

2 weeks

The tissue ingrowth extended along the entire length of the tubes and had an hour-glass appearance. Large numbers of fibroblasts and macrophages were evident. Fibrillar material, indistinguishable from the fibrillar material noted in corresponding control tubes, could be detected in the tissue ingrowth. Striated muscle fibres were demonstrated within the tissue with the PTAH stain. There was no clear evidence of readily identifiable Surgicel in the tissue or in the macrophages.

3 weeks

Tissue ingrowth consisted mainly of striated muscle fibres, adipose tissue and connective tissue. Macrophages were scattered between fat cells, and fibrillar material was seen beneath the tissue ingrowth. This was indistinguishable from that seen in appropriate control specimens.

4 weeks

Essentially the same as 3 weeks, but an increased number of striated muscle fibres and adipose cells were noted.

6 weeks

A further increase in muscle fibres and adipose tissue was observed. There was still some fibrillar material either side of the tissue ingrowth.

8 weeks

By this time, the striated muscle had been largely replaced with adipose tissue. Fibrillar material was still present.

12 and 26 weeks

The tissue ingrowth consisted mainly of adipose tissue and was similar in appearance to that observed at 8 weeks.

The percentage tissue ingrowth into the tubes was determined for all the post-implantation time periods excluding 12 and 26 weeks. At no stage was there a statistically significant difference between the Surgicel and control implants.

Histological examination of the tissue sections obtained from intrafemoral Surgicel implants revealed the following:

2 days

Large Surgicel fibres were clearly evident within the tube lumina. Erythrocytes, mononuclear cells, and polymorphs were evident among the

fibres of the implant material. Many of the macrophages exhibited a foamy appearance; polymorphs were present in small numbers.

7 days

Large Surgicel fibres were clearly visible within the lumina of all specimens. There was a granulation tissue ingrowth in which macrophages and erythrocytes were seen in large numbers, as well as among the fibres. Many macrophages exhibited a foamy appearance.

2 weeks

Surgicel fibres were not identifiable. Haematopoietic bone marrow was evident within the tube lumina. Bone spicules were also evident among the marrow cells and along the walls of the tubes. A pale-staining eosinophilic material containing many inflammatory cells, including macrophages and a few lymphocytes, was evident in one area. Many of the macrophages exhibited a foamy appearance, but no particulate matter could be identified within these cells.

3 Weeks

Spicules of woven bone were prominent within the tube lumina, and a thin layer of bone lined the tube walls. Haematopoietic marrow constituted the bulk of the tissues within the tube. Surgicel fibres were not seen in any of the tissue sections but foci of fibrillar material were identified among marrow cells and woven bone. Detailed examination of these foci indicated the presence of numerous macrophages, some of which exhibited a markedly enlarged, foamy appearance.

4 and 6 Weeks

Haematopoietic bone marrow occupied the lumina of all tubes, the walls of which were lined with a thin layer of bone. There were no signs of the implant material but many large macrophages were seen.

8 Weeks

Haematopoietic marrow and fat cells occupied the tube lumina and a thin layer of bone lined the walls. There was no evidence of the implant material, nor of the foamy macrophages.

12 and 26 Weeks

Features were essentially similar to those observed at 8 weeks. The observations of Garach (1982) on the persistence of Surgicel in bone wounds are in general accord with those of Skoog (1967), Thilander and Stenström (1969), Engdahl (1972), and Uddströmer (1978). There was no overt evidence that Surgicel either retarded (Engdahl, 1972; Uddströmer, 1978) or enhanced bone healing (Skoog, 1967; Thilander and Stenström, 1969).

Garach (1982) concluded from his series of observations that Surgicel was an absorbable haemostatic agent the absorption of which seemed to be more rapid from muscle than from cancellous haematopoietic marrow-containing bone. This result is in accord with the findings of Hurwitt et al. (1960) and Jantet and Rob (1960). Surgicel was found to be completely absorbed from haematopoietic bone marrow by 4 weeks post-implantation which is in agreement with the results obtained by Skoog (1967).

Garach (1982) indicated that the process by which Surgicel is absorbed by tissues was not clear, and that no conclusions could be drawn from his study for the following reasons: no enzyme histochemical assays were performed to indicate altered enzyme activity in response to implanted Surgicel; nor could particles of the implant material be identified in macrophages. Enlarged , vacuolated (foamy) macrophages were clearly seen in relation to implanted Surgicel in this study and the author proposed that these cells could have played a role in absorption of the implant material, but that electron microscopic studies would be required to delineate such.

Garach also concluded that the experimental model used in his investigation appeared to be suitable for the study of the biological effects of implantable local haemostatic agents.

2.7 Conclusion

It is apparent that the mechanisms of absorption of Surgicel by the body have yet to be established. One of the greatest difficulties impeding such research has been the inability of investigators to consistently identify Surgicel in vivo. There is clearly a need to develop techniques for positive identification of Surgicel in implant experiments to ensure that the absorption process is being truly monitored. Thus, the literature relating to absorption times of the material in animal models must be viewed with some caution. It was with an appreciation of this requirement that the following study was designed in order to investigate the mechanism/s of absorption of Surgicel in rat muscle.

CHAPTER THREE

MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

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1. IMPLANT MATERIALS

1.1 Surgicel

Surgicel is manufactured by Surgikos, a member of the Johnson and Johnson group of companies. It is presented as a 6.5 cm^2 strip (1.3 cm x 5.1 cm) of yellowish knitted gauze (Figure 4) which is sterile and wrapped in a foil overwrap (Figure 5). It has a faint caramel-like smell. Different microscopical appearances of the material are shown in Figure 6 (unprocessed and unstained), and Figures 7 and 8 (scanning electron microscope).

1.2 Healon(R)

Healon was used as a positive control material in one of the investigations to be described. It is a sterile, non-pyrogenic, viscoelastic preparation of a highly purified, non-inflammatory, high molecular weight (>1 x 10^6) fraction of sodium hyaluronate (Balazs, 1979). It contains 10 mg/ml of sodium hyaluronate dissolved in physiological sodium chloride-phosphate buffer (pH 7.2 ± 0.2), and is used in ophthalmic surgery. It is presented for use in a syringe.

1.3 Polytetrafluorethylene Tubing

Lengths of polytetrafluorethylene tubing (Teflon*) of outer diameter 1.45 mm and inner diameter 1.19 mm were used as carriers for the implant materials in the present study (Figure 9).

(R) Pharmacia, New Jersey

* Brand No. T2 1195-0047 : supplied by John Morris (Pty.) Ltd., Sydney

2. PREPARATION OF MATERIALS FOR IMPLANTATION

The techniques for pre-implantation preparation of materials followed those employed by Garach (1982) and were as follows:

Prior to placing the test material into the Teflon tubing, 6 cm lengths of the latter were enclosed in Guardian Hospital Autoclave bags and autoclaved in a Smith Pressure Steriliser. Pressure-sensitive autoclave tape (Scotch, Australia) was used as an indicator of adequate steam penetration. The tubes were stored in these bags until use.

Since Surgicel can not be autoclaved, strict sterility procedures were adopted. Presurgical handling of the material was undertaken in an Oliphant Laminar Flow Unit (Model HLF-6-L) during which time special body drapes and sterile gloves were worn, and sterilized instruments used.

Short lengths of Surgicel-filled Teflon tubes (S-tubes) were prepared for implantation as follows: the gauze was cut into strips and gently pulled through the 6 cm lengths of presterilized tubing using a 3/0 Black Silk Suture (Figure 10). The tube was subsequently cut into lengths of approximately 3 mm with a scalpel (Figure 11). This procedure was repeated until a sufficient number of tubes for each experiment were obtained. Empty Teflon tubes of similar length, used as controls, were also prepared from autoclaved tubing (C-tubes). Batches of S-tubes and C-tubes were prepared on the same day for each experiment and stored in sterile plastic containers prior to implantation.

Healon was prepared for implantation (H-tubes) using the following technique: sterilized Teflon tubes were placed in a sterile test-tube and Healon was injected into and around the Teflon tubes. The test tube was then sealed with Nescofilm[R] and stored at -4°C.

3. EXPERIMENTAL ANIMALS

The rat was selected as the experimental animal in this study. The rationale behind this choice included ready availability of the animal, suitability of housing facilities, and the compatibility of the size of the animal with the planned surgical procedures. In addition, a previous study (Garach, 1982) involving Surgicel employed the rat as the experimental animal.

Young female Sprague-Dawley Albino rats of average weight 196 g were obtained from the Waite Agricultural Research Institute, Adelaide. After implantation, all animals to be sacrificed at any one designated post-implantation period were kept in the same cage and appropriately marked. All animals were fed a standard diet (Appendix 1) and provided with water ad libitum.

4. CHEMICAL CHARACTERIZATION OF SURGICEL

Three 10 mg samples of dry Surgicel were placed in 4M Gu.HCl for 5 hours. A fibrous residue was subsequently dissolved by 0.3N sodium hydroxide overnight. Samples from the 4M Gu.HCl and sodium hydroxide solutions were assayed for the presence of uronic acid according to the technique described in Appendix 3.

R Nippon Shoji Kaisha Ltd., Osaka, Japan.

5. SURGICAL IMPLANTATION TECHNIQUE

5.1 Method of Anaesthesia

All implantations were performed on rats under general anaesthesia which was induced by inhalation of a mixture of nitrous oxide, oxygen and Fluothane (I.C.I., Australia) and maintained with an intramuscular injection, into the hind limbs, of ketamine hydrochloride (Ketalar : Parke-Davis, Sydney, Australia) in a dose of 0.2 ml / 100g body weight.

5.2 Operative Technique

When surgical anaesthesia was obtained, the animal was immobilized on a wooden operating table. The entire ventral surface of the rat was cleansed and sterilized with a sterile aqueous solution of 0.5% cetrimide and 0.05% chlorhexidine (Pharmacy Dept., Royal Adelaide Hospital, Adelaide, Australia).

Instruments used in the operative procedure are listed and illustrated in Appendix 2. Following a skin incision, muscles of the anterior chest wall were exposed using Kelly scissors. A small incision, contiguous with the orientation of muscle fibres, established a crevice in the muscle into which the appropriate tube was inserted using College tweezers (Figures 12 and 13). Subsequently, muscle was sutured with a single 6/0 nylon suture which, in addition to providing wound closure, also served as a localising marker at the time of recovery of tubes from the animals (Figure 14). Primary closure of skin incisions was effected with 3/0 black silk sutures (Figure 15).

6. RECOVERY OF SPECIMENS - GENERAL

At the designated post-implantation recovery periods, animals were sacrificed by an inhalation overdose of nitrous oxide/Fluothane.

Access to the implantation sites was gained via a skin incision over the anterior chest wall, using Kelly scissors. The muscle containing the implant was dissected out en bloc and processed according to the specific requirements of each experiment (see later).

7. URONIC ACID ASSAYS

7.1 Recovery of Specimens for Assay

Blocks of tissue containing the implant were dissected from the animals. Each block was then placed on a clear perspex petri dish and the tube and its contents were carefully dissected out of the muscle using transillumination.

7.2 Uronic Acid Isolation Procedures

Each implant was placed in 2 ml of 4M guanidinium hydrochloride in 0.5M sodium acetate at pH 5.7 (4M Gu.HCl). This solution contained the following protease inhibitors: 0.1 ml Tyrasylol(R) (1000 Kallikrein inactivation units) per litre solution of 0.1M ethylene-diamino-tetra-acetic acid (E.D.T.A.) ; 1M 6-amino hexanoic acid (caproic acid) ; 0.005M benzamidine hydrochloride. Specimens were extracted in test-tubes containing this solution for a minimum of 8 hours.

(R) Bayer Pharm., Haywards Heath, Sussex

The implant was then digested in 2 ml of a papain solution [in 1 mg/ml sodium acetate buffer containing 0.01M cystein hydrochloride (Sigma Chem. Co.) and 0.01M E.D.T.A.] and placed in a 60°C oven for 24 hours.

Following 4M Gu.HCl extraction and papain digestion, extracts were mixed with 4 volumes of cold absolute ethanol and allowed to stand overnight. A white precipitate which subsequently formed was centrifuged, and resuspended in 2 ml 0.5M sodium acetate. Following resuspension, the residual precipitate did not readily dissolve : these samples were again centrifuged at 3500 r.p.m. in a Roto-Uni 80W centrifuge and the soluble supernatant was retained for uronic acid assay.

7.3 Uronic Acid Assay Technique

Uronic acid assays were performed on all experimental specimens, and pure standards of glucuronic acid (25, 50 and 100 g/ml), according to the method of Blumenkrantz and Asboe-Hansen (1973) (Appendix 3).

7.4 Experimental Protocols

7.4.1 First Experiment

Nine rats were implanted with S-tubes, H-tubes (used as positive controls) and C-tubes (used as negative controls) as indicated in Table 2.

TABLE 2 : Implantation and recovery protocol for first experiment

Recovery Time (T)			Implant	
24 hours	48 hours	7 days	Right Hand Side	Left Hand Side
Rat 1	Rat 4	Rat 7	1 x S-tube	1 x C-tube
Rat 2	Rat 5	Rat 8	1 x S-tube	1 x H-tube
Rat 3	Rat 6	Rat 9	1 x S-tube	1 x S-tube

7.4.2 Second Experiment

In the second experiment the implantation protocol was confined to assaying samples from two post-implantation time intervals. Details of implantation and recovery of specimens are shown in Table 3.

TABLE 3 : Implantation and recovery protocol for second experiment

Recovery Time (T)		Implant	
24 hours	48 hours	Right Hand Side	Left Hand Side
Rat 1	Rat 5	1 x S-tube	1 x C-tube
Rat 2	Rat 6	1 x S-tube	1 x C-tube
Rat 3	Rat 7	1 x S-tube	1 x S-tube
Rat 4	Rat 8	1 x S-tube	1 x S-tube

Uronic acid extraction and assays were performed on the recovered implants and pure glucuronic acid standards.

7.4.3 Third Experiment

A third series of implants were recovered from animals at 6 and 18 hours post-implantation according to the protocol outlined in Table 4.

TABLE 4 : Implantation and recovery protocol for third experiment

Recovery Time (T)		Implant	
6 Hours	18 Hours	Right Hand Side	Left Hand Side
Rat 1	Rat 5	2 x S-tubes	2 x S-tubes
Rat 2	Rat 6	2 x S-tubes	2 x S-tubes
Rat 3	Rat 7	2 x C-tubes	2 x C-tubes
Rat 4	Rat 8	2 x C-tubes	2 x C-tubes

All Surgicel implants recovered from Rat 1 and Rat 2 (6 hours) were placed in the same test-tube and extracted in 4M Gu.HCl. Similarly, control implants from Rat 3 and Rat 4 were extracted in another test-tube containing 4 M Gu.HCl. This procedure was also applied to implants recovered at 18 hours. Papain digestions were subsequently undertaken on the same implant groups in four more test-tubes. For uronic acid assays, two 0.2 ml samples were collected from each of the eight test-tubes. Duplicate assays were performed.

7.4.4 Fourth Experiment

Specimens were not subjected to implantation and recovery in the fourth experiment, the protocol for which is illustrated in Table 5.

TABLE 5 : Protocol for Fourth Experiment

Group	Assay Sample	Pre-assay Extraction/Digestion Solution	Time
A	8 S-tubes	4 M Gu.HCl	24 hours
		4 M Gu.HCl	48 hours
		4 M Gu.HCl	72 hours
		Papain	24 hours
B	8 C-tubes	4 M Gu.HCl	24 hours
		Papain	24 hours
C	8 samples of Surgicel "pushed out" of tubes	4 M Gu.HCl	24 hours
		Papain	24 hours
D	8 S-tubes	Hanks' B.S.S./ Bovine Albumin*	24 hours
E	Glucuronic Acid Standards (25, 50 & 100 g/ml)		
F	Blank (Sodium acetate)		

* A solution of Hanks' Balanced Salt Solution (Flow Laboratories, Virginia, U.S.A.) containing 5% by weight Bovine Serum Albumin (Commonwealth Serum Laboratories, Melbourne Australia), adjusted to pH 7 with sodium bicarbonate.

Group A tubes were extracted in 2 ml 4M Gu.HCl for 24 hours after which the solution was removed, stored for assay, and replaced with a fresh 2 ml 4M Gu.HCl. This procedure was repeated at 48 hours, and at 72 hours papain was added to the test tube (Figure 16). Group B tubes served as negative control tubes and were extracted in 4M Gu.HCl for 24 hours, after which papain was added. Group C tubes (positive controls)

were treated in the same manner prior to assay, and Group D tubes were placed in Hanks' B.S.S./Bovine Albumin, samples of which were removed after 24 hours for assay. Uronic acid assays were performed on 0.2 ml of each sample.

7.4.5 Quantitation of Assay Data

To permit valid comparison of assay data, a method of quantitation was required.

A direct correlation between Surgicel weight and length of Teflon tubing was hypothesized and an initial trial was undertaken to test this. Surgicel was pushed out of 34 S-tubes with a dental explorer on to the tray of a Mettler H54AR Balance, the weight of each piece of material being obtained in grams to 4 decimal places. The length of the corresponding tube was subsequently measured (in mm to 3 decimal places) with a Micro 2000 micrometer (Moore and Wright, Sheffield, England).

Results were correlated on a frequency distribution table and assimilated with a standard statistical computer programme.

A second trial was undertaken in the same manner but using 40 tubes packed with "tightly twisted Surgicel" in an attempt to obtain a more uniform density of Surgicel in the tubing. Results were also assimilated with a standard statistical computer programme.

In a third trial, 19 tubes were conventionally filled with Surgicel and exhaustively hydrated in Hanks' B.S.S./Bovine Albumin for 12 hours after which any excess Surgicel protruding from either end of the tube

(Figure 17) was removed with a sharp scalpel. The following parameters were then measured:

(1) Length of tube

(2) Weight of tube containing Surgicel

(3) Weight of Surgicel

(4) Weight of tube

These were correlated on frequency distribution tables and assimilated with a standard statistical computer programme.

The high correlation between weight of hydrated Surgicel and tube length was used for calculation of the initial hydrated implant weight. In order to measure the degree of hydration of Surgicel, and to thus facilitate calculation of its dry weight prior to implantation, 8 randomly cut test samples of Surgicel were weighed, placed in the Hanks' B.S.S./Bovine Albumin solution for 12 hours, and weighed again. The average percentage weight increase was 421% (Appendix 4).

7.4.6 Fifth Experiment

Surgicel and control tubes were implanted and recovered from 16 rats according to the protocol outlined in Table 6.

All implants were exhaustively hydrated in Hanks' B.S.S./Bovine Albumin solution (see above) for 12 hours and any excess Surgicel was removed with a sharp scalpel prior to implantation.

TABLE 6 : Implantation and recovery protocol for fifth experiment

Recovery Time (T)	No. of Rats	Implant	
		Right Hand Side	Left Hand Side
6 Hours	2	2 x S-tubes	2 x S-tubes
	2	2 x C-tubes	2 x C-tubes
18 Hours	2	2 x S-tubes	2 x S-tubes
	2	2 x C-tubes	2 x C-tubes
24 Hours	2	2 x S-tubes	2 x S-tubes
	2	2 x C-tubes	2 x C-tubes
48 Hours	2	2 x S-tubes	2 x S-tubes
	2	2 x C-tubes	2 x C-tubes

Eight S-tubes and eight C-tubes were recovered at each post-implantation time. Each set of eight tubes was placed in a separate test-tube and extracted sequentially in 4M Gu.HCl at 24, 48 and 72 hours (Figure 18). Uronic acid assays were subsequently performed on samples of all extractions, and appropriate standards. Values obtained for each set of sequential extractions (24, 48 and 72 hours) were added together and reflected the amount of uronic acid present in each group of eight implanted tubes.

The wet weight of Surgicel prior to implantation was extrapolated for each set of eight tubes from measurements of tube lengths. Data so derived was standardized for a 10 mg dry weight sample (Appendix 4).

8. HISTOLOGY : SURGICEL

After chemical demonstration of a quantitative loss of polyanhydroglucuronic acid from the Surgicel implant system as a function of time, histological and histochemical investigations were undertaken in order to both identify Surgicel morphologically and to follow its fate in the rat tissues.

8.1 Implantation Protocol

Tubes were not exhaustively hydrated prior to implantation for histology as quantitation of data was unnecessary. Implantation and recovery proceeded according to the protocol illustrated in Table 7.

After sacrifice, muscle blocks containing the implants were dissected out and placed in the appropriate fixation solution (see below), as were three S-tubes and one C-tube which had not been implanted (for transmission electron microscopic examination at T = 0).

TABLE 7 : Implantation and recovery protocol for Surgicel histology

Recovery Time (T)				Implant	
6 hrs.	18 hrs.	24 hrs.	48 hrs.	Right Hand Side	Left Hand Side
OPTICAL MICROSCOPY					
Rat 1	Rat 2	Rat 3	Rat 4	1 x S-tube	1 x S-tube
TRANSMISSION ELECTRON MICROSCOPY					
Rat 5	Rat 6	Rat 7	Rat 8	1 x S-tube	1 x C-tube

8.2 Optical Microscopy

8.2.1 Determination of Selective Staining Affinity of Alcian Blue for Surgicel in vitro

The Critical Electrolyte Concentration (CEC) after which selective staining of Surgicel by Alcian Blue 8GX (G.T. Gurr, London) ceased in a model experiment using filter papers was determined according to the method of Scott and Dorling (1965), and is described in Appendix 5.

8.2.2 Processing of Specimens for Optical Microscopy

Specimens of muscle containing implants were fixed in 10% neutral buffered formalin and processed for optical microscopy (Appendix 5). Sections of 7 µm were obtained on a Leitz* Rotary Microtome according to the orientation shown in Figure 19. They were subsequently floated onto chromic acid cleansed gelatinized glass slides.

* Ernst Leitz Wetzlar GMBH, Wetzlar, Federal Republic of Germany.

8.2.3 Staining of Sections

Representative sections from the centre of all tubes were stained with haematoxylin and eosin. Additional sections were stained with Alcian Blue 8GX according to the method of Scott and Dorling (1965) at 8 different $MgCl_2$ concentrations. Details of all staining procedures are given in Appendix 5.

8.2.4 Examination of Sections

All sections were examined in a Leitz* Orthoplan Largefield Light Microscope. Haematoxylin and eosin sections were examined for the presence of Surgicel, Surgicel-like material and tissue reaction. Alcian Blue sections were compared in order to detect a CEC of $MgCl_2$ *in vivo* for the affinity of Alcian Blue for Surgicel. Quantitative confirmation of this CEC was obtained by densitometrically scanning the 6 hour histologic sections. Photographs of sections were taken with a Leitz Vario-Orthomat camera using Ilford** Pan F (50 ASA) 35 mm black and white film.

8.3 Transmission Electron Microscopy

8.3.1 Preparation of Specimens for Transmission Electron Microscopy

All muscle specimens were fixed in 2.5% glutaraldehyde, trimmed, and post-fixed in osmium tetroxide. After correct orientation and embedding in TAAB(R) epoxy resin, sections were obtained on a Reichert

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** Ilford Ltd., Essex, U.K.
R TAAB Laboratories Equipment Ltd., Berkshire, England.

OM3 ultramicrotome. Initial thick sections were stained with Toluidine Blue and subsequent ultrathin silver sections were mounted on TAAB HR24 200 mesh High Grade grids (Cu/Rh, Ni, Au) and stained with uranyl acetate and lead. Detailed processing methods are described in Appendix 5.

8.3.2 Examination of T.E.M. Specimens

All specimens were examined in a JEOL 100S electron microscope. The following criteria were used in examination and photography of specimens:

(1) Identification of Surgicel at T = 0 hours

(2) Identification of Surgicel in implanted specimens

(3) Presence of Surgicel at T = 6, 18, 24 and 48 hours post-implantation

(4) Location of Surgicel at these post-implantation times.

9. HISTOLOGY : FIBROUS RESIDUE

In the chemical characterization of Surgicel referred to in Section 4, a fibrous residue was noted after dissolution of the material in 4M Gu.HCl. This experiment was designed in order to investigate the presence and morphology of this fibrous material, in both the light and electron microscopes, at various post-implantation times.

9.1 Implantation Protocol

All tubes were placed in 4M Gu.HCl for 72 hours prior to implantation, which proceeded according to Table 8.

TABLE 8 : Implantation and recovery protocol for fibrous residue histology

RECOVERY TIME (T)				IMPLANT
6 hrs.	18 hrs.	24 hrs.	48 hrs.	
OPTICAL MICROSCOPY				
Rat 1	Rat 2	Rat 3	Rat 4	2 x S-tubes
TRANSMISSION ELECTRON MICROSCOPY				
Rat 5	Rat 6*	Rat 7	Rat 8	2 x S-tubes

* Died. No T.E.M. specimen recovered.

9.2 Optical Microscopy

After recovery, specimens were fixed and processed for optical microscopy. Sections of 7 µm thickness were obtained and stained with haematoxylin and eosin, and Alcian Blue at a $MgCl_2$ concentration of 0.15M (Appendix 5). Haematoxylin and eosin sections were examined for the presence of fibrous material and tissue reaction. Alcian Blue sections were examined for the presence of intensely and selectively stained material at this $MgCl_2$ concentration.

9.3 Transmission Electron Microscopy

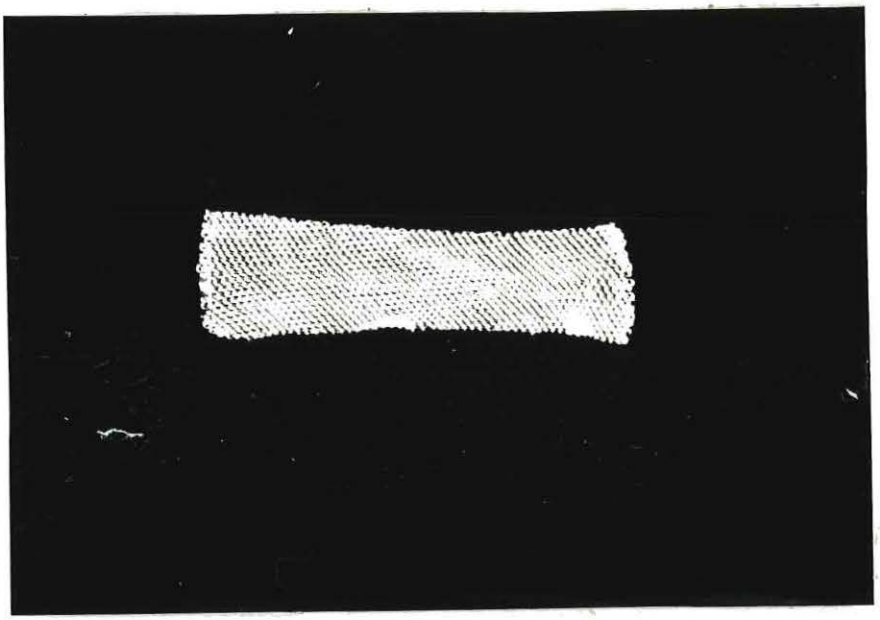
Specimens for T.E.M. were fixed, processed, stained and viewed according to the methods previously described. Criteria used in examination of sections included:

(1) The appearance of the fibrous material at T = 0 hours

(2) The presence of this fibrous material extracellularly or intracellularly at T = 6, 18, 24 and 48 hours post-implantation.

FIGURE 4: Photograph of unwrapped Surgicel. Note the knitted nature of the gauze.

FIGURE 5: Photograph showing a package of Surgicel.



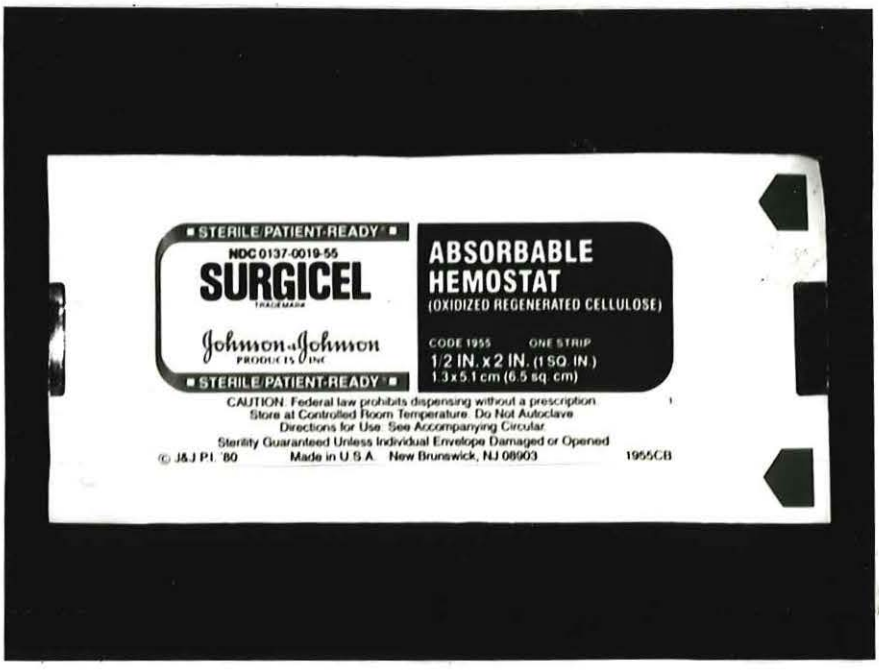
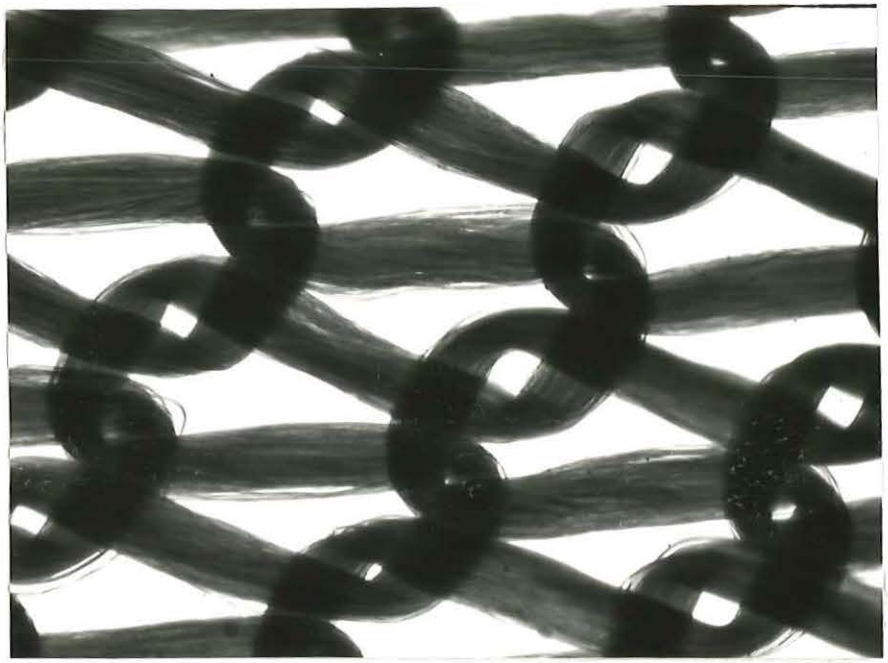

STERILE/PATIENT-READY
NDC 0137-0019-55
SURGICEL
TRADEMARK
Johnson & Johnson
PRODUCTS, INC
STERILE/PATIENT-READY
ABSORBABLE
HEMOSTAT
(OXIDIZED REGENERATED CELLULOSE)
CODE 1955 ONE STRIP
1/2 IN. x 2 IN. (1 SQ. IN.)
1.3 x 5.1 cm (6.5 sq. cm)
CAUTION: Federal law prohibits dispensing without a prescription.
Store at Controlled Room Temperature. Do Not Autoclave.
Directions for Use: See Accompanying Circular.
Sterility Guaranteed Unless Individual Envelope Damaged or Opened
© J&J PI. '80 Made in U.S.A. New Brunswick, NJ 08903 1955CB

FIGURE 6: Photomicrograph of unstained Surgicel gauze. (original magnification x 28)

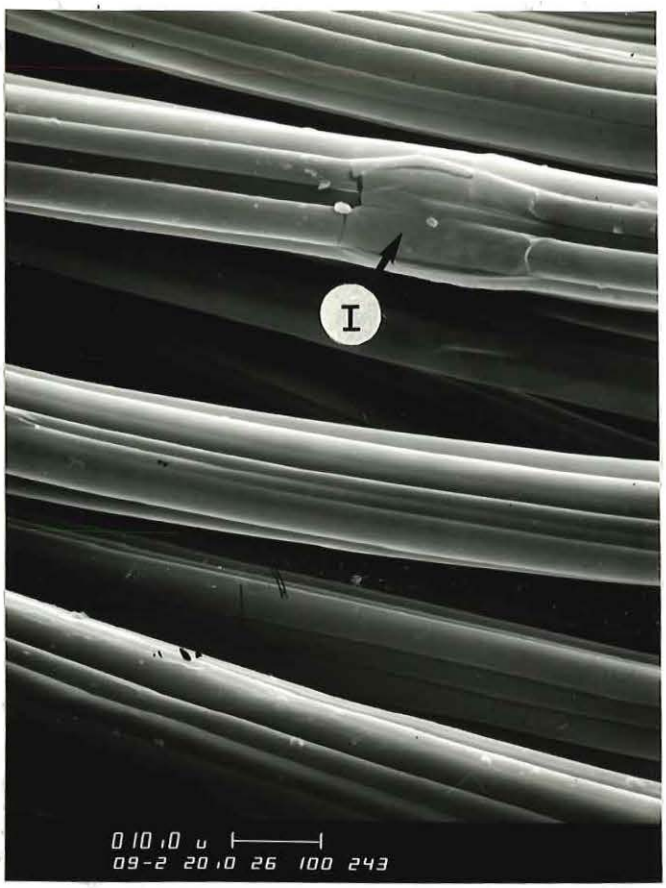
FIGURE 7: Scanning electron micrograph of Surgicel gauze. Note its fine fibrillar structure.




100,0 u
04-1 20,0 26 100 240

FIGURE 8: Scanning electron micrograph of Surgicel gauze. Small irregularities (I) may be observed along the fibres.

FIGURE 9: Photograph of Teflon (polytetrafluorethylene) tubing of outer diameter 1.45 mm and inner diameter 1.19 mm, used in the present investigation.


I
010.0 u
09-2 20.0 26 100 243

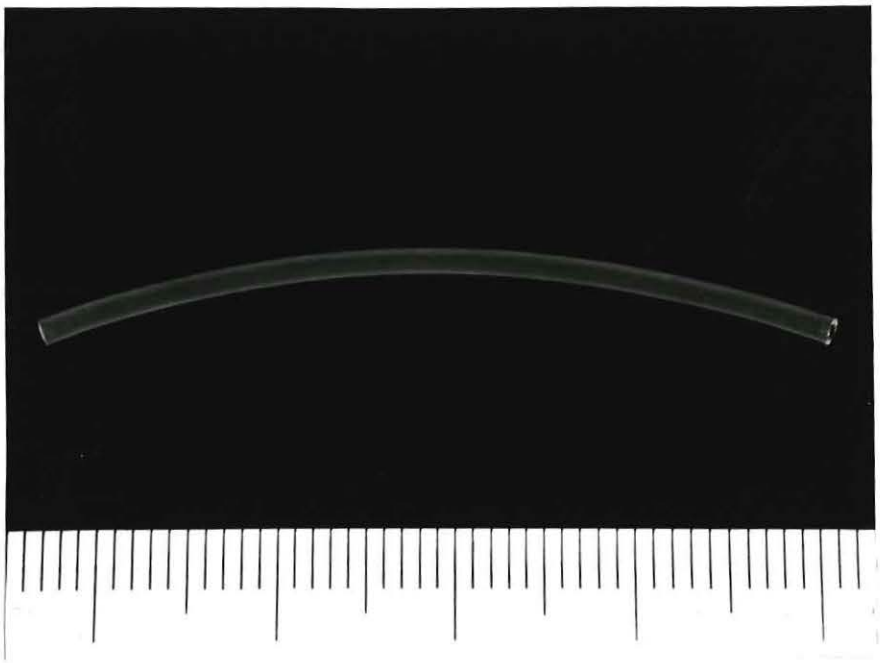
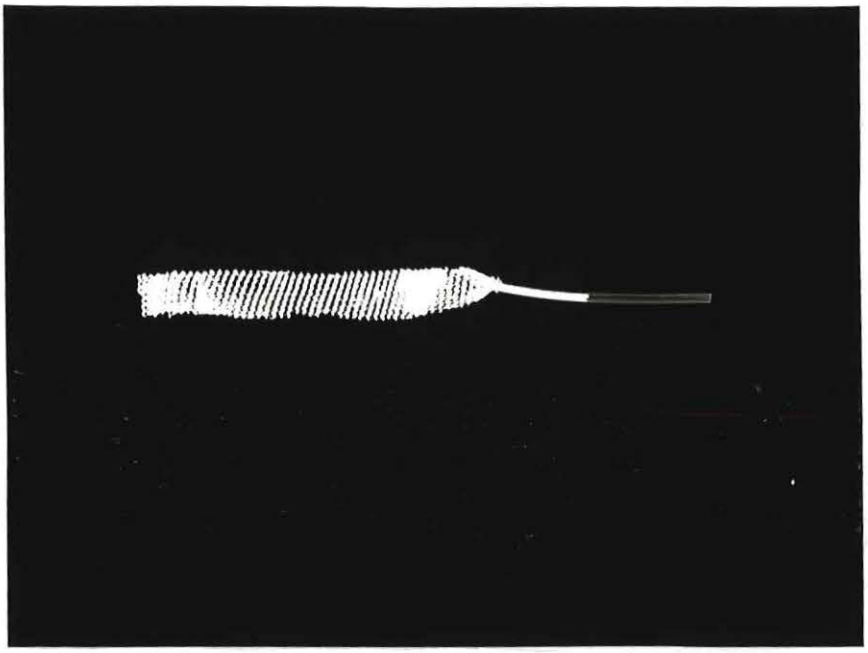


FIGURE 10: Photograph illustrating Surgicel gauze being drawn into the Teflon tubing.

FIGURE 11: Photograph illustrating a length of Surgicel-filled Teflon tubing ready for implantation.



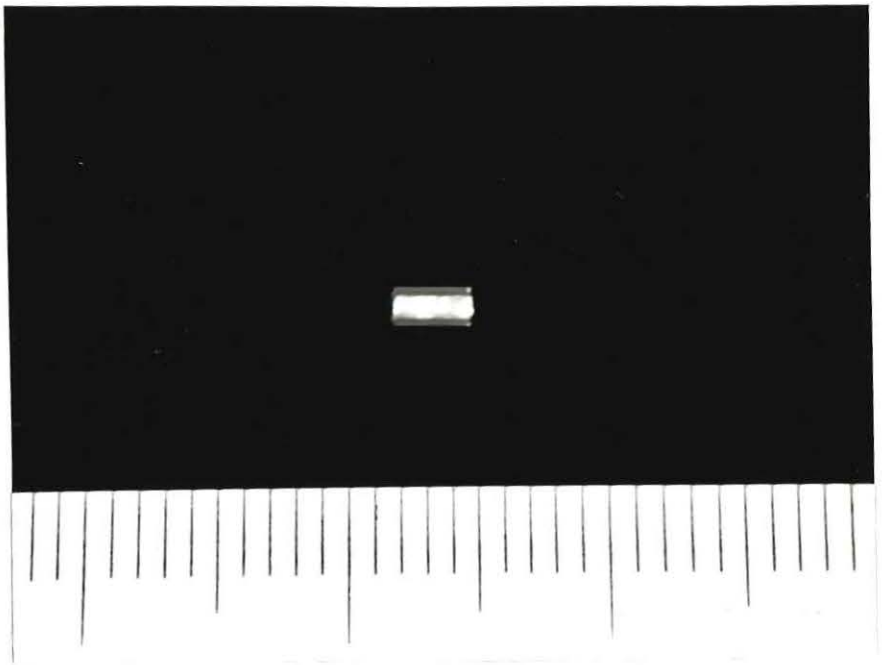
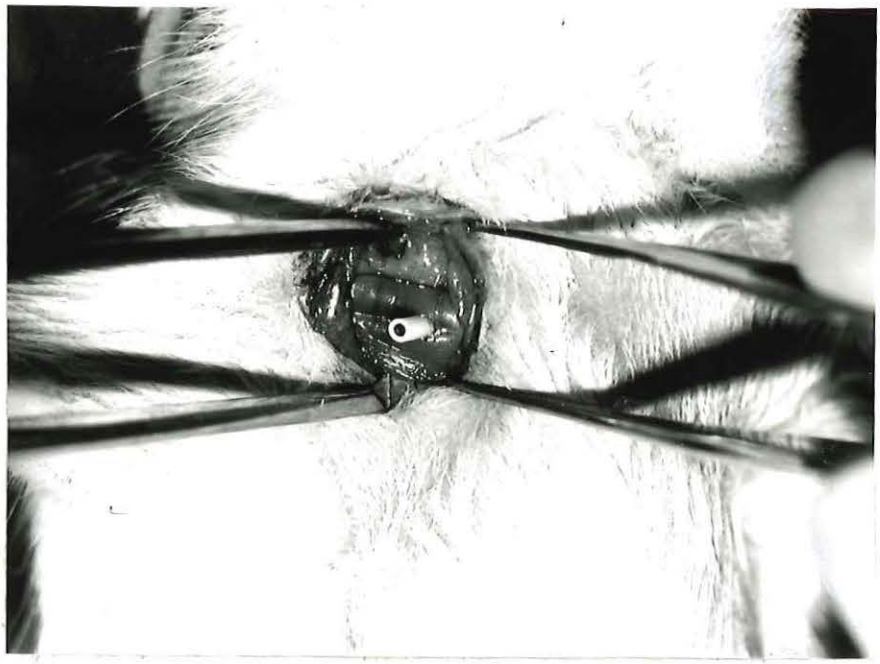


FIGURE 12: Photograph illustrating a crevice prepared in the anterior chest wall muscles of the rat. The Surgicel-filled tube is positioned above the crevice, prior to insertion.

FIGURE 13: Photograph showing location of the Surgicel-filled tube in the muscle crevice.



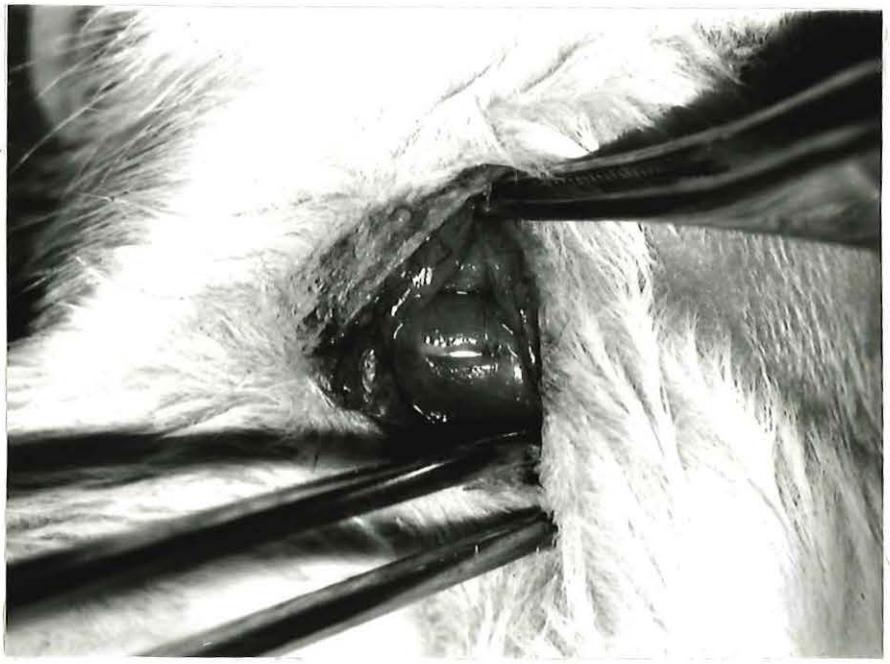
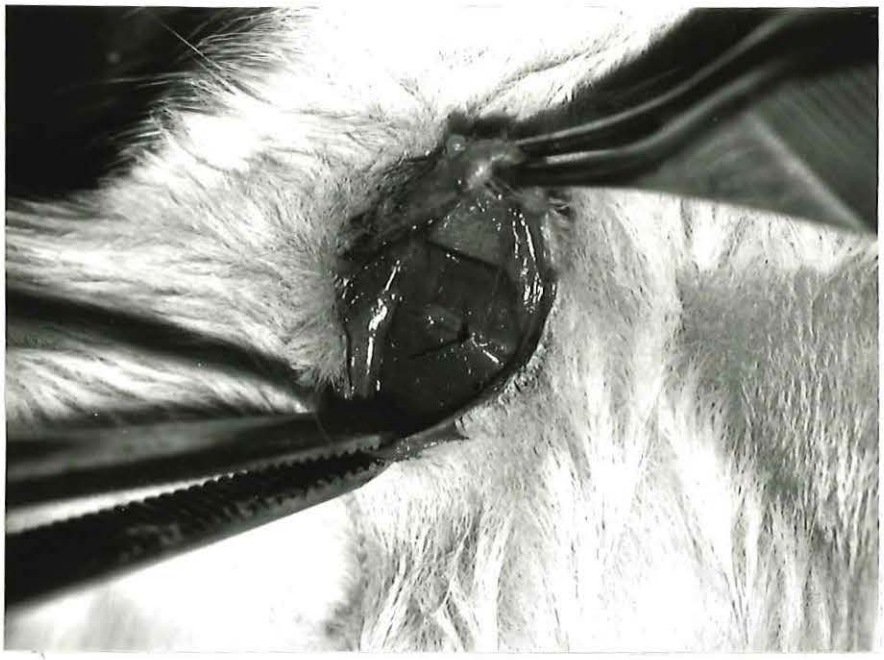
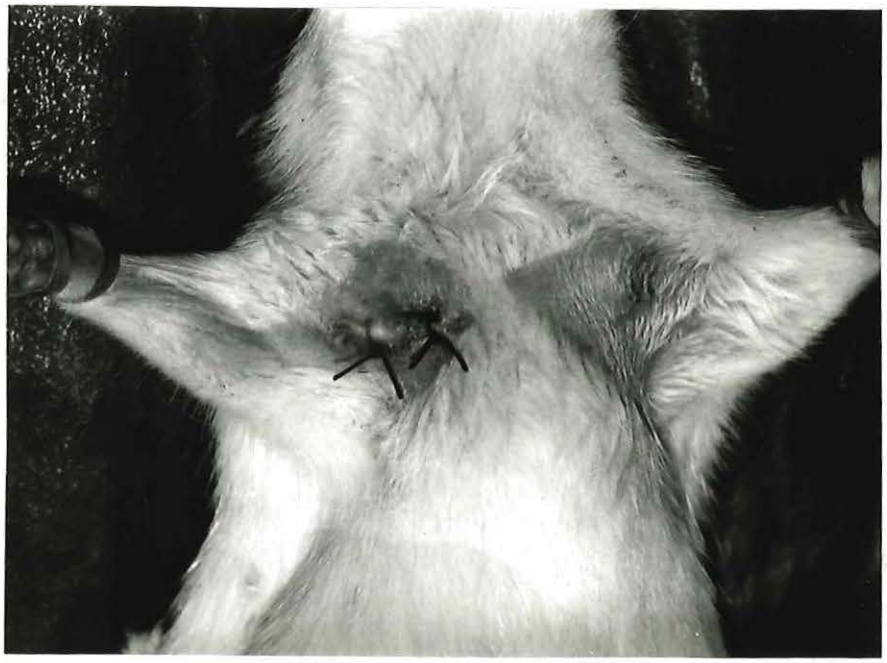


FIGURE 14: Photograph illustrating closure of the muscle crevice with a 6/0 nylon suture.

FIGURE 15: Photograph showing closure of the skin wound with 3/0 black silk sutures.





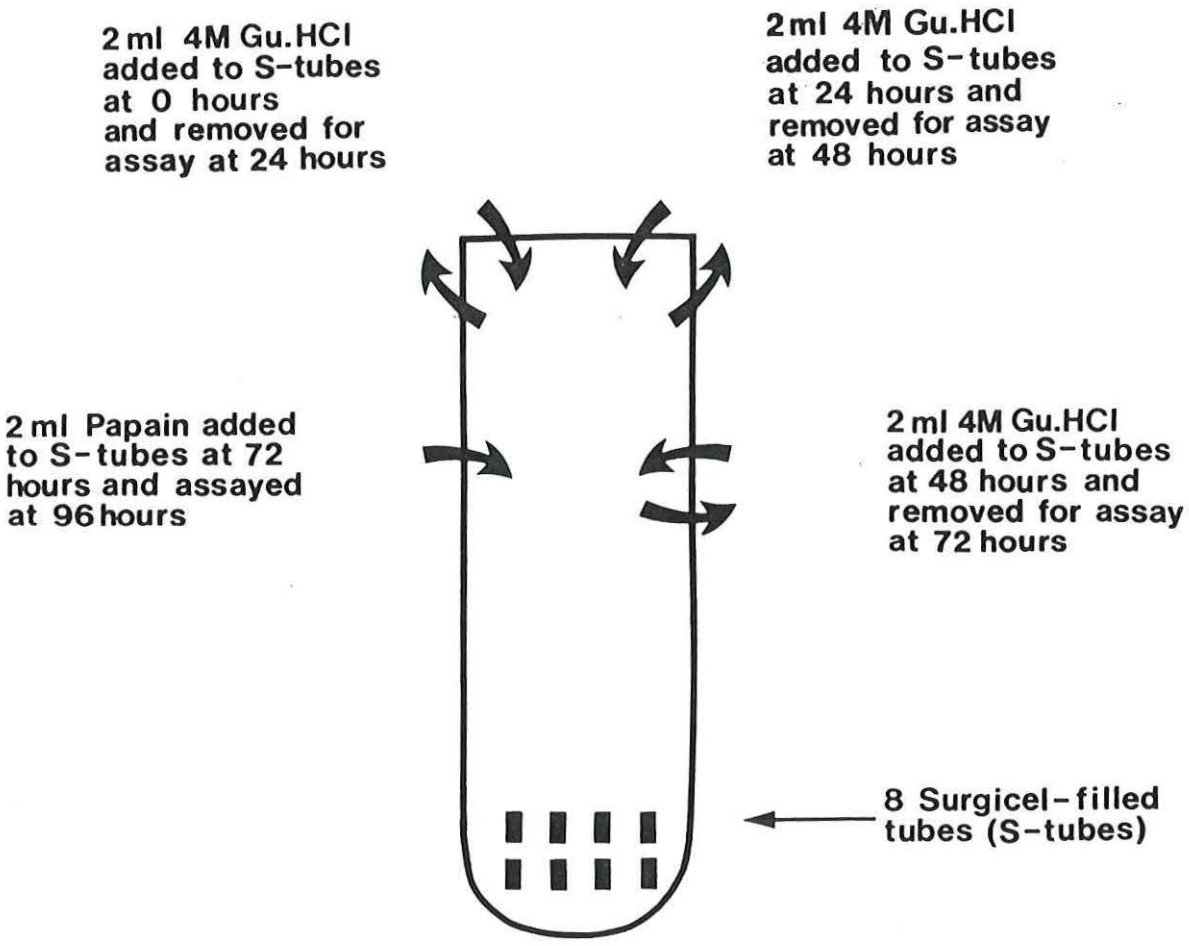


FIGURE 16: Diagram illustrating sequential extraction of samples in 4M Gu.HCl for fourth assay experiment.

FIGURE 17: Photograph illustrating the appearance of a Surgicel-filled tube after exhaustive hydration in Hanks' B.S.S./Bovine Albumin solution.
Note the excess material swelling out of the tube ends. This was removed with a scalpel prior to implantation of the tube.

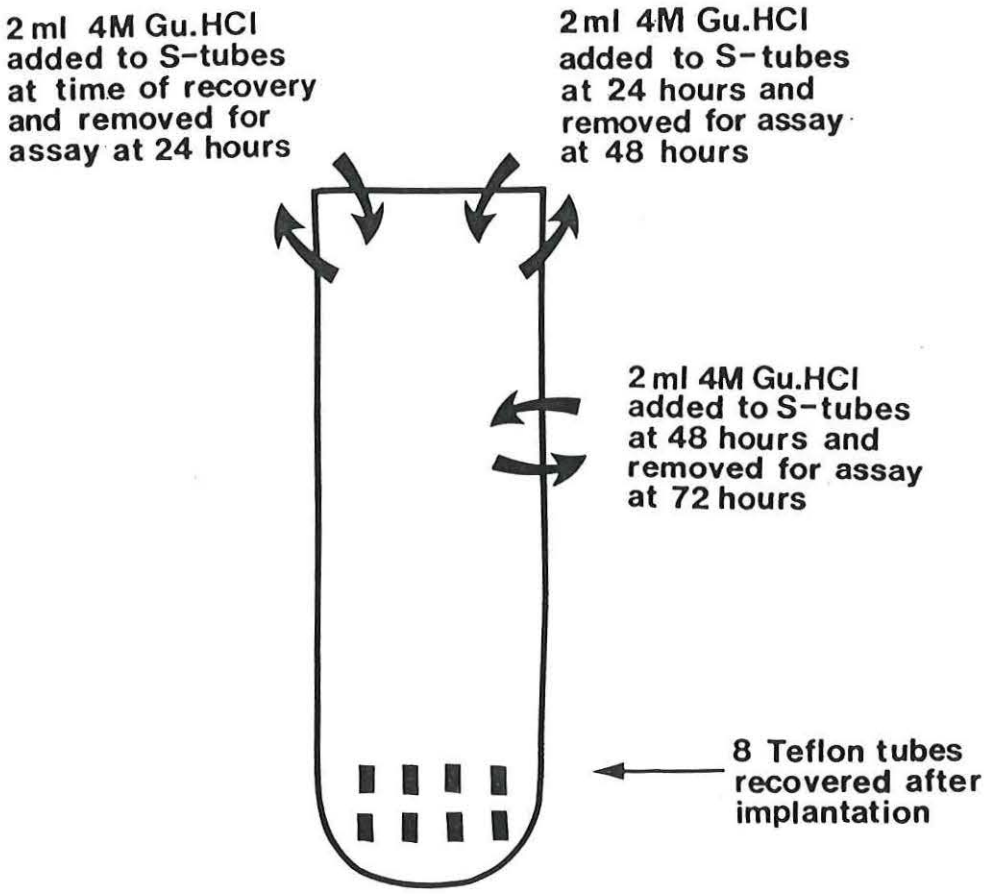


FIGURE 18: Diagram illustrating sequential extraction of implanted tubes in 4M Gu.HCl for fifth assay experiment.

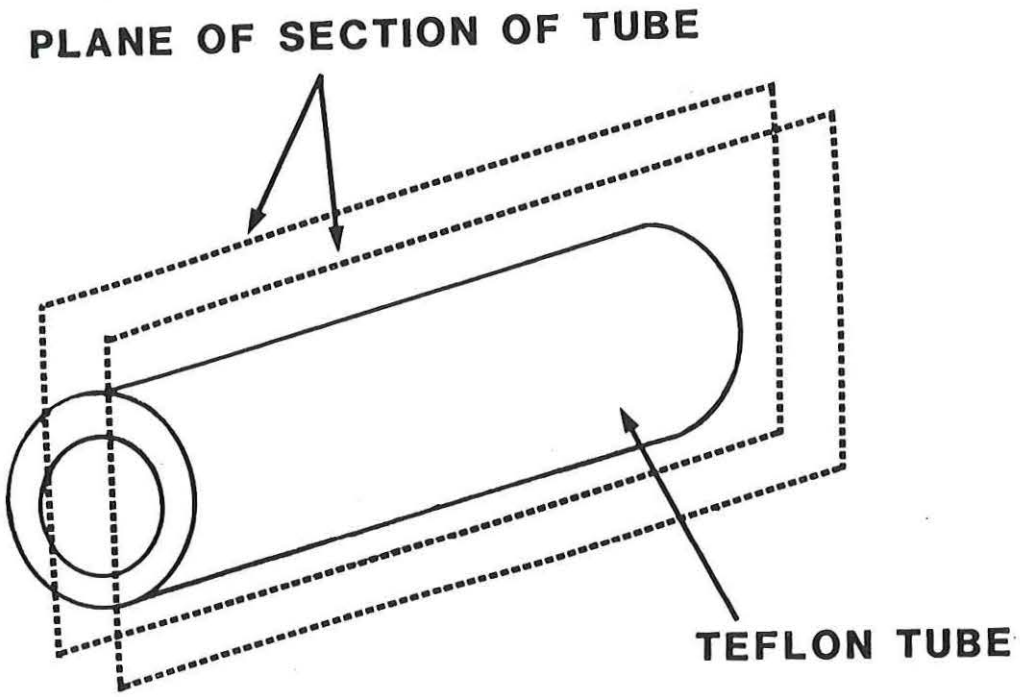


FIGURE 19: Diagram illustrating the plane of histologic section of specimens with respect to the Teflon tube.

CHAPTER FOUR

RESULTS

CHAPTER 4

RESULTS

1. Chemical Characterization of Surgicel

2. Uronic Acid Assays
   - 2.1 First Experiment
   - 2.2 Second Experiment
   - 2.3 Third Experiment
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   - 2.5 Quantitation of Assay Data
   - 2.6 Fifth Experiment

3. Histology : Surgicel
   - 3.1 Optical Microscopy
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4. Histology : Fibrous Residue
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1.0 Chemical Characterization of Surgicel

Uronic acid assay results revealed that a 10 mg (10,000 µg) dry sample of Surgicel yielded 5250 µg uronic acid, extracted in 4M Gu.HCl, and a further 3575 µg uronic acid, hydrolysed by 0.3N sodium hydroxide. Thus, the total uronic acid present in a 10 mg sample of Surgicel was 8,820 µg of which 59.5% was extractable by 4M Gu.HCl, and the residual 40.5% hydrolysed by 0.3N sodium hydroxide.

2.1 First Experiment

Absorbances obtained for pure glucuronic acid standards are illustrated graphically in Figure 20. Values for Group A, B and C implants are shown in Figures 21, 22 and 23 respectively.

2.2 Second Experiment

Figure 24 illustrates the absorbances obtained for pure glucuronic acid standards. Values for 24 hours post-implantation are shown in Figure 25, and those for 48 hours post-implantation in Figure 26.

2.3 Third Experiment

Figure 27 shows the absorbances obtained for pure glucuronic acid standards. Values for 6 and 18 hours post-implantation are illustrated in Figure 28.

2.4 Fourth Experiment

Absorbances obtained for pure glucuronic acid standards are illustrated in Figure 29. Values for Groups A,B,C and D samples are shown in Figure 30.

2.5 Quantitation of Assay Data

Results from the first two quantitation trials are illustrated in Figures 31 and 32 respectively. The correlation between length of tube and weight of tube in the third quantitation trial is shown in Figure 33 between length of tube and weight of Surgicel in Figure 34, and between length of tube and weight of tube containing Surgicel in Figure 35.

2.6 Fifth Experiment

Absorbances obtained for pure glucuronic acid standards are illustrated in Figure 36. Optical density recordings for each recovery period are illustrated in Figure 37 (values indicate the sum of sequential 4M Gu.HCl extractions). Quantitated data (see Appendix 4) for a standard 10 mg dry weight of Surgicel implanted are shown in Figure 38.

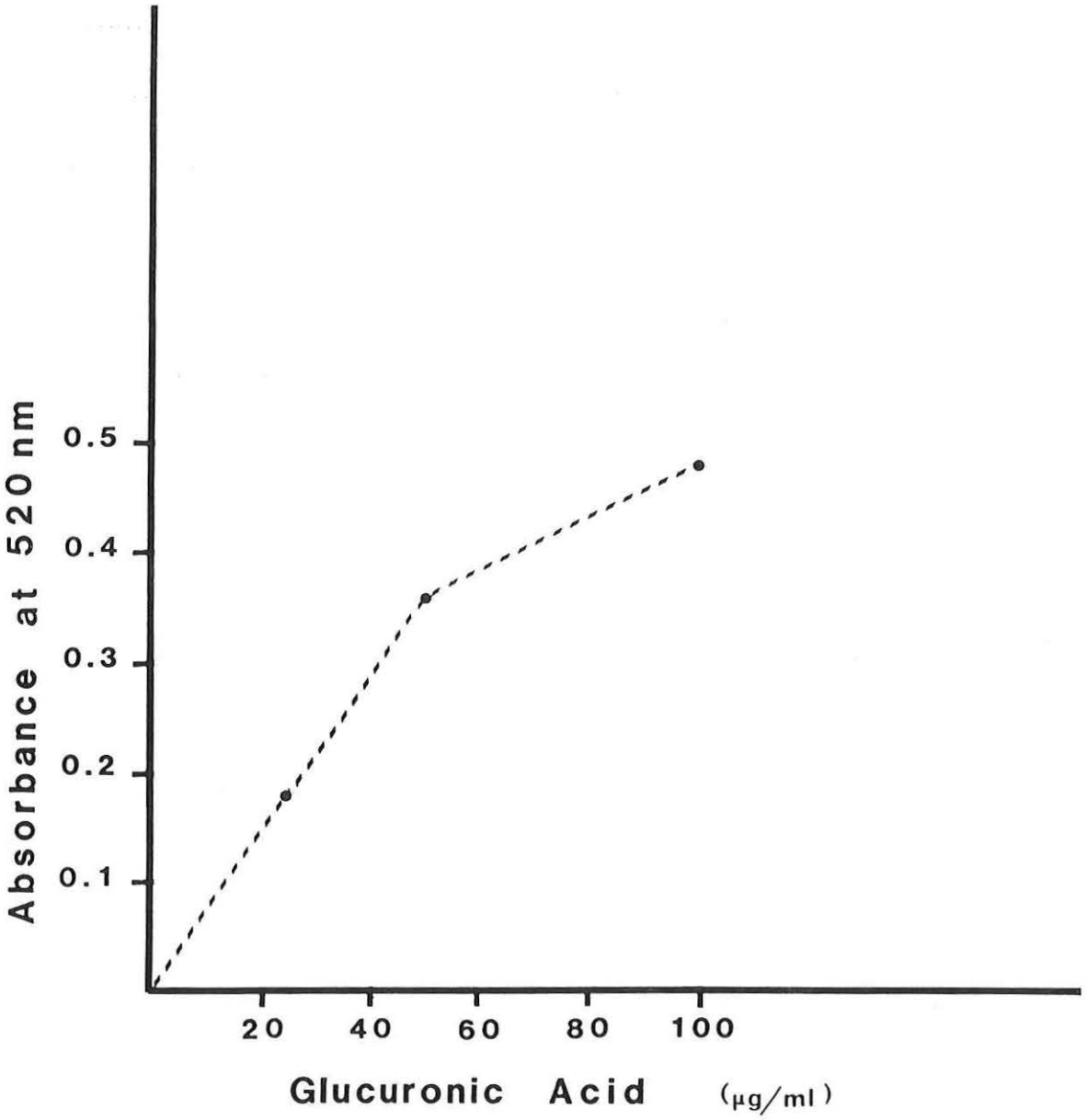


FIGURE 20: First uronic acid assay. Absorbance values of pure glucuronic acid standards.

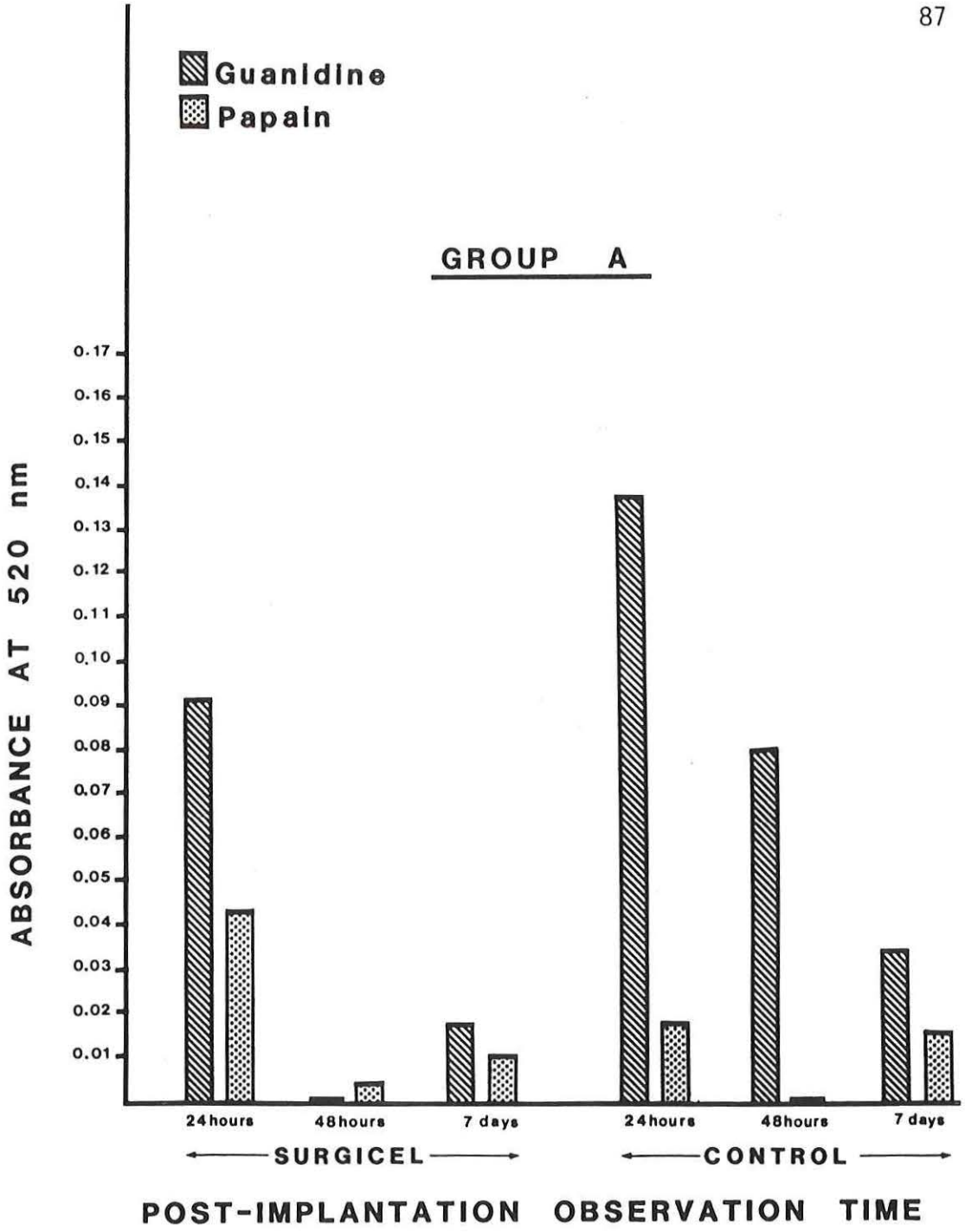


FIGURE 21: First uronic acid assay. Absorbance values of Group A implants at 24 and 48 hours, and 7 days post-implantation.

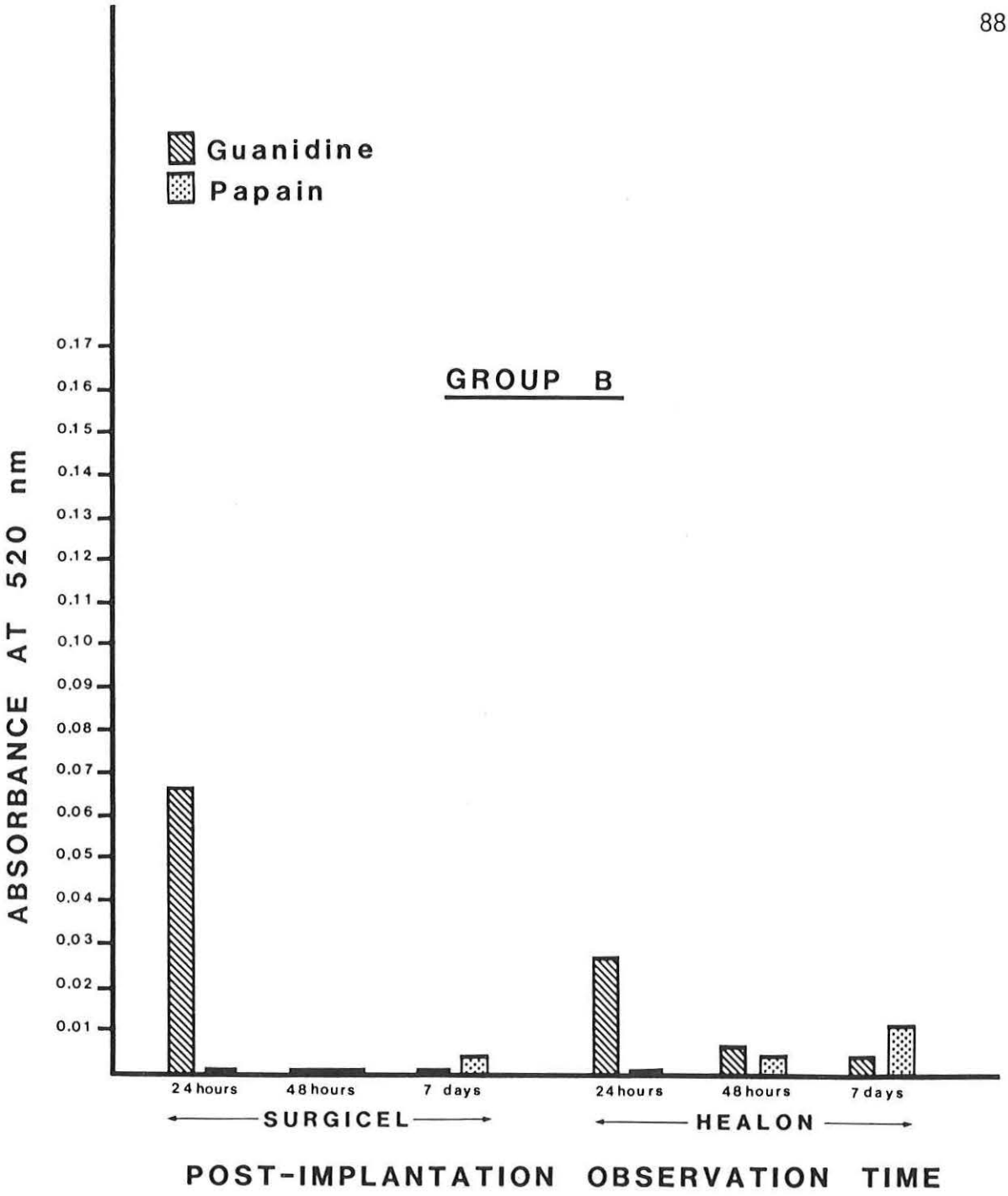


FIGURE 22: First uronic acid assay. Absorbance values of Group B implants at 24 and 48 hours, and 7 days post-implantation.

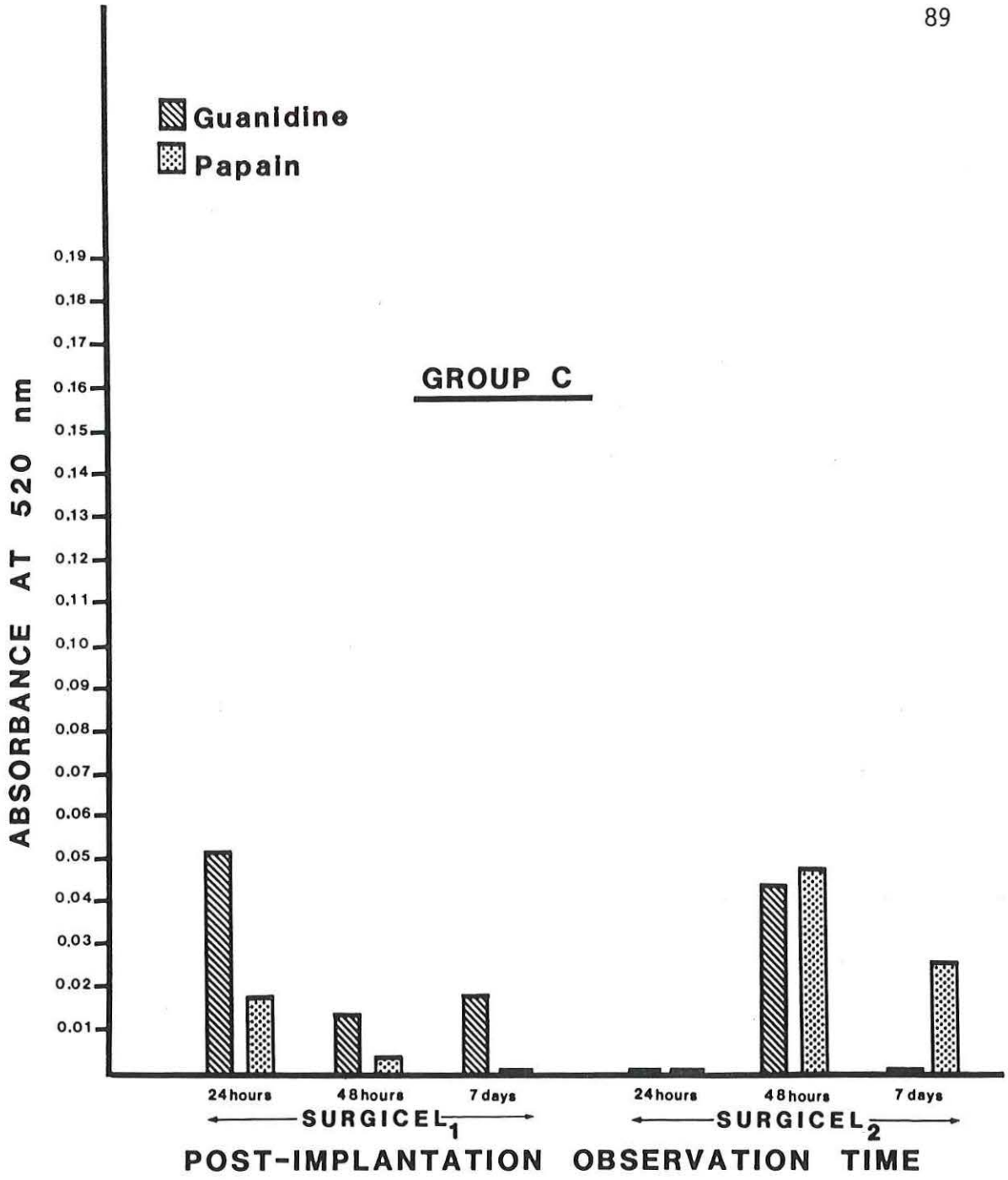


FIGURE 23: First uronic acid assay. Absorbance values of Group C implants at 24 and 48 hours, and 7 days post-implantation.

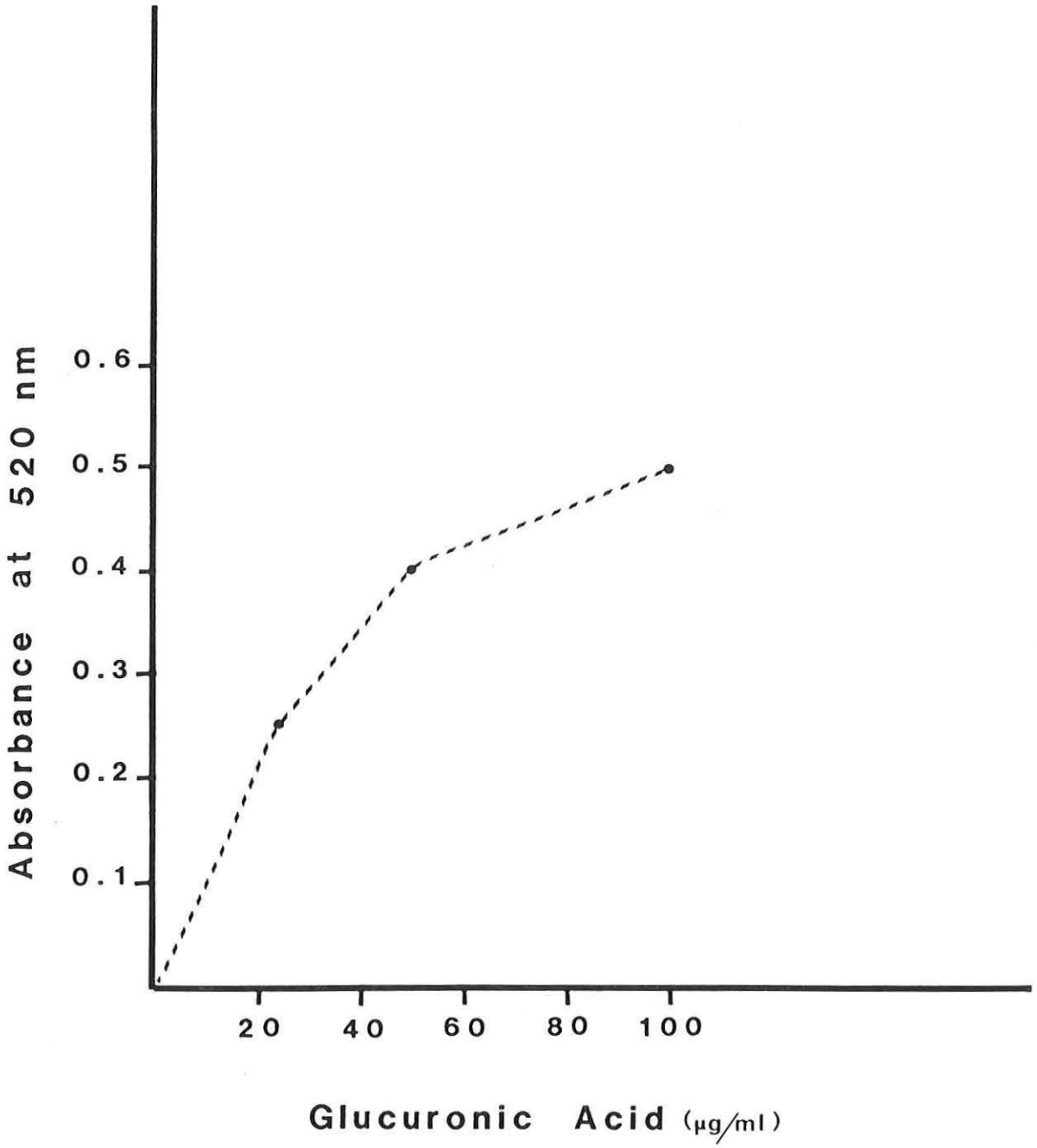


FIGURE 24: Second uronic acid assay. Absorbance values of pure glucuronic acid standards.

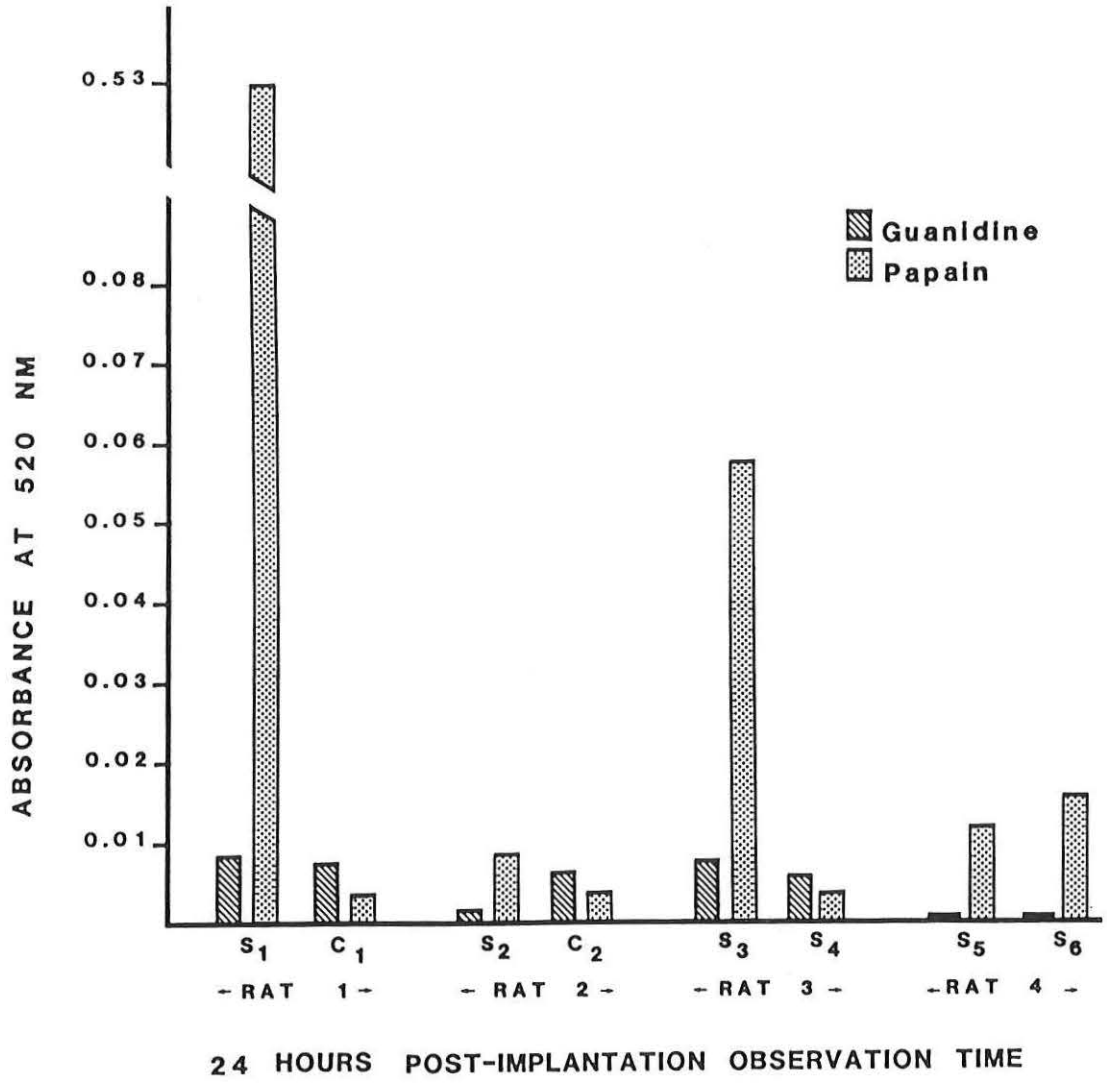


FIGURE 25: Second uronic acid assay. Absorbance values of Surgicel and control implants at 24 hours post-implantation.

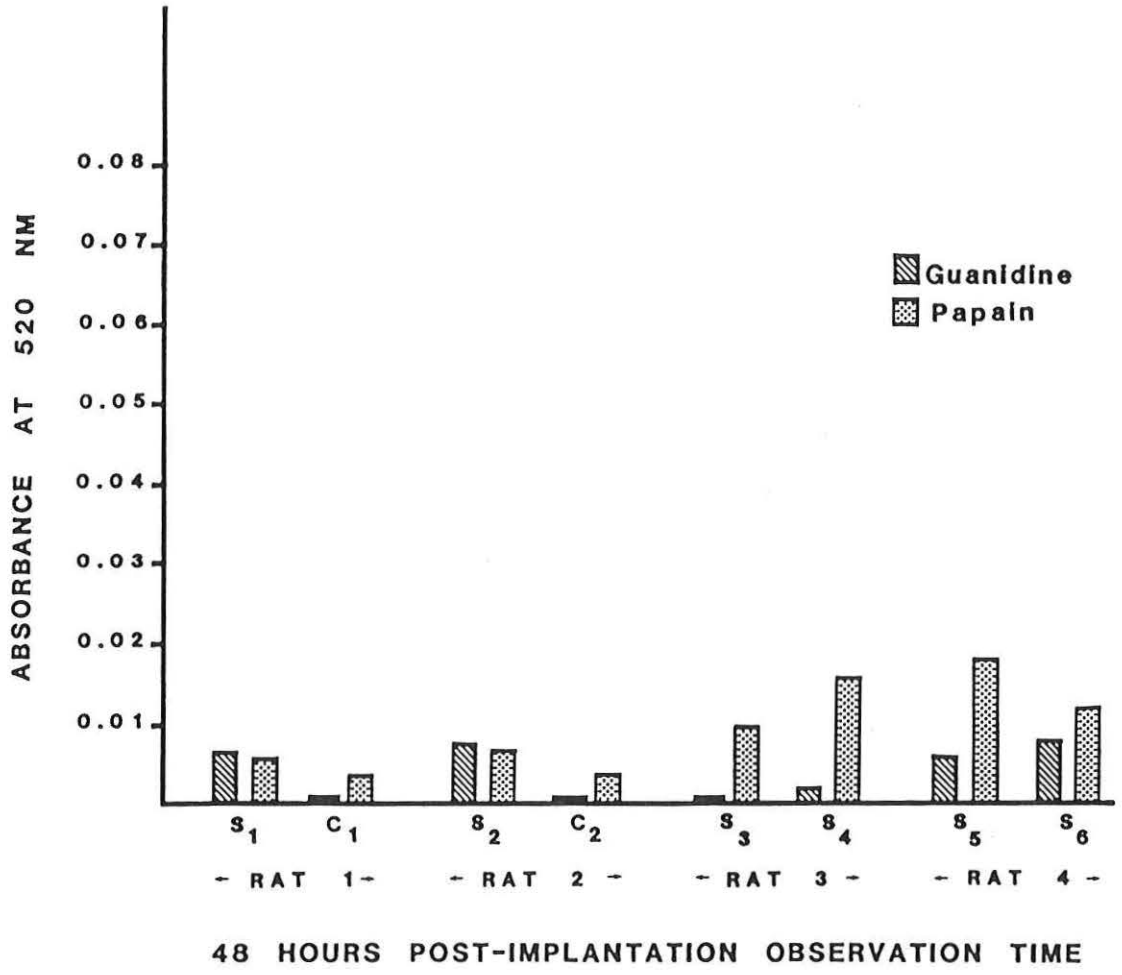


FIGURE 26: Second uronic acid assay. Absorbance values of Surgicel and control implants at 48 hours post-implantation.

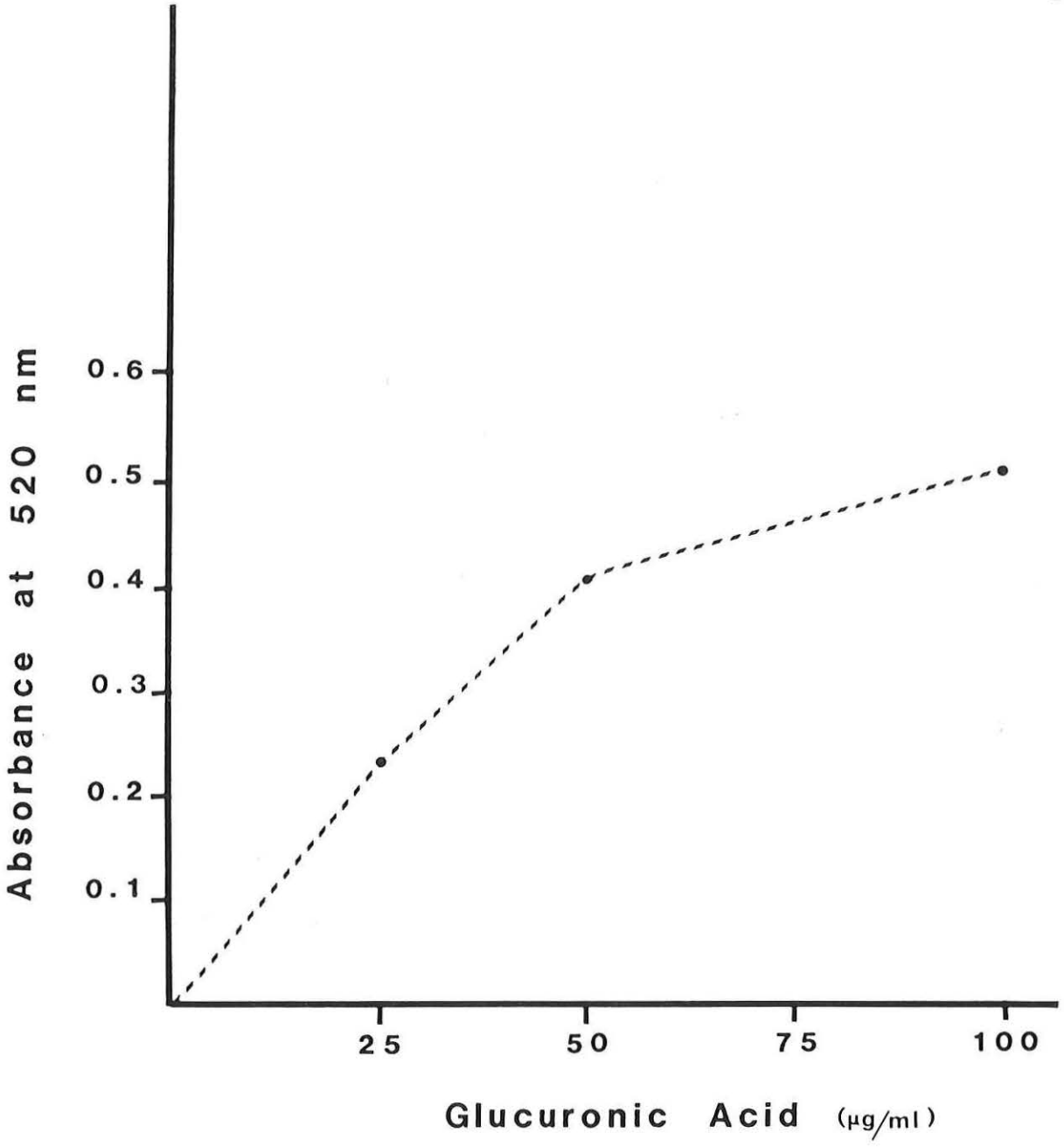


FIGURE 27: Third uronic acid assay. Absorbance values of pure glucuronic acid standards.

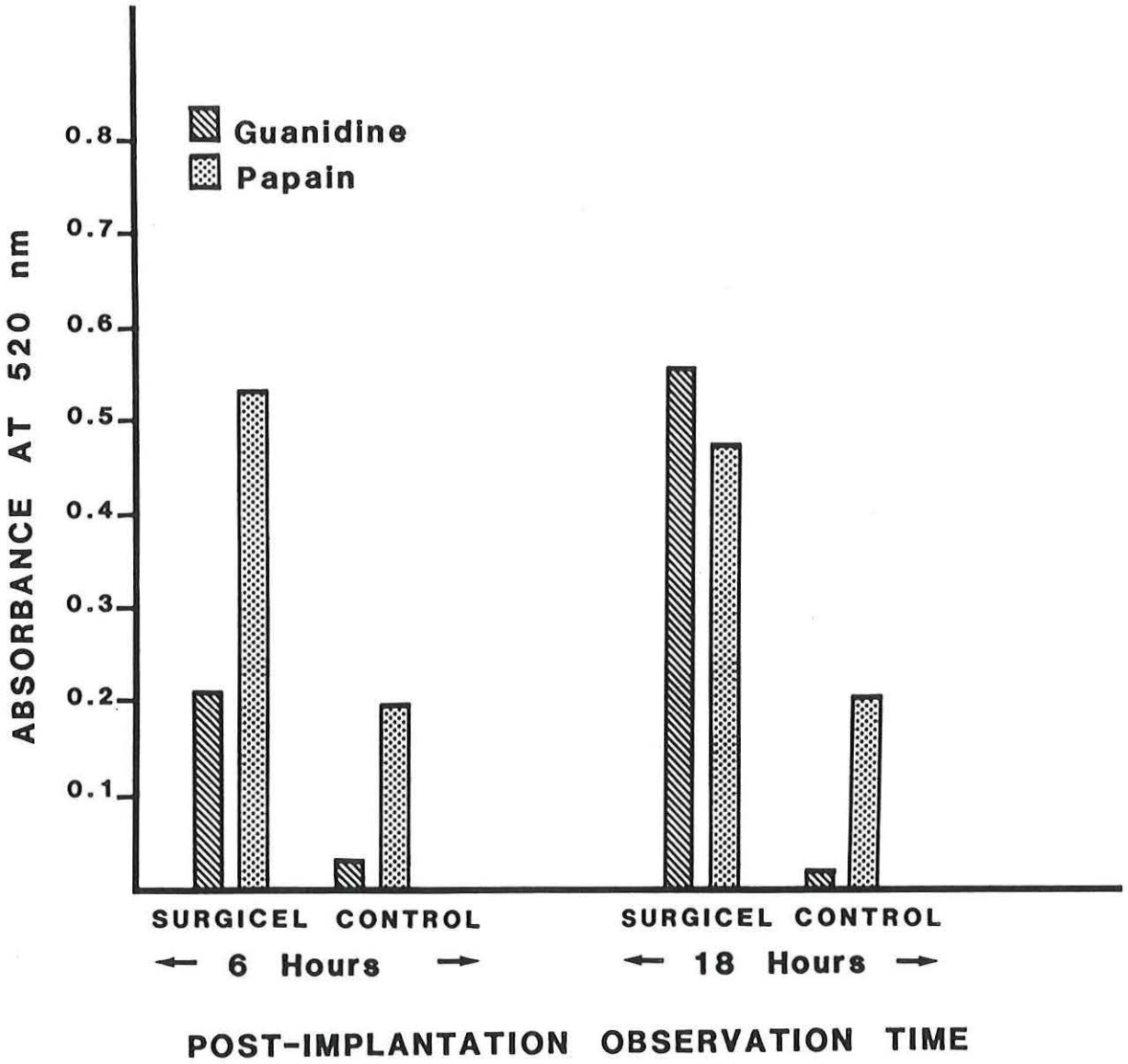


FIGURE 28: Third uronic acid assay. Absorbance values for Surgicel and control implants at 6 and 18 hours post-implantation. (Duplicate assays were performed; results were averaged).

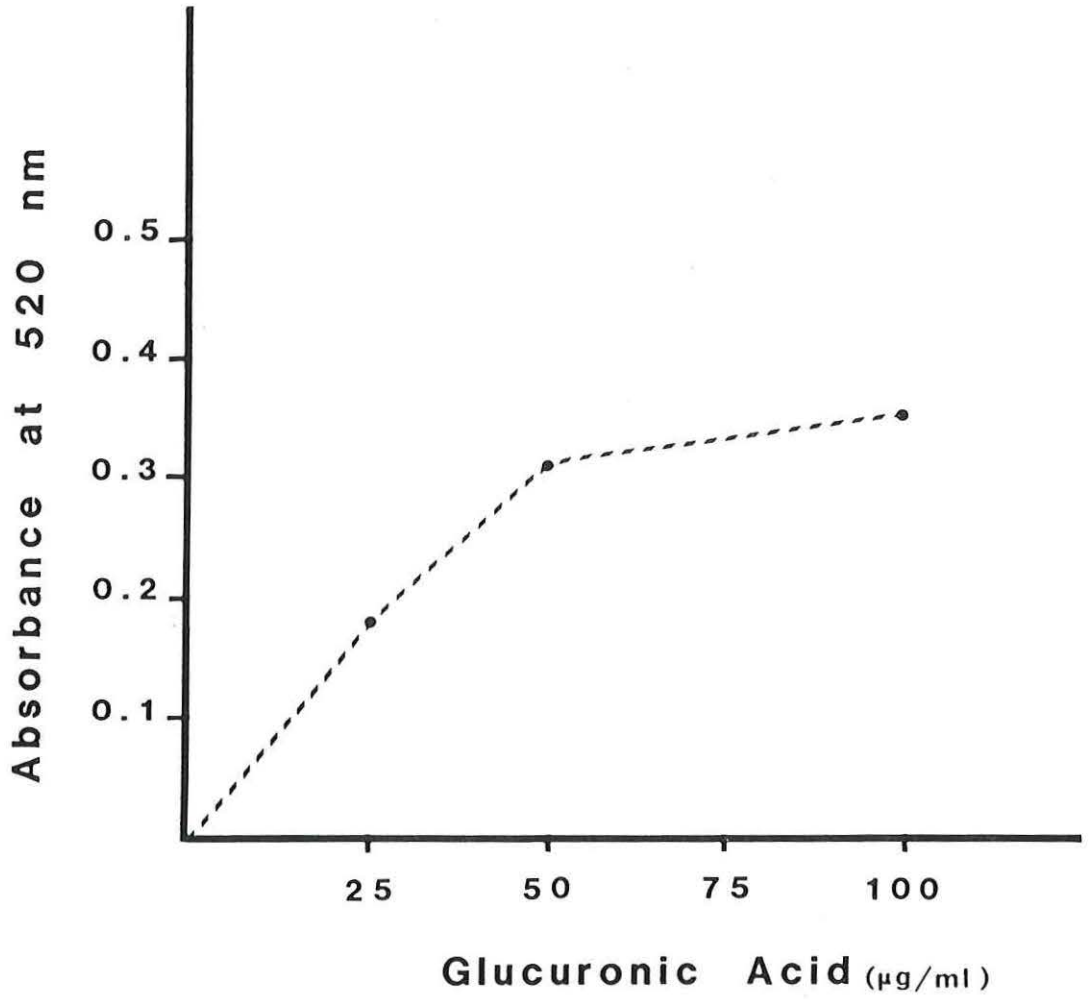


FIGURE 29: Fourth uronic acid assay. Absorbances for pure glucuronic acid standards.

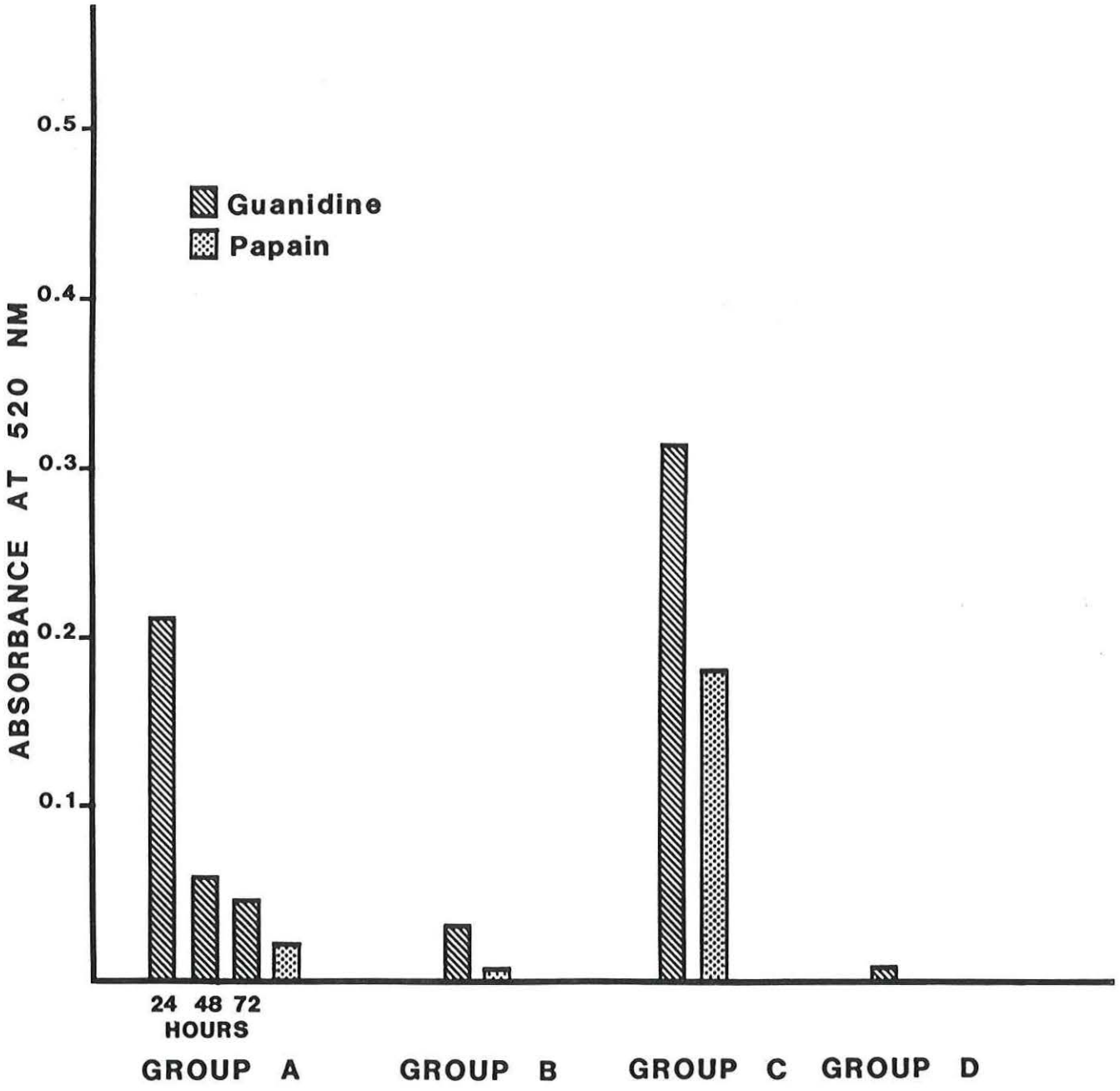


FIGURE 30: Fourth uronic acid assay. Absorbance values for Group A (Surgicel tubes, sequentially extracted), Group B (Empty control tubes), Group C (Surgicel samples removed from tubes) and Group D (Surgicel tubes hydrated in Hanks' B.S.S./Bovine Albumin) samples.

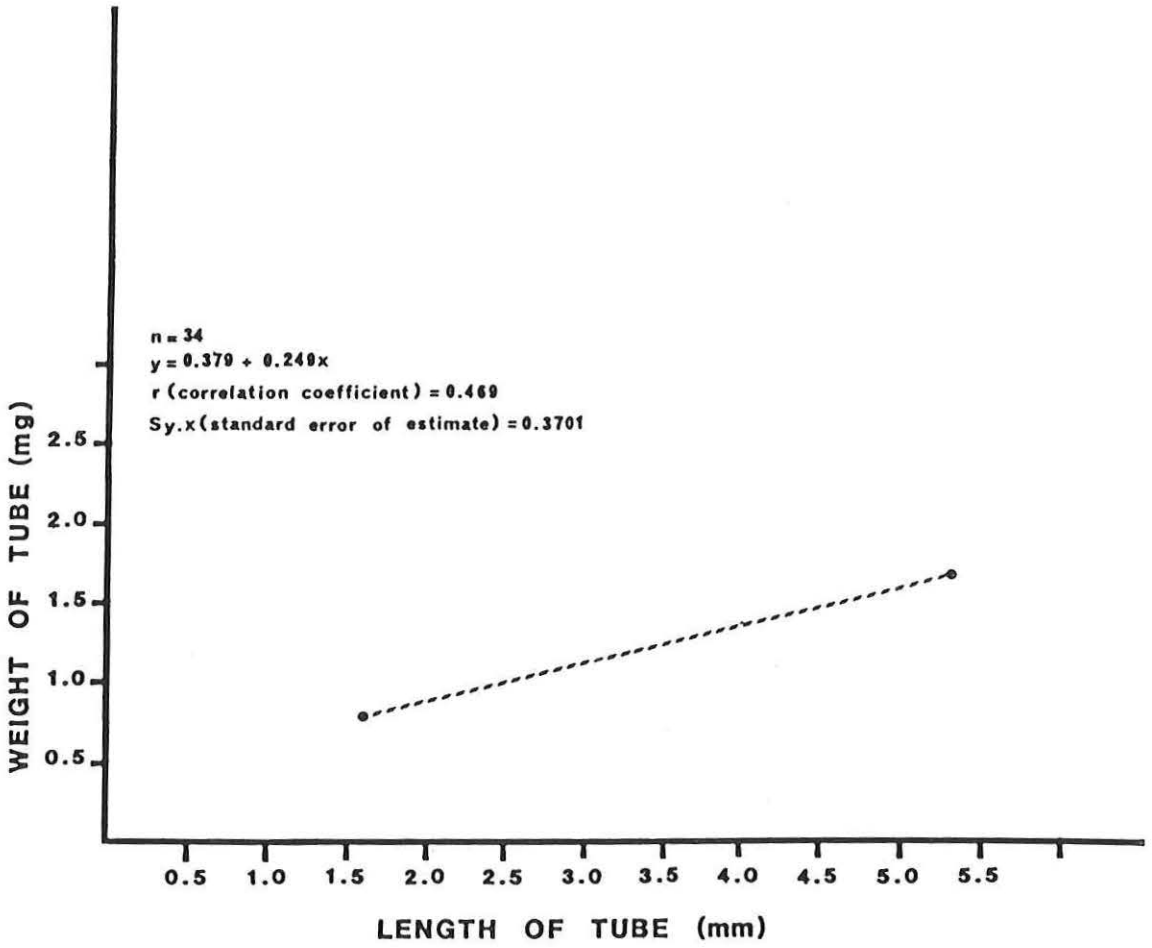


FIGURE 31: Quantitation of assay data.
Correlation between Surgicel weight and tube length (Trial #1).

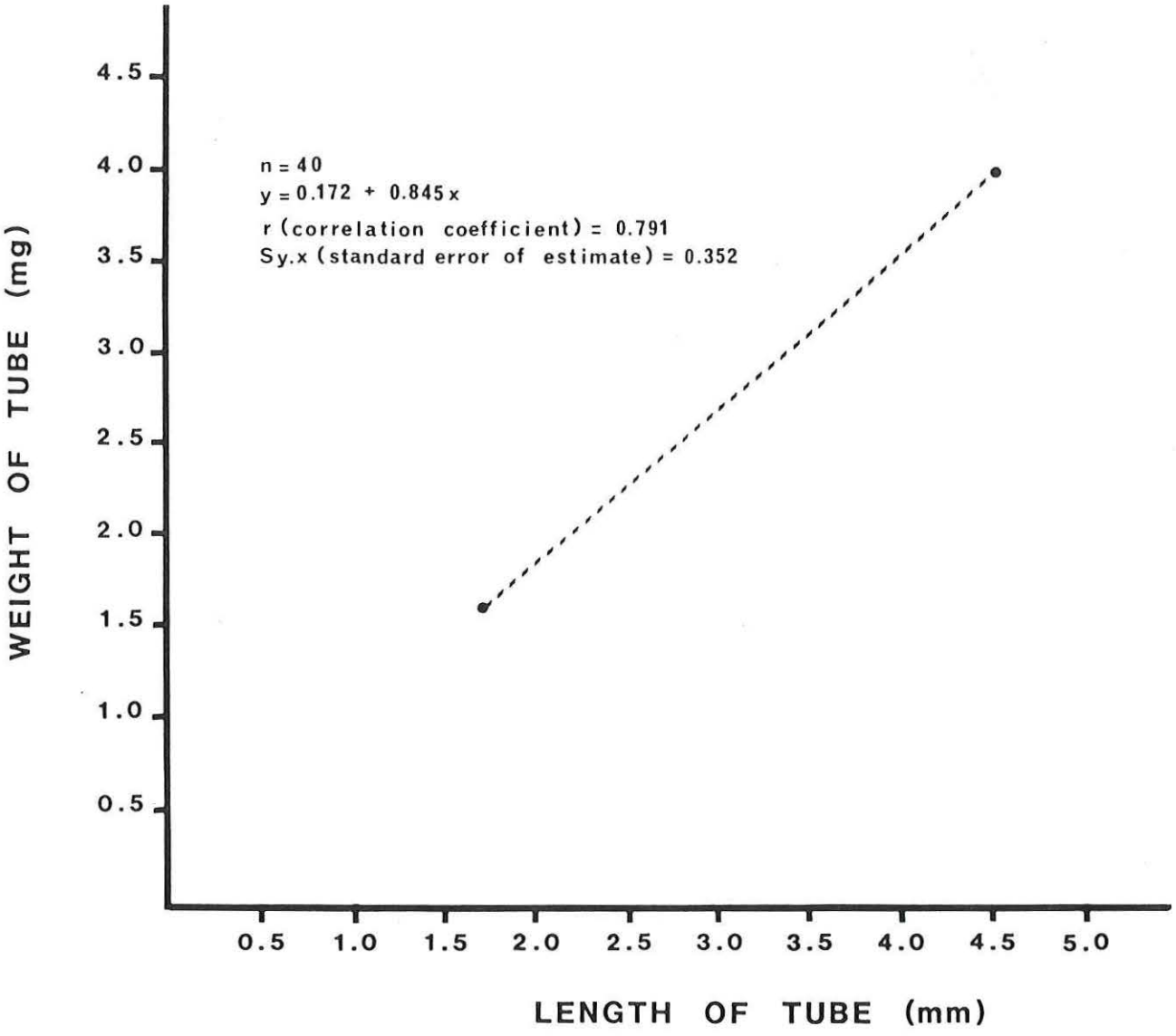


FIGURE 32: Quantitation of assay data.
Correlation between Surgicel weight and tube length (Trial #2).

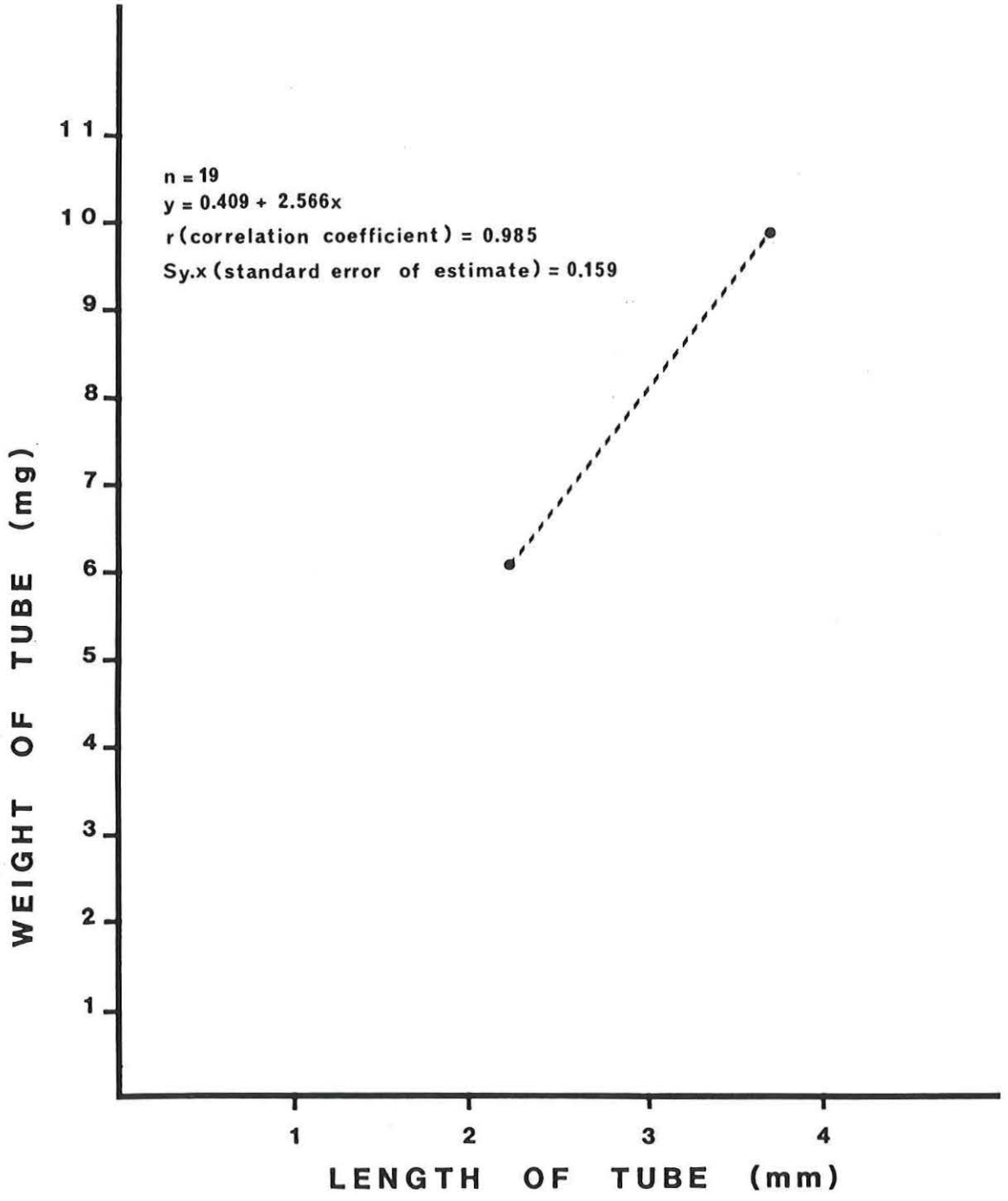


FIGURE 33: Quantitation of assay data.
Correlation between length and weight of tube (Trial #3).

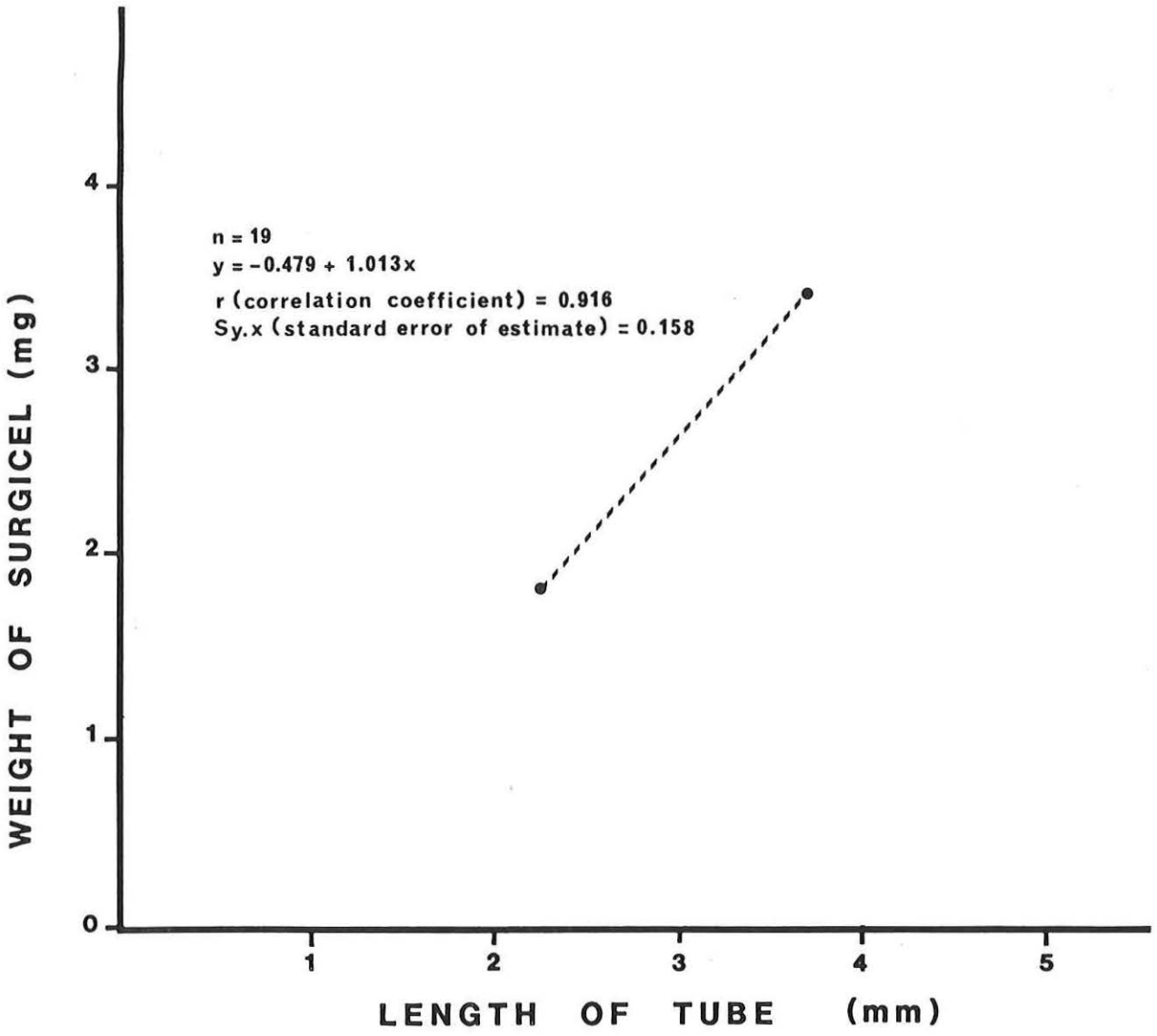


FIGURE 34: Quantitation of assay data.
Correlation between tube length and Surgicel weight (Trial #3).

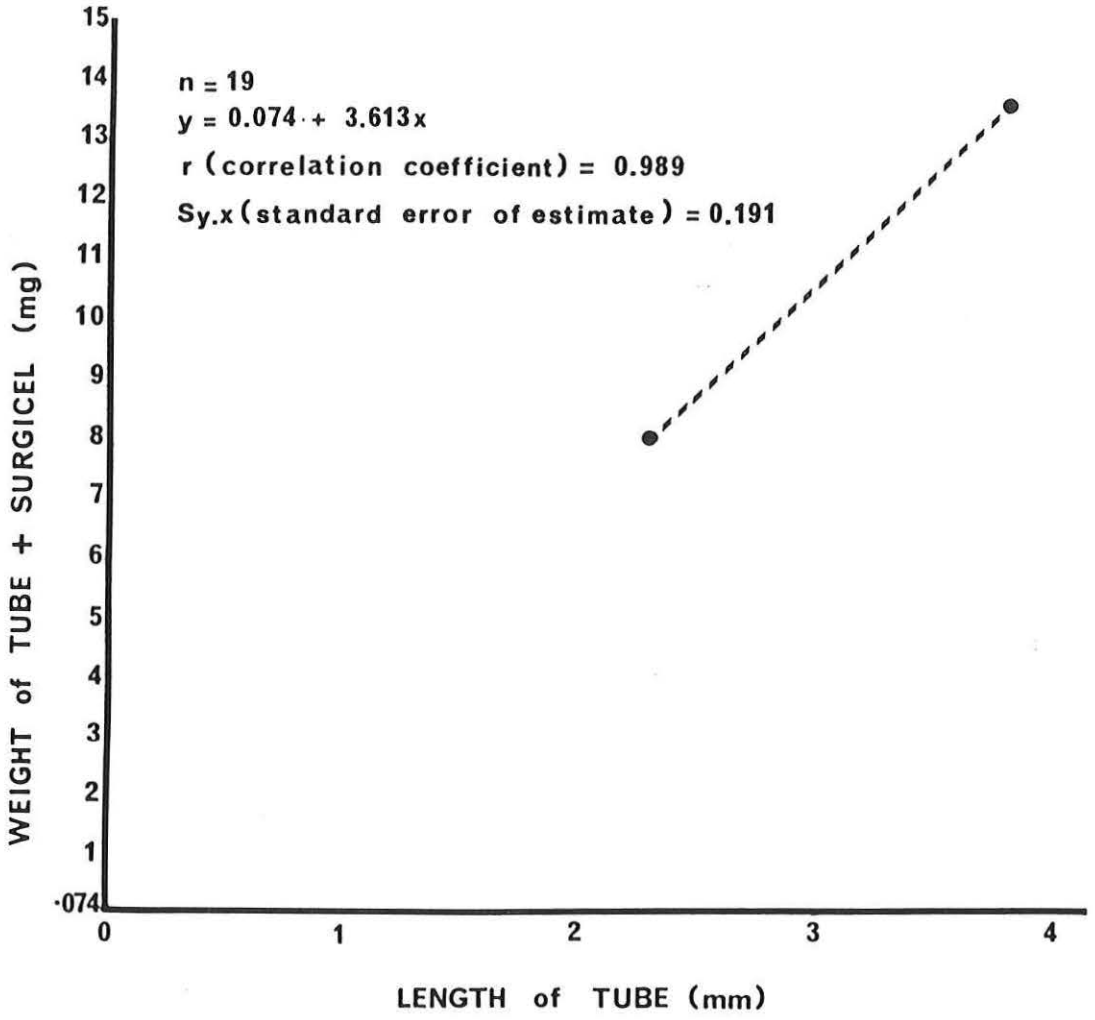


FIGURE 35: Quantitation of assay data.
Correlation between tube length and weight of tube containing Surgicel (Trial #3).

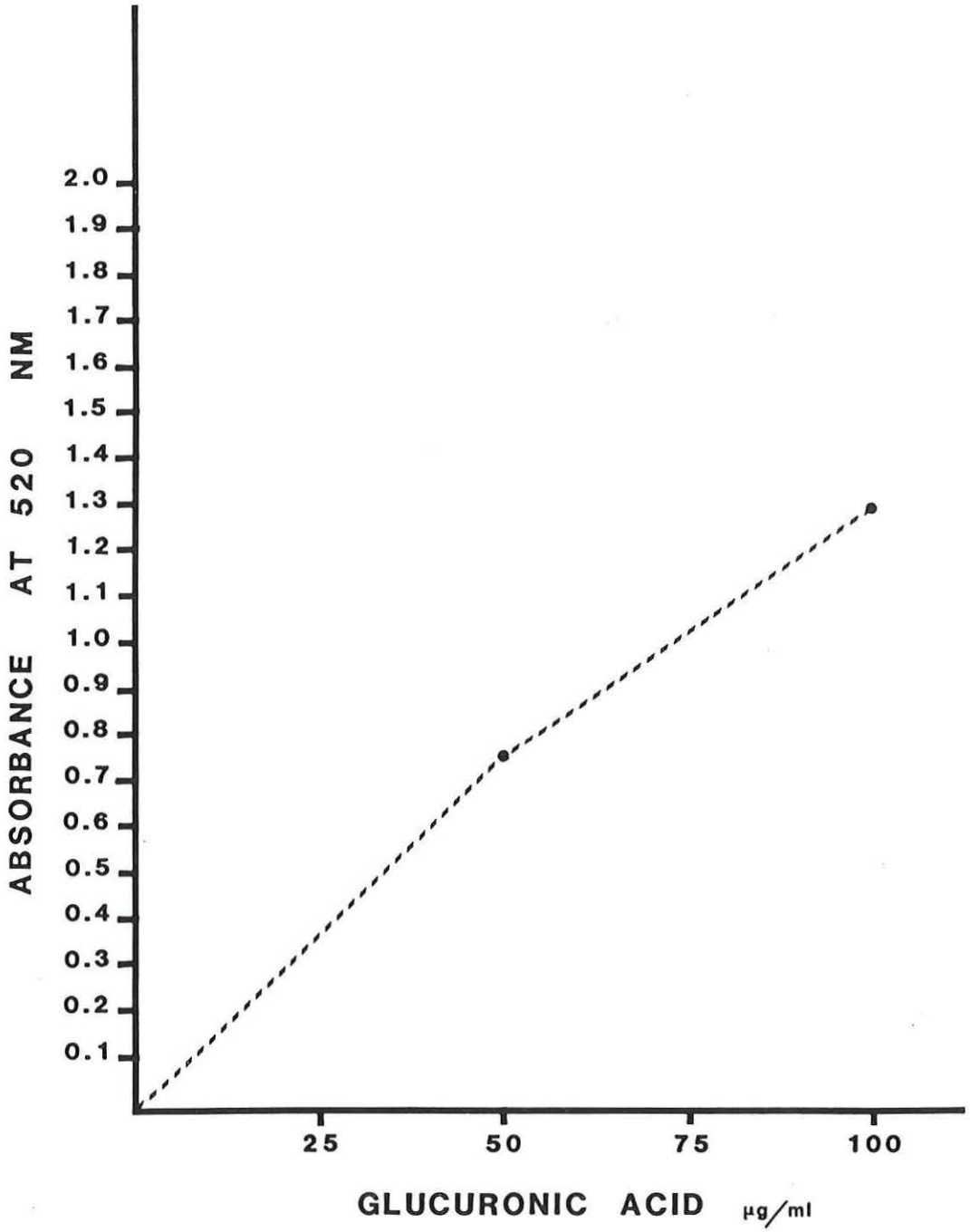


FIGURE 36: Fifth uronic acid assay.
Absorbances for pure glucuronic acid standards.

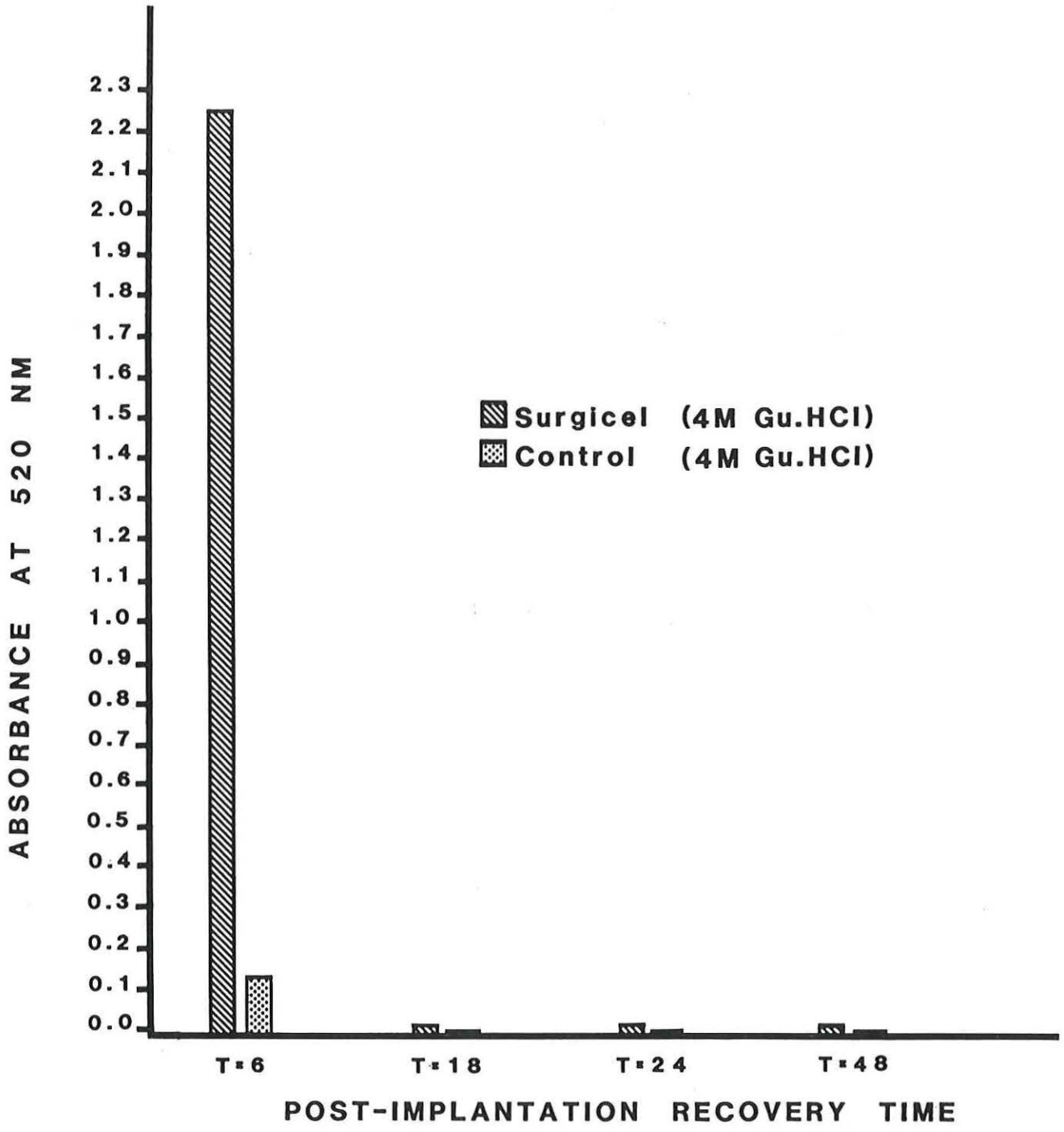


FIGURE 37: Fifth uronic acid assay.
Absorbances for Surgicel and control implants at 6, 18, 24 and 48 hours post-implantation.

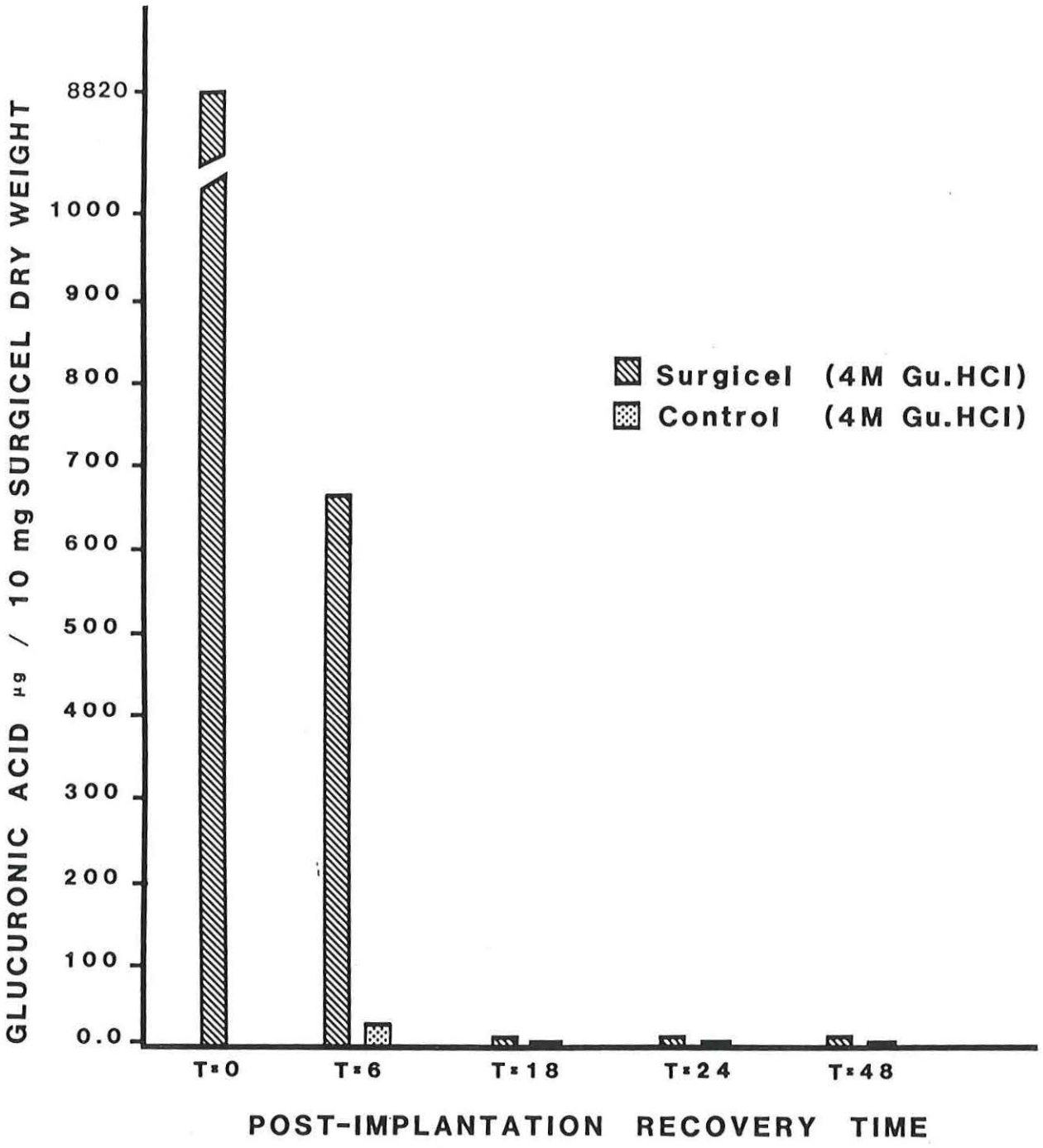


FIGURE 38: Fifth uronic acid assay.
Quantitated data, indicating the amount of uronic acid remaining (for a standard 10 mg dry weight of Surgicel implanted) after recovery at 6, 18, 24 and 48 hours post-implantation. T = 0 values were calculated from earlier chemical characterization of Surgicel (see Chapter 4, Section 1).

3. HISTOLOGY : SURGICEL

3.1 Optical Microscopy

3.1.1 Haematoxylin and Eosin

The appearance of sections stained with haematoxylin and eosin at the different recovery periods was as follows:

CONTROL SECTIONS

6 Hours : Figure 39 shows the appearance of the control sample at 6 hours post-implantation. The tube lumen appeared empty except for a small amount of fibrin deposition. Occasional perivascular concentrations of polymorphonuclear leukocytes (PMNs) were noted in the surrounding muscle (Figure 40).

18 Hours : At 18 hours, the tube lumen appeared empty (Figure 41). An inflammatory infiltrate, consisting of PMNs, plasma cells, lymphocytes and an occasional eosinophil, contained within an eosinophilic fibrinous network, surrounded the tube space (Figure 42). These cells, particularly PMNs, were also evident in perivascular concentrations within the surrounding muscle.

24 Hours : Figure 43 indicates the appearance of the control implant at 24 hours. Plasma cells and lymphocytes appeared more numerous and macrophages were also evident around the tube space (Figures 44 and 45). Perivascular concentrations also consisted of these cells. PMNs were present, but less numerous than at 18 hours.

48 Hours : At 48 hours, granulation tissue appeared to be growing into the tube lumen (Figure 46). Macrophages and plasma cells dominated the cellular infiltrate at either end of the tube (Figure 47).

SURGICEL SECTIONS

6 Hours : At 6 hours post-implantation, the tube lumen was filled with a deeply basophilic fibrillar material not observed in control sections (Figure 48). Eosinophilic, fibrinous material was also evident around the tube and PMNs were present in very small numbers, predominantly perivascularly.

18 Hours : The appearance of Surgicel at 18 hours is shown in Figure 49. The tube lumen contained an amorphous, weakly basophilic vacuolated coagulum (Figure 50) characterized by the presence of a cellular infiltrate, consisting predominantly of PMNs. These cells were also present around the tube, and perivascularly.

24 Hours : Figure 51 indicates the appearance of Surgicel at 24 hours post-implantation. The tube lumen was infiltrated by large numbers of PMNs and numerous extravasated erythrocytes (Figure 52). Macrophages were also noted at either end of the tube and within the lumen.

48 Hours : The appearance of the Surgicel implant at 48 hours post-implantation is shown in Figure 53. Ingrowing granulation tissue was detected at both ends of the tube and plasma cells, foamy macrophages and PMNs were evident (Figures 54, 55 and 56).

3.1.2 Staining Affinity of Alcian Blue for Surgicel *in vitro*

The appearance of Surgicel-spotted filter papers stained with Alcian Blue at various concentrations of $MgCl_2$ is demonstrated in Figure 57. Staining of the central Surgicel spot ceased after a critical electrolyte ($MgCl_2$) concentration (CEC) of 0.15M.

3.1.3 Alcian Blue

CONTROL SECTIONS

6 Hours : The appearance of an implanted empty tube after 6 hours, stained with Alcian Blue at the CEC is shown in Figure 58. No darkly staining material was evident.

18, 24 and 48 Hours : Control implants at these post-implantation times exhibited an appearance similar to that observed at 6 hours, after staining with Alcian Blue at the CEC (Figure 59).

SURGICEL SECTIONS

6 Hours : Differential staining of tube contents was noted in sections stained with Alcian Blue at the lower electrolyte ($MgCl_2$) concentrations. A densitometric scan (Figure 60) of sections stained at all $MgCl_2$ concentrations revealed that the CEC for Surgicel *in vitro* was 0.15M $MgCl_2$. Figures 61 and 62 illustrate the appearance of Surgicel fibres within the tube lumen at 6 hours, stained with Alcian Blue at the CEC.

18 Hours : At the CEC, no darkly-staining fibres were evident in the tube lumen, which was filled with a pale-staining fibrinous material.

24 Hours : At the CEC, the tube lumen was occupied by an "hour-glass" shaped ingrowth containing fibrinous material and cells. No Alcian Blue positive fibres were detected.

48 Hours : Figure 63 indicates the appearance of a Surgicel implant at the CEC. There was a tissue ingrowth into the tube lumen and a fibrinous deposition. No darkly-staining fibres were evident.

(Collated observations recorded for Alcian Blue experiments are summarized in Table 9).

	Magnesium chloride concentration (M)									
	0	.05	.1	.15	.2	.3	.55	.8	1.0	1.25
FILTER PAPER	++	++	++	+++	±	−	−	−	−	−
6 CONT	−		−	−	−	−	−	−	−	−
18 CONT	−		−	−	−	−	−	−	−	−
24 CONT	−		−	−	−	−	−	−	−	−
48 CONT	−		−	−	−	−	−	−	−	−
6 SURG	+++		+++	+++	+	±	±	±	−	−
18 SURG	−		−	−	−	−	−	−	−	−
24 SURG	−		−	−	−	−	−	−	−	−
48 SURG	−		−	−	−	−	−	−	−	−
6 SURG 4MG				−						
18 SURG 4MG				−						
24 SURG 4MG				−						
48 SURG 4MG				−						

TABLE 9 : Staining intensity of intraluminal contents/filter papers with Alcian Blue at different electrolyte ($MgCl_2$) concentrations.

CONT = Empty control tube
SURG = Surgicel tube
SURG 4MG = Fibrous residue tube

3.2 Transmission Electron Microscopy

CONTROL SECTIONS

6 Hours : Sections of the tube lumen at 6 hours post-implantation revealed the presence of cell debris and a fine fibrillar-granular material, having an appearance consistent with that of fibrin (Figure 64). Sections taken at the ends of the tube showed erythrocytes, collagen, fibroblasts and tissue debris (Figures 65 and 66).

18 Hours : Sections from the middle of the tube lumen appeared devoid of any cellular infiltrate. Fibrinous material and an occasional phagocytic cell were observed towards the end of the tube lumen (Figures 67 and 68).

24 Hours : Sections taken from several representative areas of the tube lumen at 24 hours revealed the presence of a fine, fibrillar-granular material (Figure 69). No cells were detected.

48 Hours : Macrophages, and, to a lesser extent, PMNs, were the only cells observed in the tube lumen at 48 hours (Figures 70 and 71). Macrophage phagosome contents are shown at a greater magnification in Figures 72 and 73.

SURGICEL SECTIONS

0 Hours : The ultrastructural appearance of native Surgicel, fixed and processed for T.E.M., is demonstrated in Figures 74-76.

6 Hours : Figures 77-80 demonstrate the appearance of Surgicel at 6 hours. The material exhibited a morphology similar to that observed at 0 hours. However, at this later post-implantation time, small fragments of the material appeared to be lying free from the main structure, resulting in a "moth-eaten" appearance (Figures 79 and 80).

18 Hours : Phagocytes (predominantly PMNs) and an extracellular fibrillar material were the predominant features of the tube lumen at 18 hours post-implantation (Figures 81-84). PMN phagosome contents, at higher magnifications (Figures 85 and 86), appeared amorphous, rather than fibrillar in nature (compare with Figures 79 and 80). Pale inclusions, faintly reminiscent of Surgicel (see Figure 76), were present in the cytoplasm of these cells. Such areas did not appear to be membrane-enclosed.

24 Hours : At this time period, macrophages, containing abundant phagocytic vacuoles, predominated in a fibrillar, extracellular matrix (Figure 87). Detailed examination of phagolysosome contents (Figures 88 and 89) revealed a Surgicel-like material which was also evident extracellularly (Figure 90).

48 Hours : Macrophages and PMNs were evident in a fibrinous extracellular environment at 48 hours (Figures 91 and 92). Macrophages were characterized by large phagocytic vacuoles, containing Surgicel-like material (Figures 93-96). Myelin bodies were frequently observed in phagolysosomes. Similar structures were not demonstrable in PMNs.

(Observations recorded for T.E.M. sections are collated in Table 10).

	Presence of Macrophages	Presence of PMNs	Intracellular Surgicel-like material: Macrophages	Intracellular Surgicel-like material: PMNs	Extracellular Surgicel-like material
0 CONT	–	–	–	–	–
18 CONT	+	–	–	–	–
24 CONT	–	–	–	–	–
48 CONT	++	+	–	–	–
0 SURG	–	–	–	–	+++
6 SURG	–	–	–	–	+++
18 SURG	–	++	–	–	+
24 SURG	++	+	++	±	+
48 SURG	++	+	+++	–	–
0 SURG 4MG	–	–	–	–	+++
6 SURG 4MG	–	–	–	–	+++
18 SURG 4MG					
24 SURG 4MG	++	+	++	–	–
48 SURG 4MG	++	+	+++	–	–

TABLE 10 : Characteristics of Transmission Electron Microscope sections.

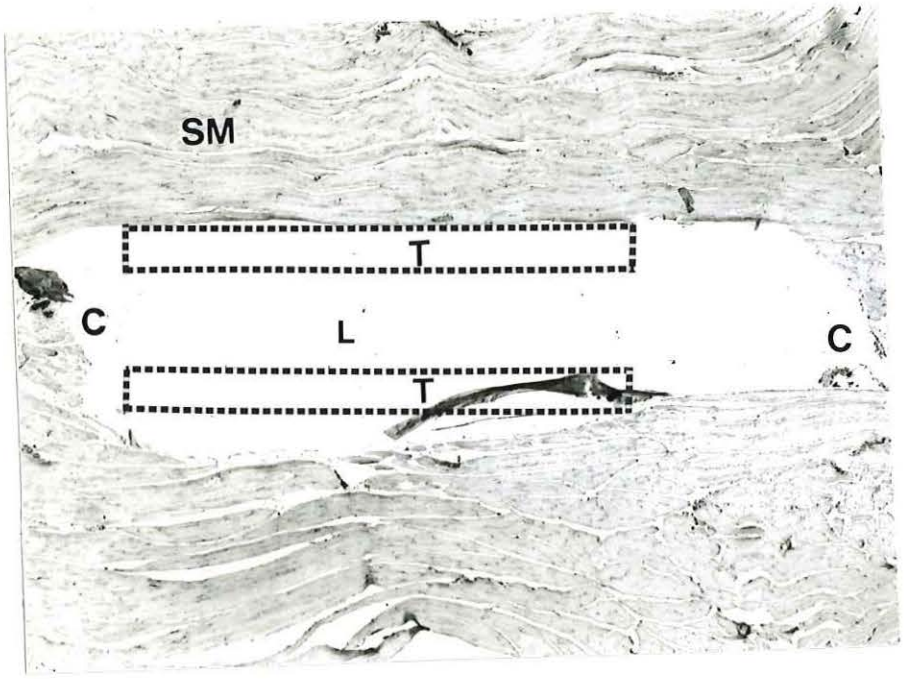
CONT = Control tube
SURG = Surgicel tube
SURG 4MG = Fibrous residue tube

FIGURE 39: Control implant T = 6.
Photomicrograph showing the surgically created crevice (C) in striated muscle (SM). T represents artefact space due to loss of the Teflon tube during sectioning. The tube lumen (L) appears empty, except for a small amount of fibrin deposition.
(H & E, original magnification x 20).

Note: throughout this thesis the following conventions are used:

(1) H & E - haematoxylin and eosin
(2) Magnifications refer to original microscopic magnifications

FIGURE 40: Control implant T = 6.
Photomicrograph showing striated muscle (SM) near the Teflon implant. Polymorphonuclear leukocytes (PMNs) are evident.
(H & E x 400).


SM
T
C
L
C
T

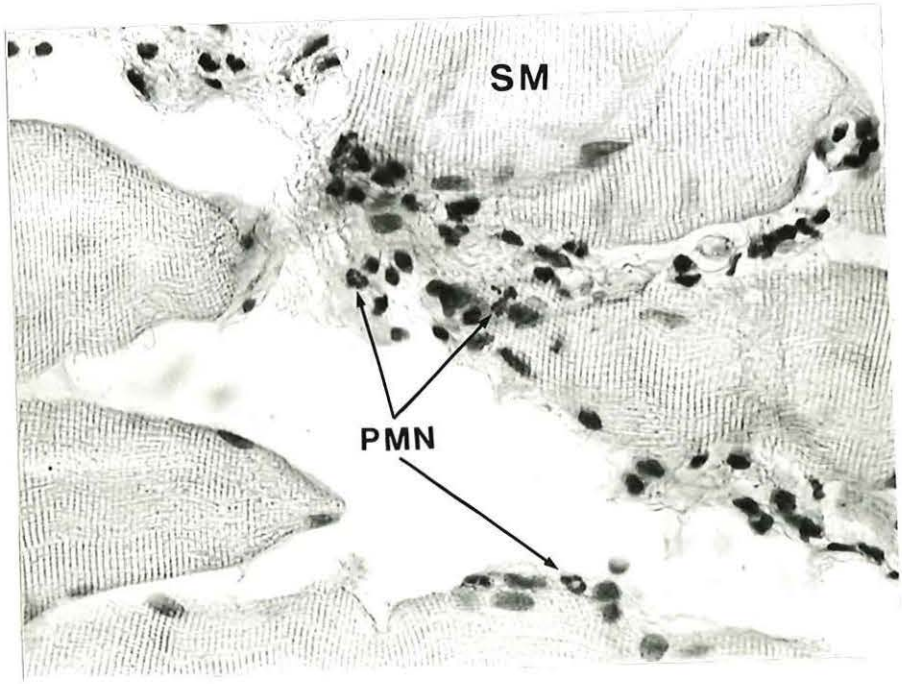
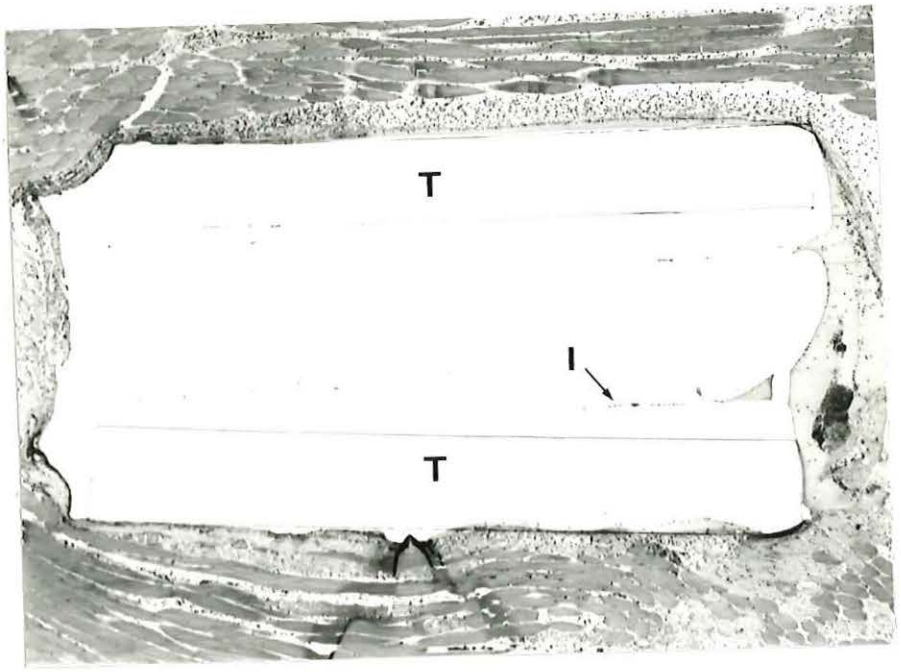

SM
PMN

FIGURE 41: Control implant T = 18.
Photomicrograph illustrating the relationship between the tube (T) and surrounding tissues. Note early ingress (I) of inflammatory cells into the tube lumen.
(H & E x 28).

FIGURE 42: Detail of Figure 41.
A mixed inflammatory infiltrate appears interposed between the tube wall (T) and muscle fibres (M).
(H & E x 312).


T
I
T

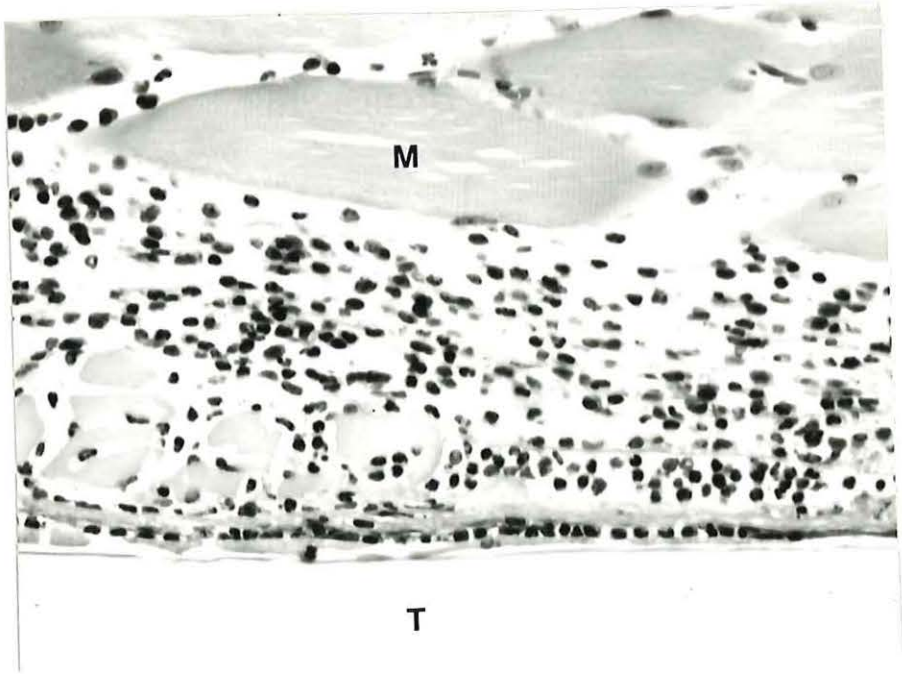
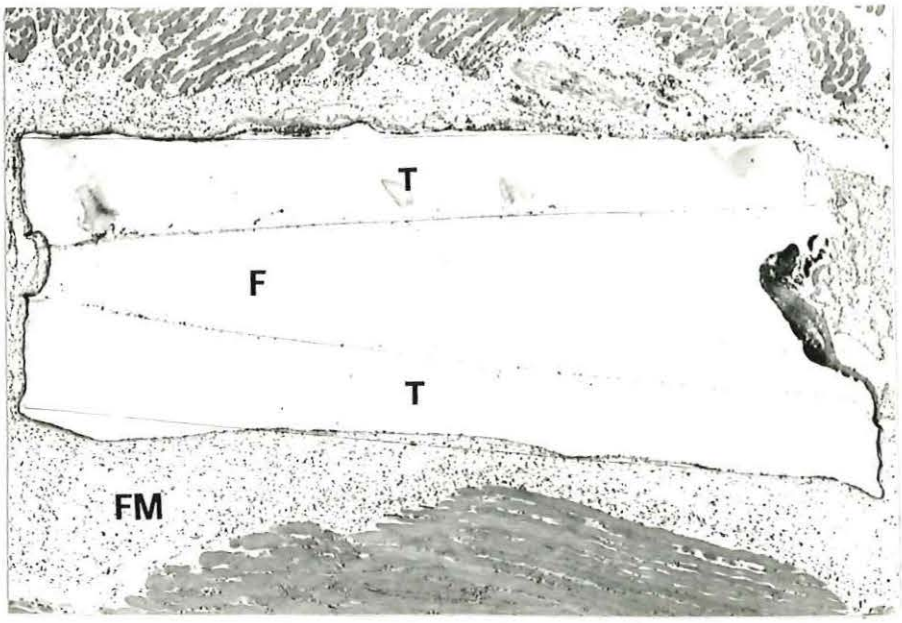

M
T

FIGURE 43: Control implant T = 24.
Photomicrograph showing fibrin (F) deposition in the tube lumen. The tube (T) appears to be surrounded by an oedematous fibrillar matrix (FM).
(H & E x 28).

FIGURE 44: Detail of Figure 43.
Chronic inflammatory cells predominate in the oedematous fibrillar matrix at the end of the tube.
(H & E x 312).


T
F
T
FM

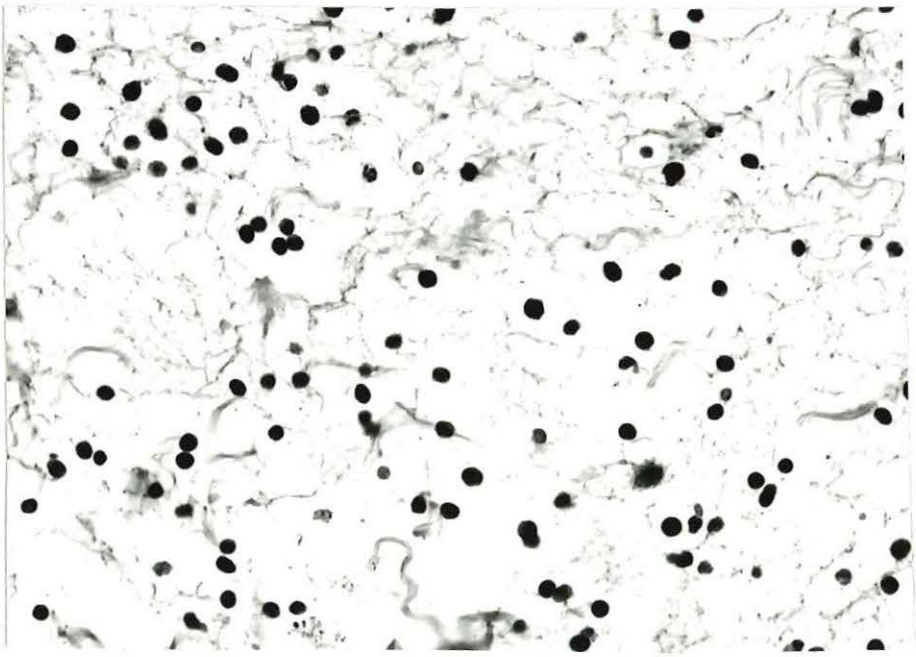


FIGURE 45: Detail of Figure 43.
Chronic inflammatory cells in an organizing fibrillar matrix.
(H & E x 268).

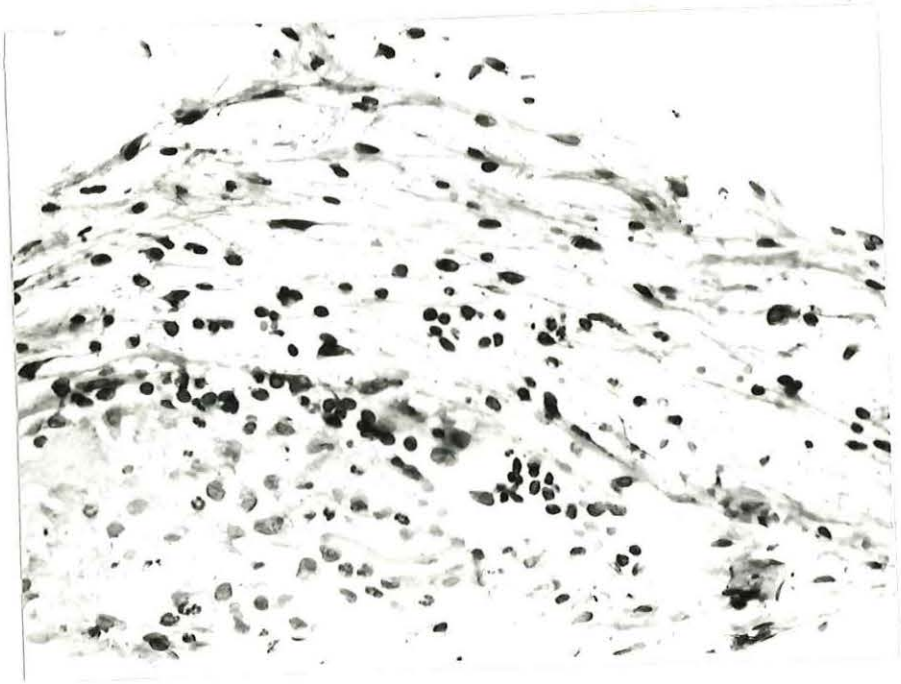
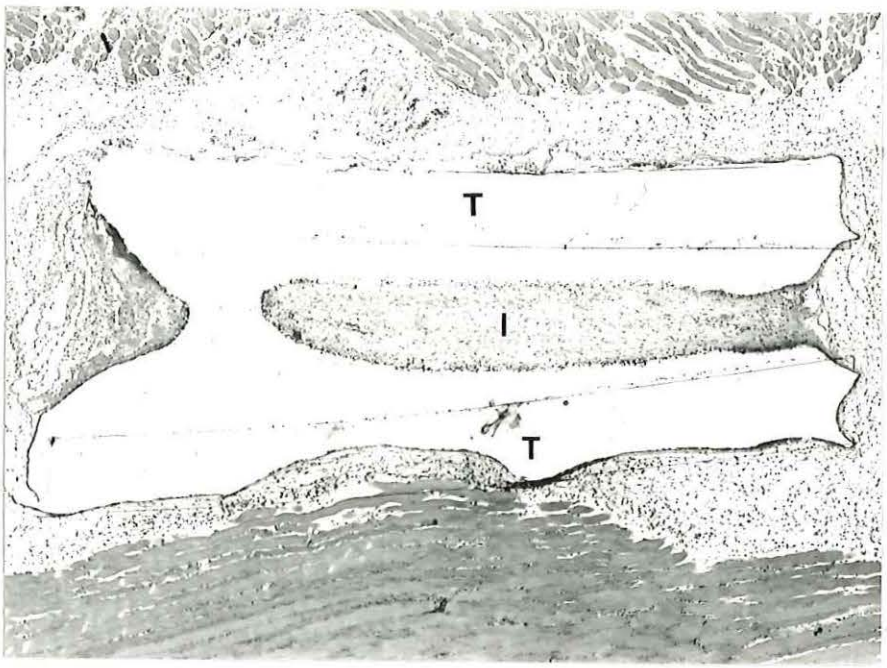


FIGURE 46: Control implant T = 48.
Photomicrograph showing tissue ingrowth (I) into the tube lumen. T indicates tube walls.
(H & E x 26).

FIGURE 47: Detail of Figure 46.
Foamy macrophages (M) and plasma cells (P) dominate the cellular infiltrate at either end of the tube.
(H & E x 440).


T
I
T

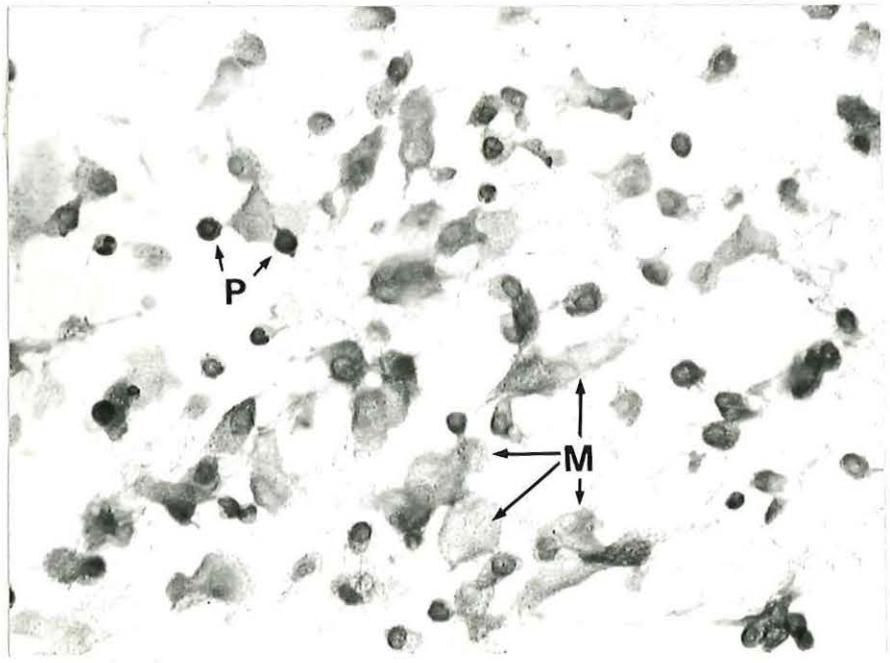

P
M

FIGURE 48: Surgicel implant T = 6.
Photomicrograph showing basophilic Surgicel fibres (S) contained within Teflon tubing (T). ES indicates extraluminal Surgicel caused by sectioning artefact. M indicates surrounding muscle.
(H & E x 48).

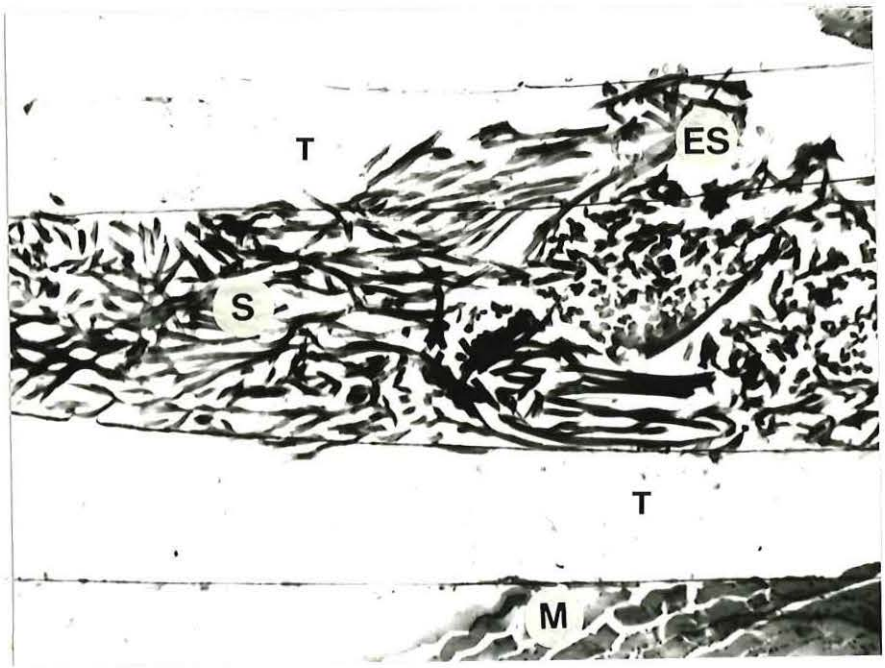

T
ES
S
T
M

FIGURE 49: Surgicel implant T = 18.
Photomicrograph showing cellular infiltration of the tube lumen. The tube (T) has been tangentially sectioned.
(H & E x 26)

FIGURE 50: Detail of luminal contents in Figure 49.
Inflammatory cells (mainly PMNs) predominate in an amorphous, weakly basophilic vacuolated coagulum.
(H & E x 480).

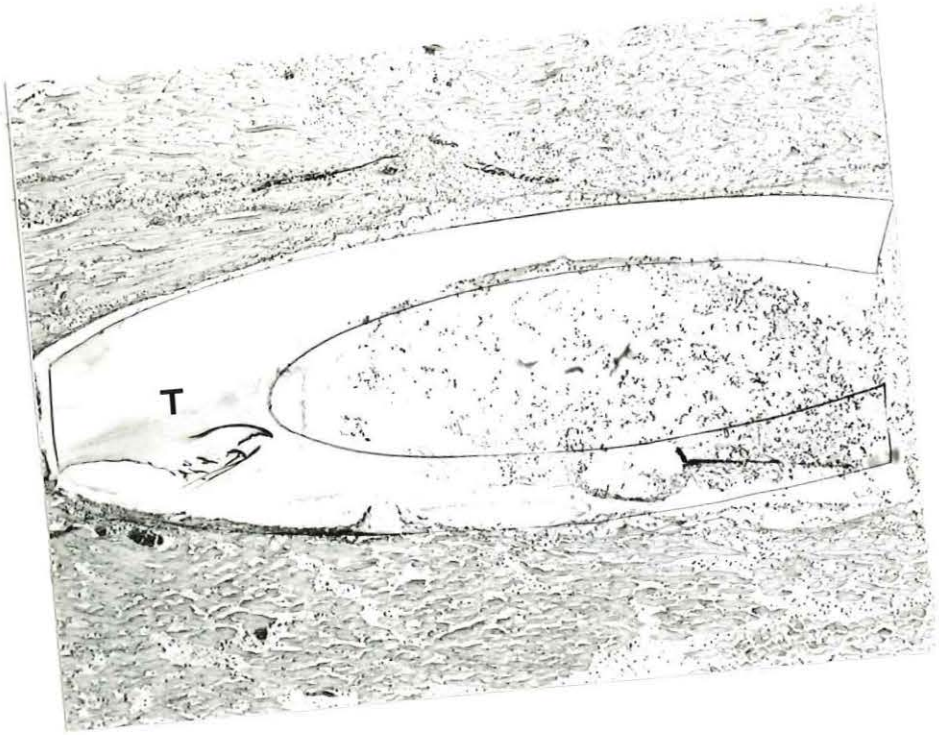
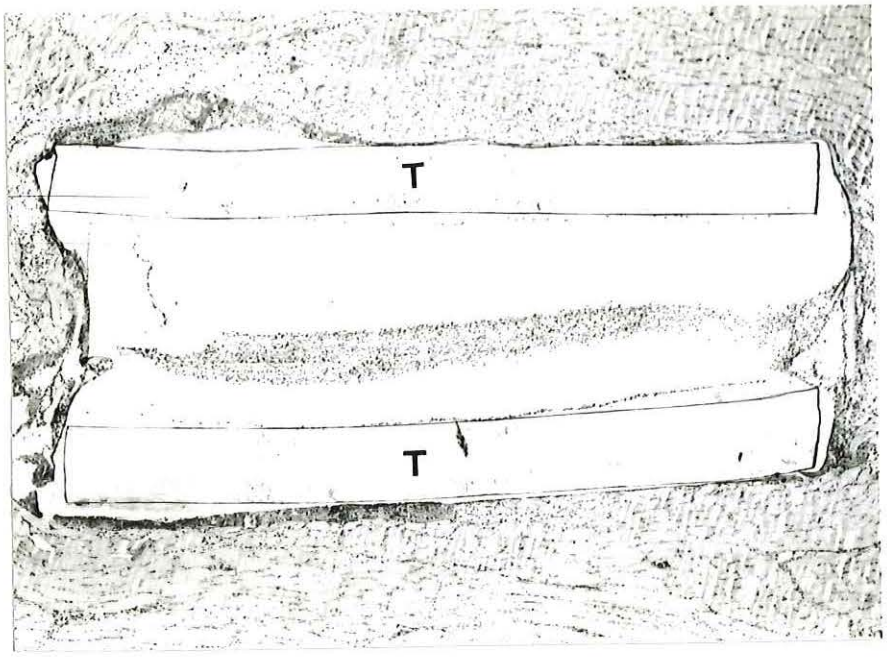

T

FIGURE 51: Surgicel implant T = 24.
Photomicrograph showing cellular invasion of the tube (T) lumen.
(H & E x 25).

FIGURE 52: Detail of intraluminal contents in Figure 51.
PMNs and extravasated erythrocytes (E) comprise the inflammatory infiltrate.
(H & E x 240).


T
T

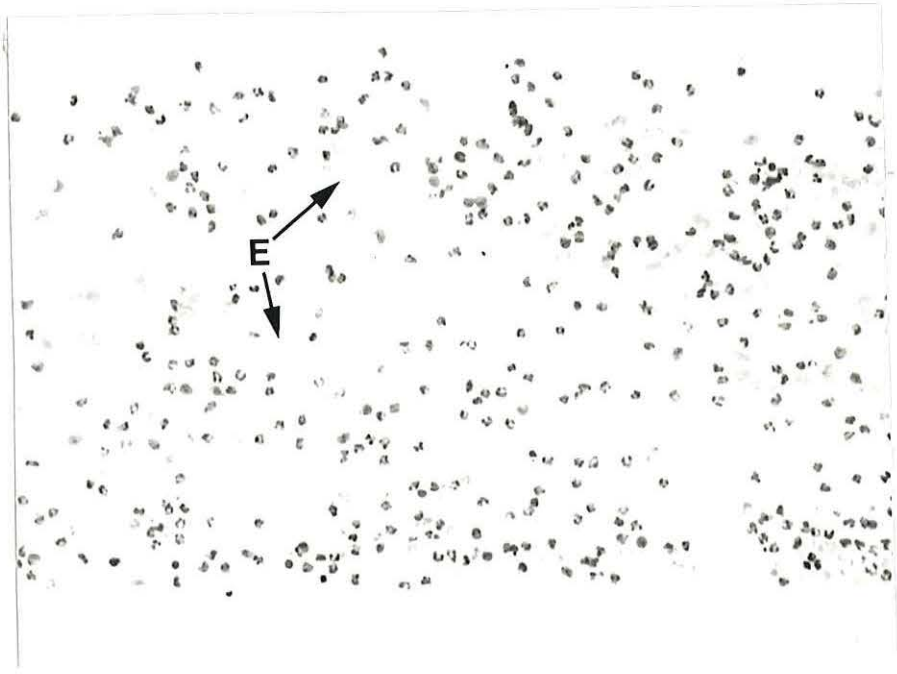
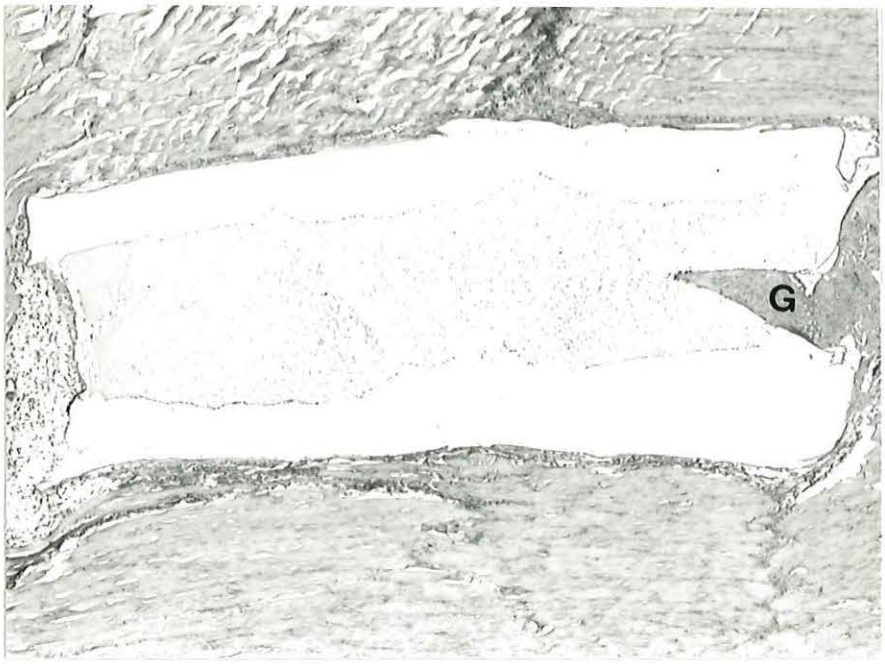
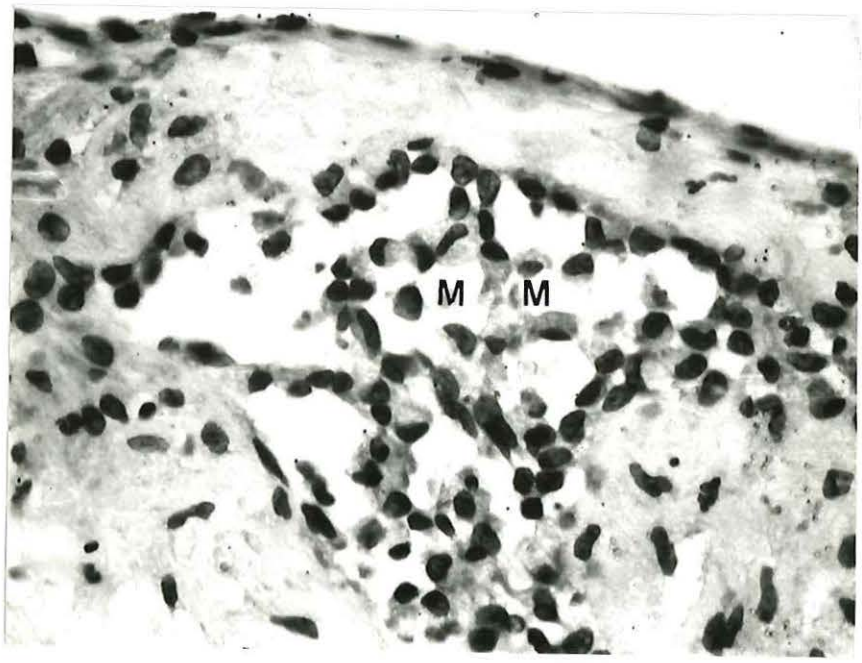

E

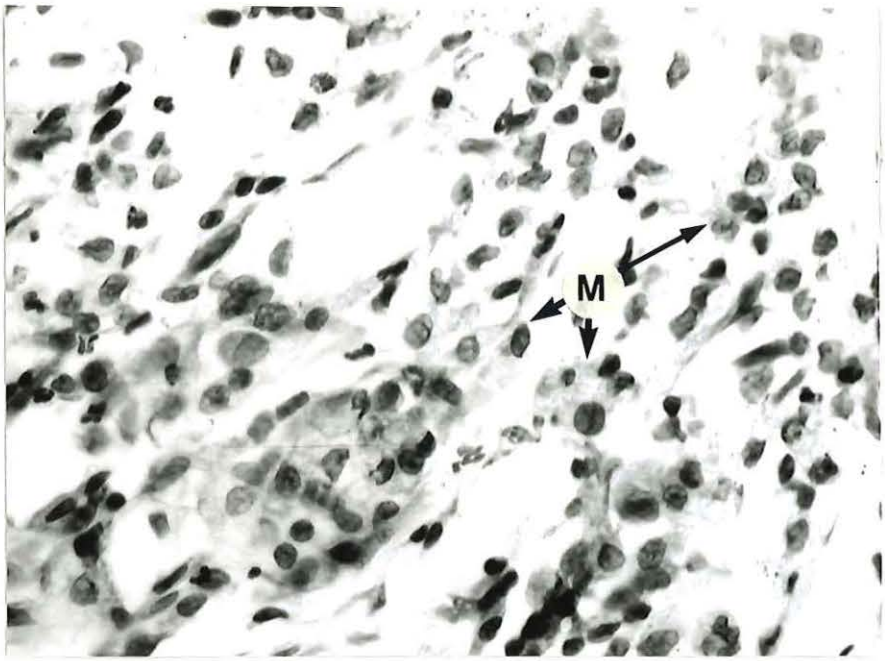
FIGURE 53: Surgicel implant T = 48.
Granulation tissue (G) appears to be growing into the tube lumen.
(H & E x 25).

FIGURE 54: Detail of Figure 53.
Foamy macrophages (M) are abundant in the granulation tissue growing into the tube lumen.
(H & E x 500).


G


M
M

FIGURES 55 and 56: Surgicel implant T = 48.
Detail of Figure 53. Large, foamy macrophages (M) are abundant in the oedematous matrix at the ends of the tube.
(H & E x 440).
(H & E x 480).


M

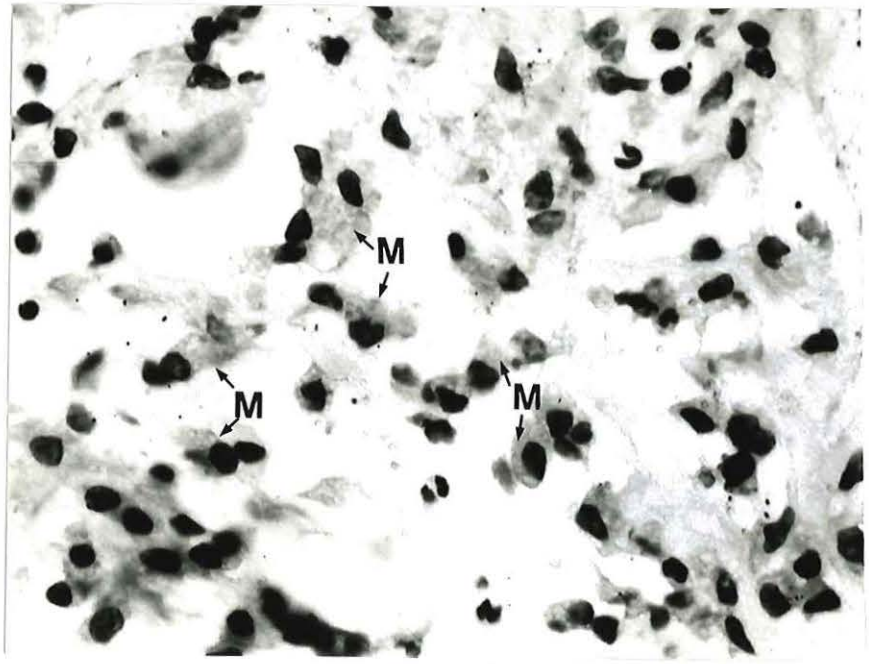

M
M
M

FIGURE 57: Alcian Blue-Filter Paper Experiment.
A differential in blue staining of the central Surgicel spot may be observed between papers stained at 0.1M and 0.2M $MgCl_2$.

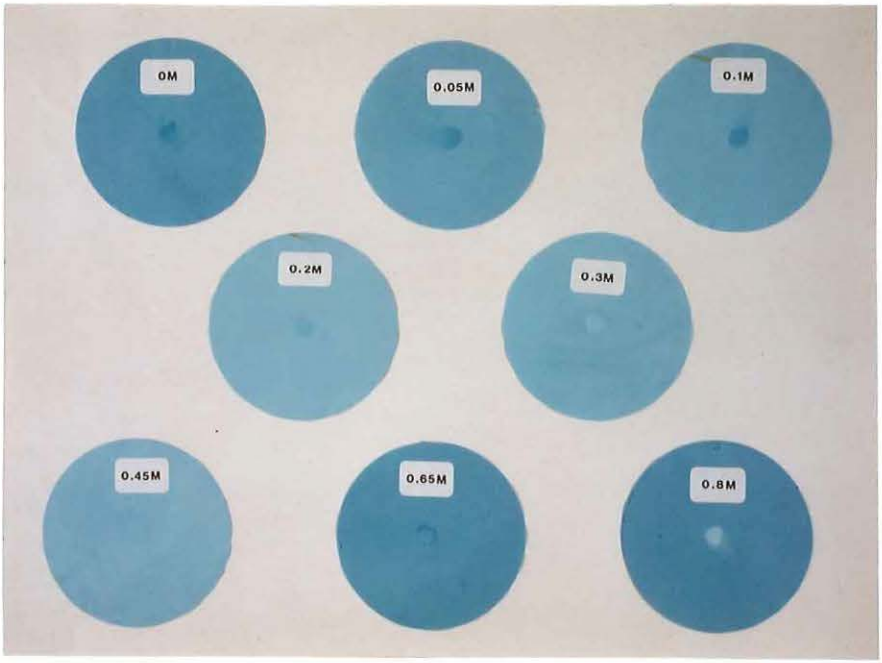
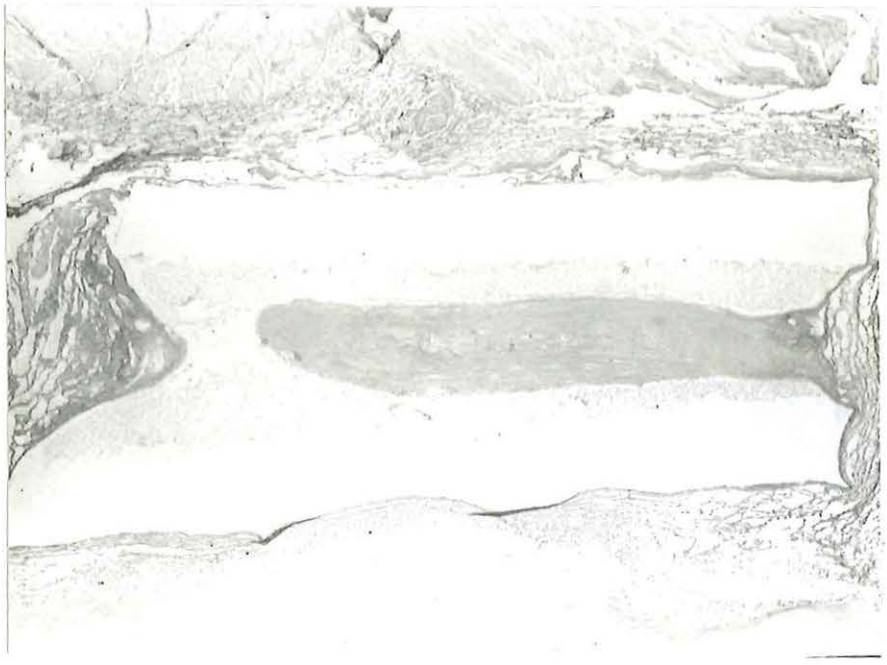

0M
0.05M
0.1M
0.2M
0.3M
0.45M
0.65M
0.8M

FIGURE 58: Control implant T = 6.
No deeply staining material is evident within the tube lumen at the Critical Electrolyte Concentration.
(Alcian Blue (0.15M $MgCl_2$) x 22).

FIGURE 59: Control implant T = 48.
No darkly staining material is evident.
(Alcian Blue (0.15M $MgCl_2$), x 26).



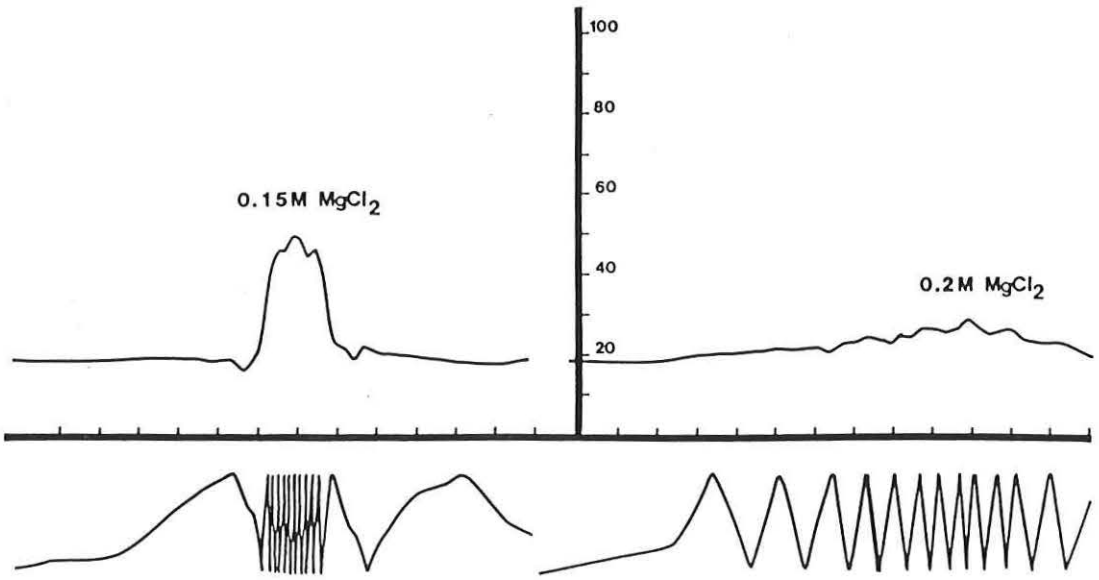


FIGURE 60: Densitometric scan of tube contents in 6 hour Surgicel specimens stained with Alcian Blue at 8 different $MgCl_2$ concentrations.
A strong staining differential is apparent between 0.15M $MgCl_2$ and 0.2M $MgCl_2$.

FIGURE 61: Surgicel implant T = 6.
Note the darkly staining Surgicel fibres (S) in contrast to the pale staining muscle. ES indicates extraluminal Surgicel caused by sectional artefact.
(Alcian Blue (0.15M $MgCl_2$) x 28).

FIGURE 62: Detail of Figure 61.
Darkly staining Surgicel fibres at the CEC.
(Alcian Blue (0.15M $MgCl_2$) x 400).

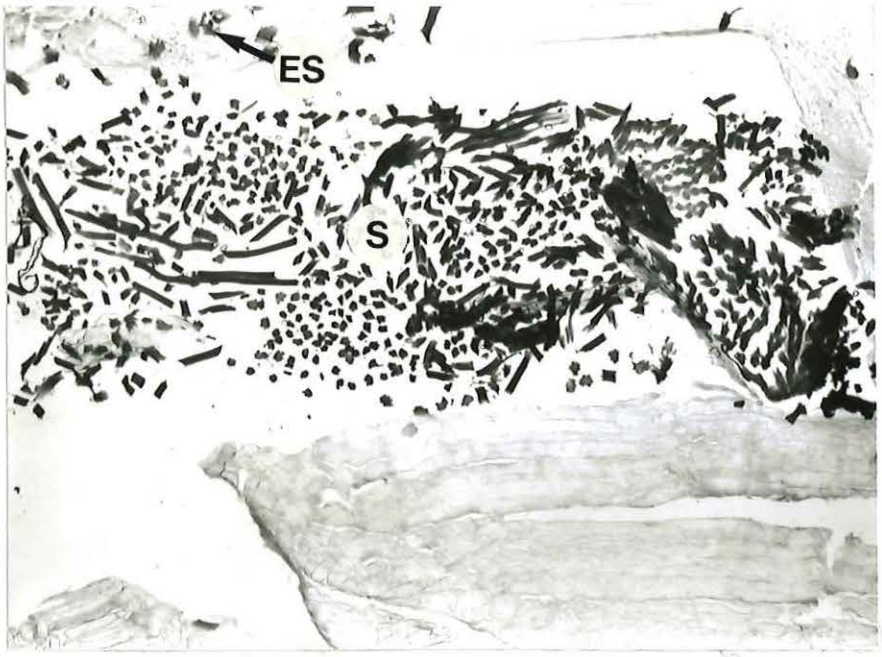
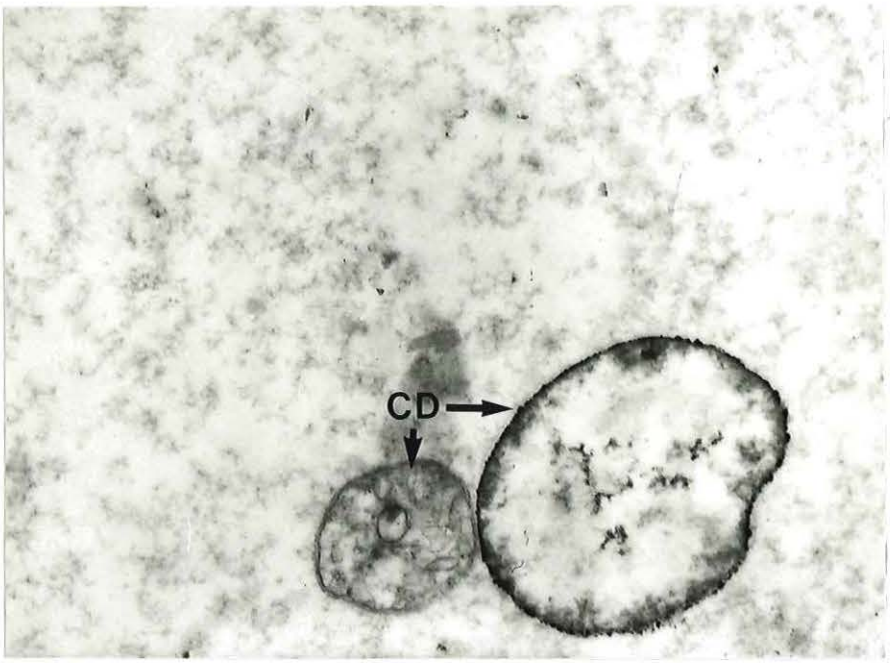

ES
S

FIGURE 63: Surgicel implant T = 48.
No darkly staining Surgicel fibres are evident at the CEC.
(Alcian Blue (0.15M $MgCl_2$) x 34).

FIGURE 64: Control implant T = 6.
Electronmicrograph showing fibrillar-granular material present in the tube lumen. CD indicates cell debris.
(TEM x 20,000).

FIGURE 65: Control implant T = 6.
Electronmicrograph showing fibroblast (F) erythrocytes (E) and tissue debris at the end of the tube.
(TEM x 5,000).


CD

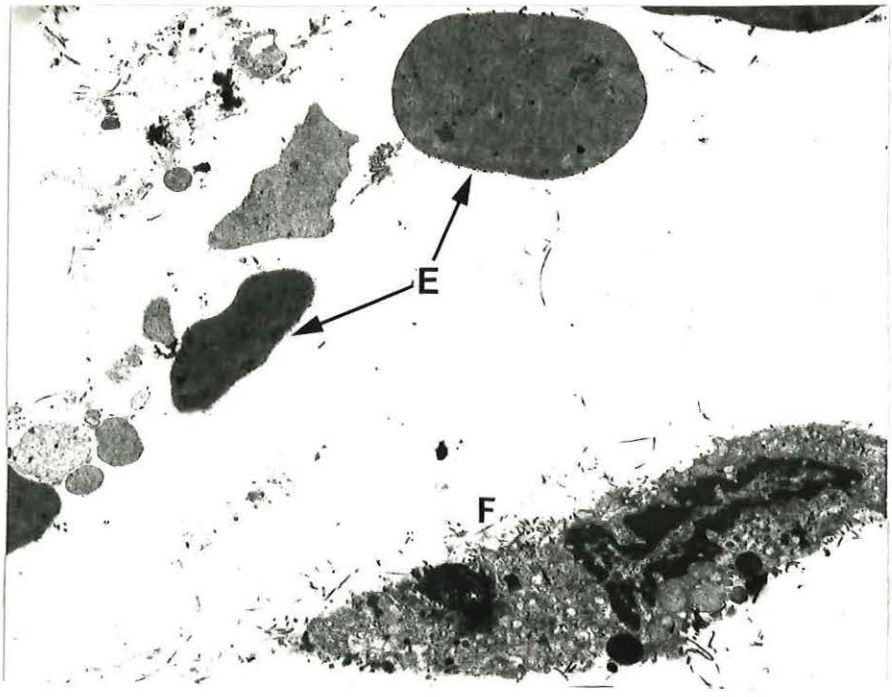

E
F

FIGURE 66: Control implant T = 6.
Electronmicrograph demonstrating erythrocytes (E) collagen (C) and tissue debris at the end of the tube.
(TEM x 30,000).

FIGURE 67: Control implant T = 18.
Electronmicrograph showing fibrinous material in the middle of the tube lumen.
(TEM x 20,000).

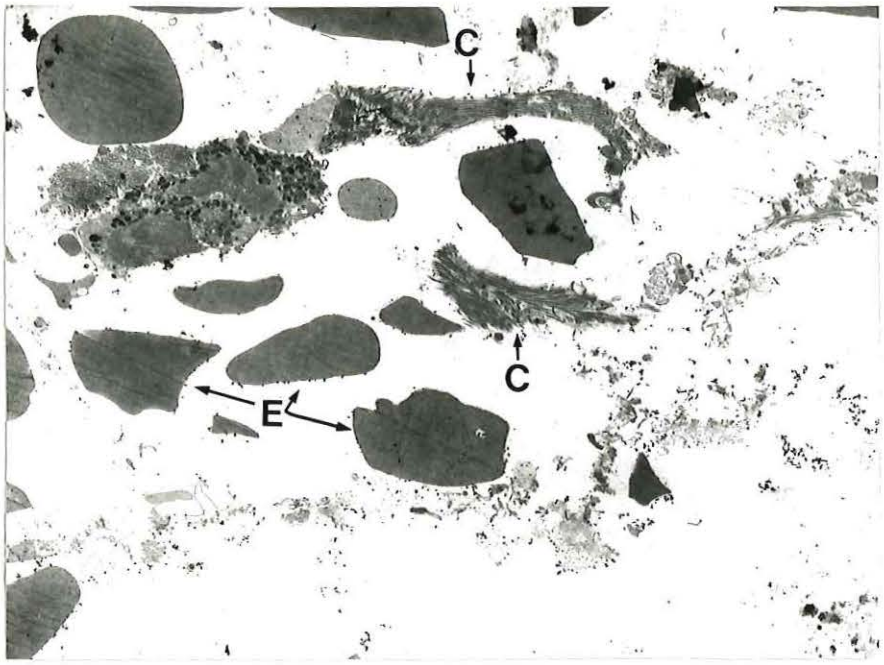
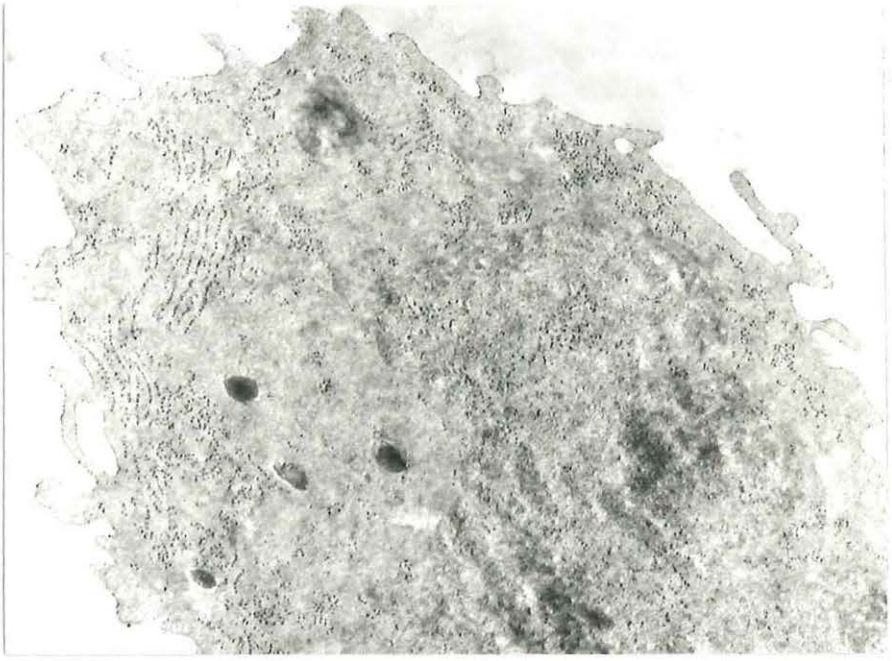

C
C
E

FIGURE 68: Control implant T = 18.
Electronmicrograph of a cell (phagocyte?) detected towards the end of the tube lumen.
(TEM x 15,000).

FIGURE 69: Control implant T = 24.
Electronmicrograph showing fine fibrillar-granular material in the tube lumen.
(TEM x 25,000).



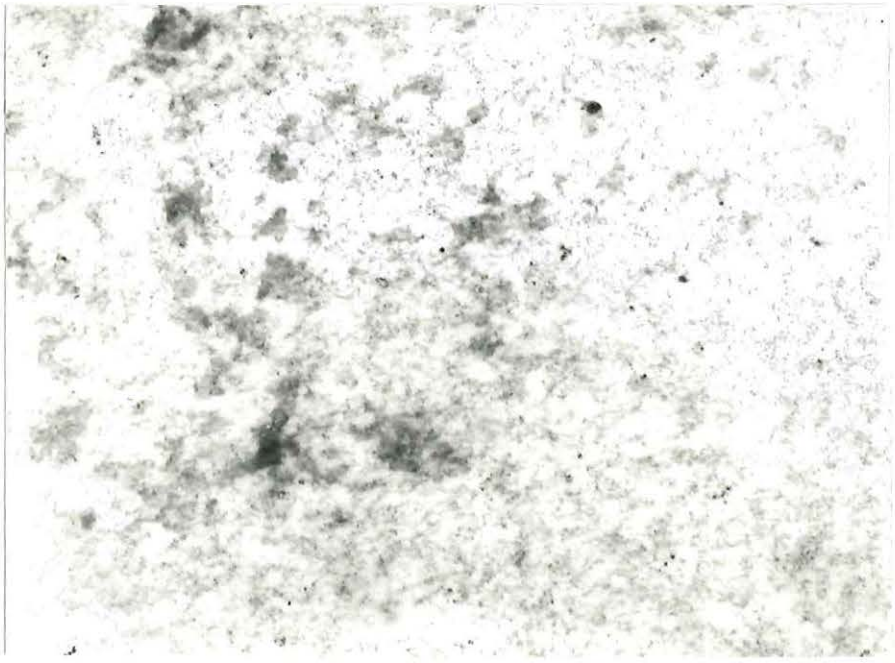
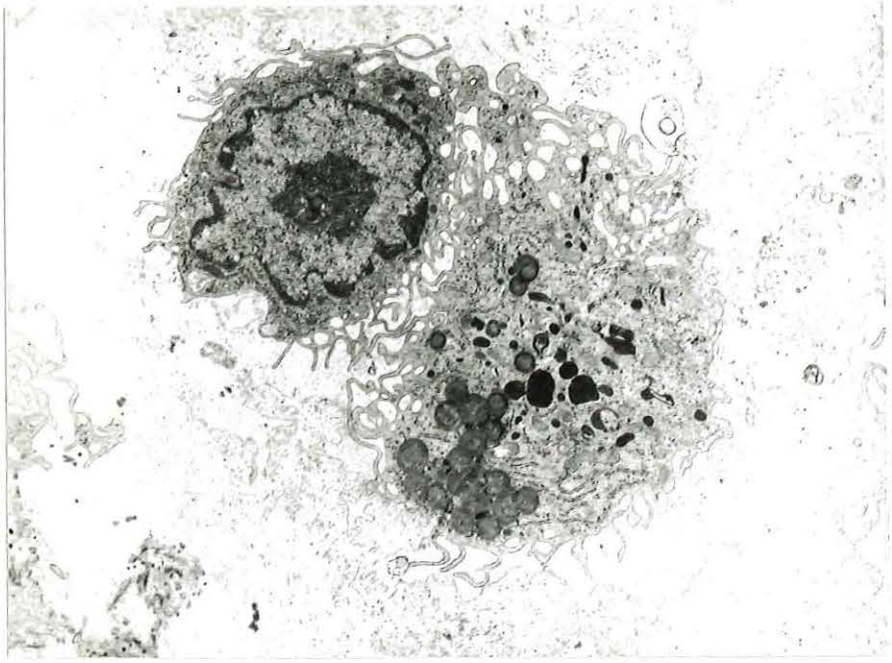


FIGURE 70: Control implant T = 48.
Electronmicrograph showing macrophages in tube lumen.
(TEM x 4,000).

FIGURE 71: Control implant T = 48.
Electronmicrograph of macrophage containing numerous small phagosomes.
(TEM x 8,000).



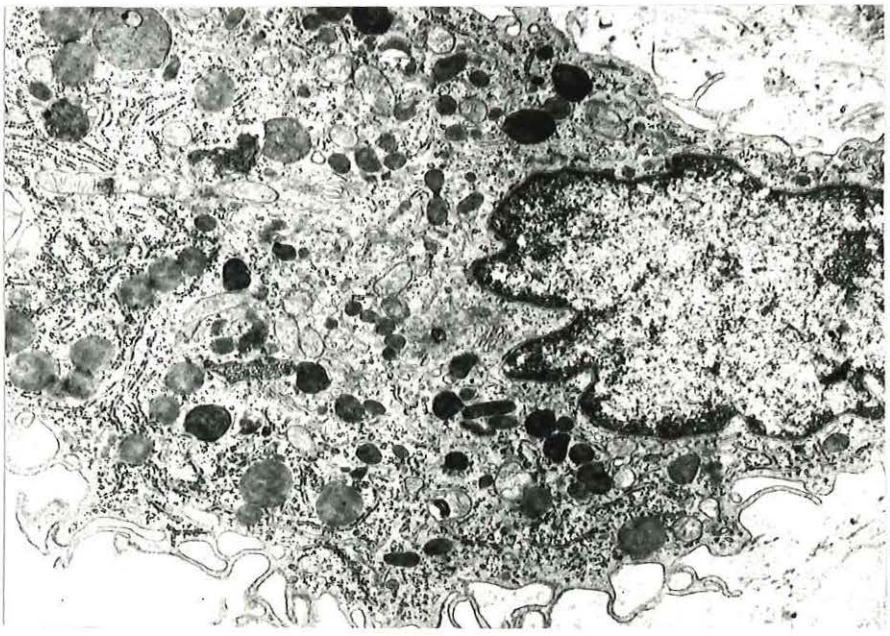
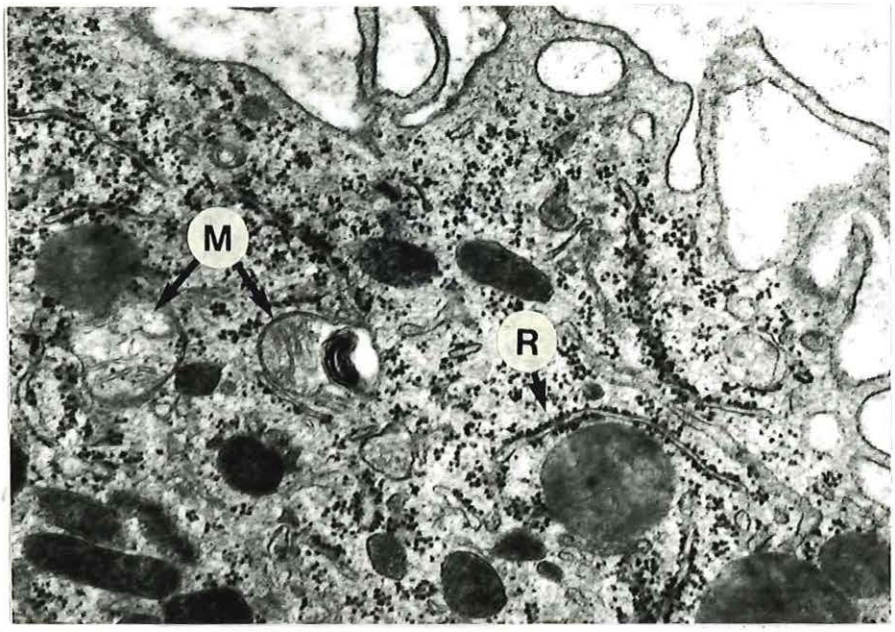


FIGURE 72: Detail of Figure 71, showing mitochondria (M) rough ER (R) and phagosome contents.
(TEM x 25,000).

FIGURE 73: Detail of Figure 71, showing phagosome contents and rough ER.
(TEM x 25,000).


M
R

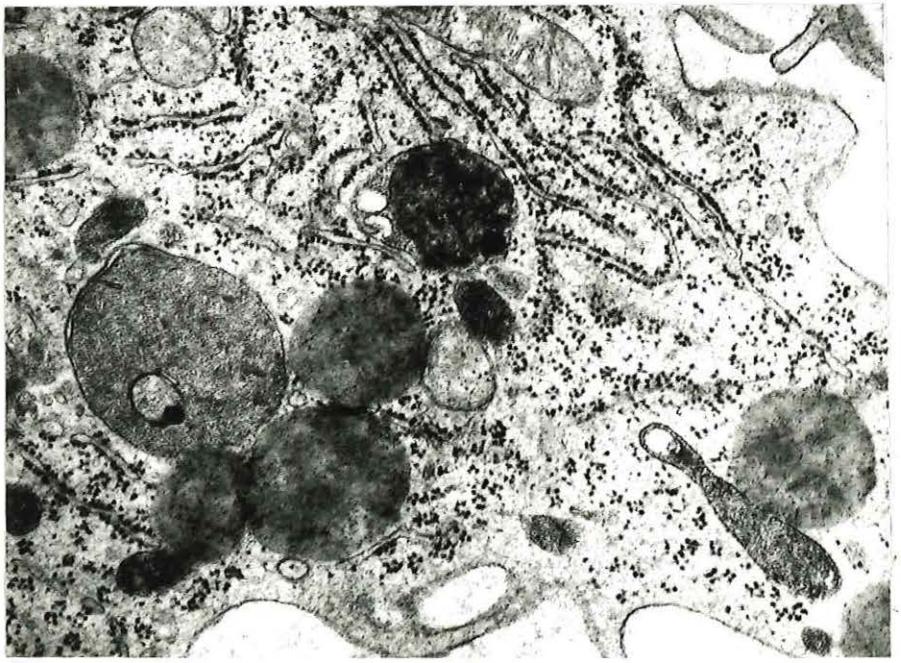
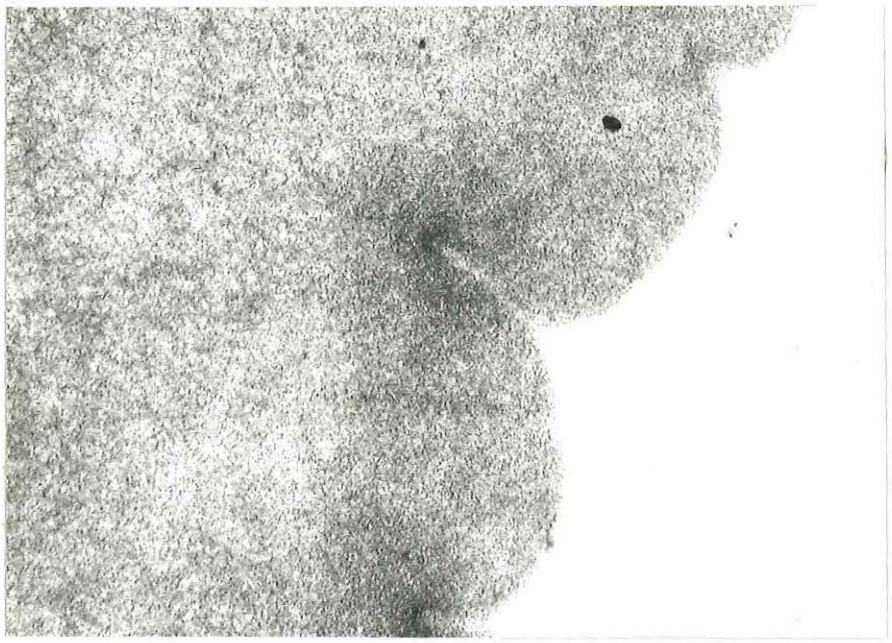


FIGURE 74: Surgicel T = 0.
Electronmicrograph showing the ultrastructural appearance of native Surgicel.
(TEM x 8,000).

FIGURE 75: Surgicel T = 0.
Electronmicrograph showing the ultrastructural appearance of native Surgicel at higher magnification.
(TEM x 10,000).



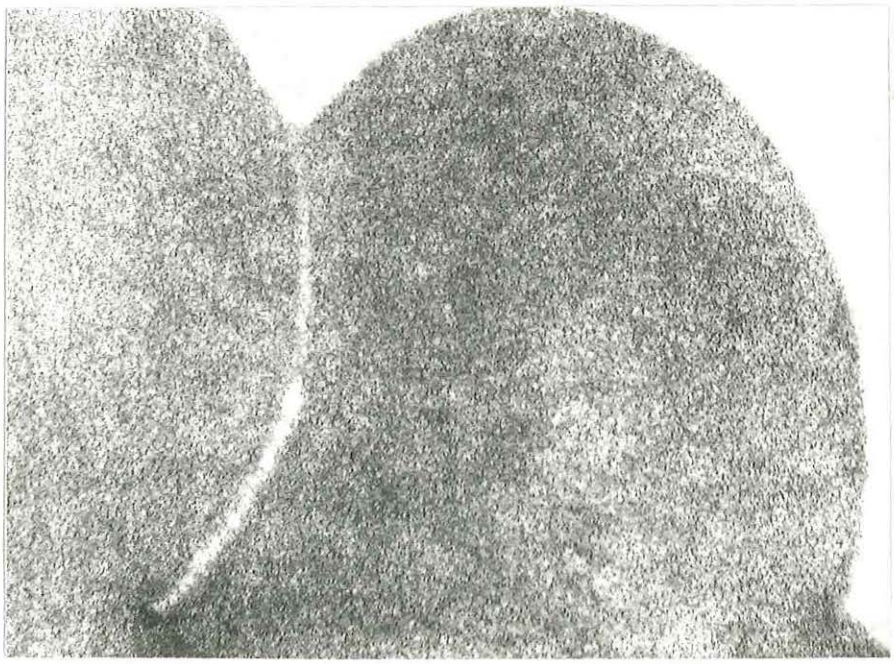
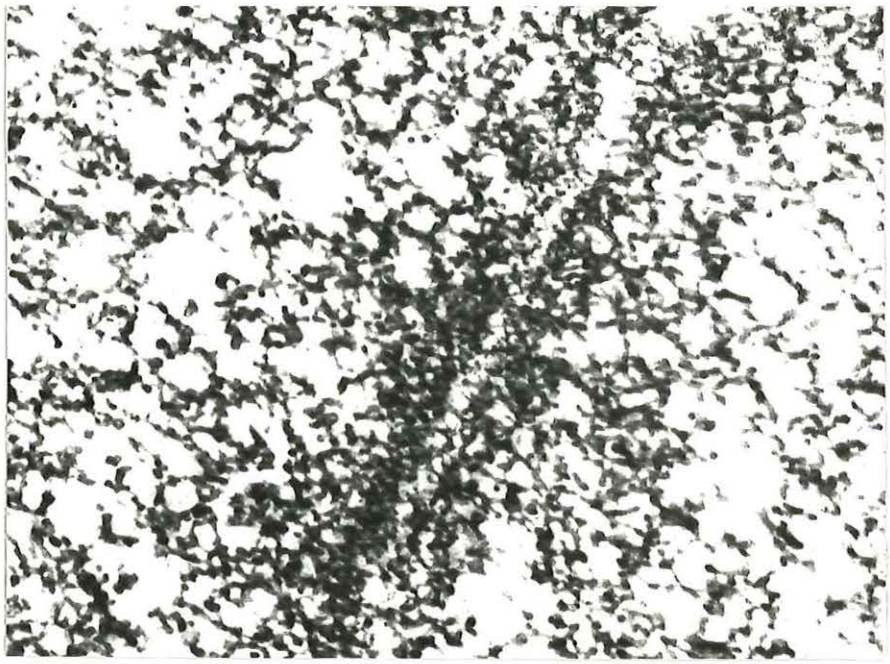
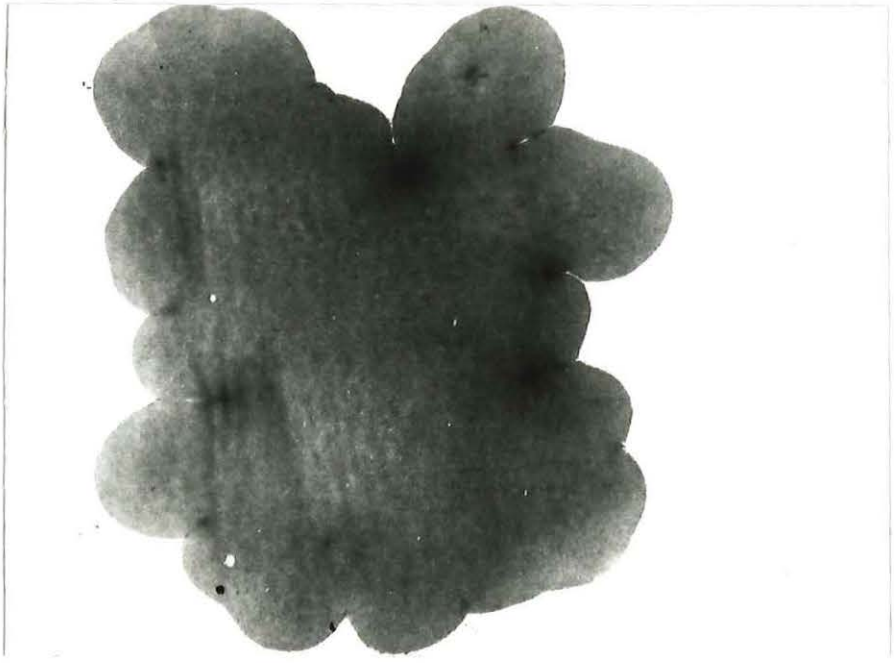


FIGURE 76: Surgicel T = 0.
Electronmicrograph showing higher magnification view of unimplanted Surgicel.
(TEM x 50,000).

FIGURE 77: Surgicel implant T = 6.
Electronmicrograph showing ultrastructural appearance of Surgicel in tube lumen. Note the lobulated structure.
(TEM x 3,000).





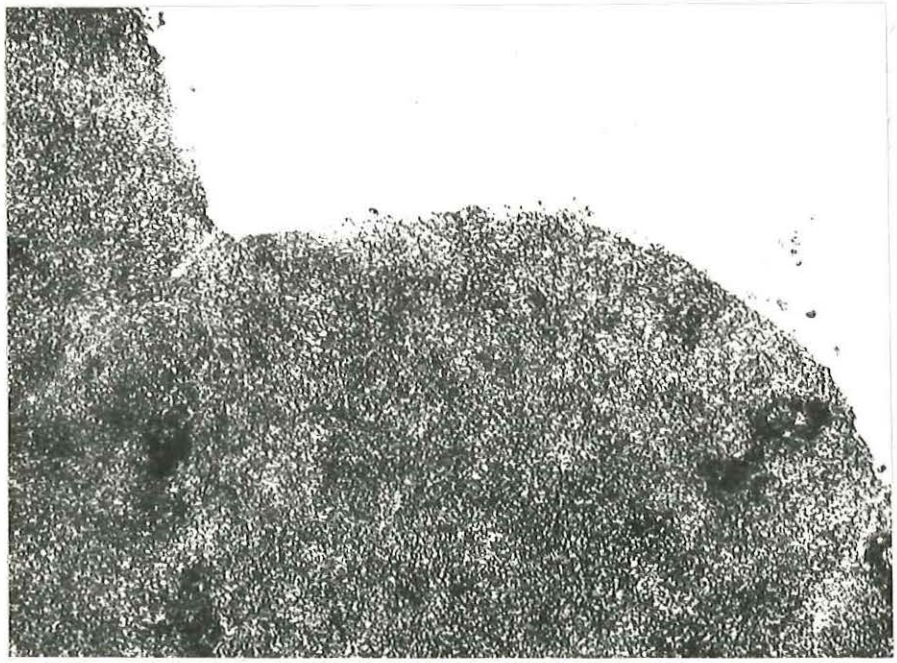
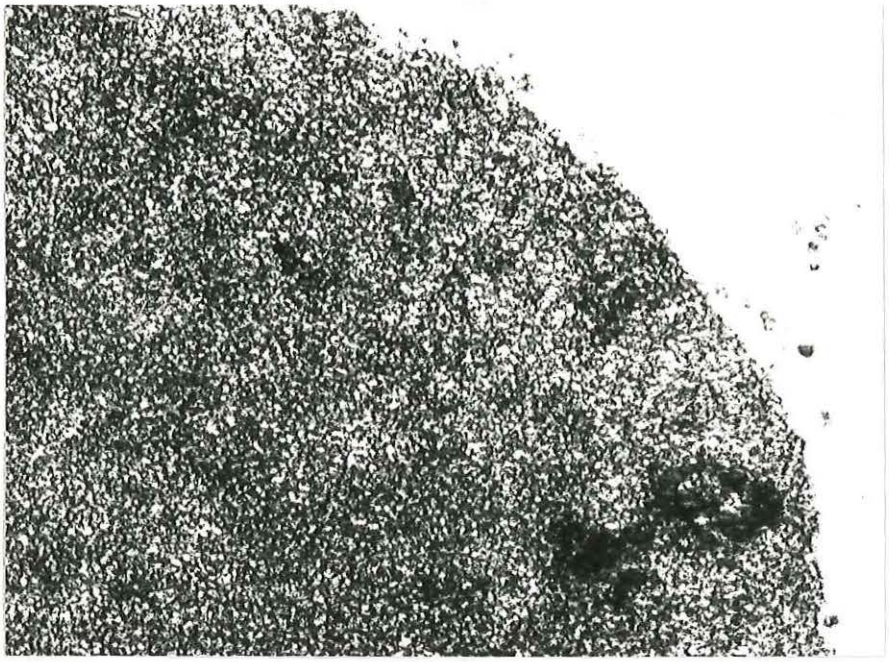


FIGURE 80: Surgicel implant T = 6.
Electronmicrograph showing higher magnification of implanted Surgicel. Note the "moth-eaten" appearance of the surface.
(TEM x 40,000).

FIGURE 81: Surgicel implant T = 18.
Electronmicrograph of tube contents showing PMNs and an extracellular fibrillar-granular material (E) reminiscent of Surgicel.
(TEM x 8,000).



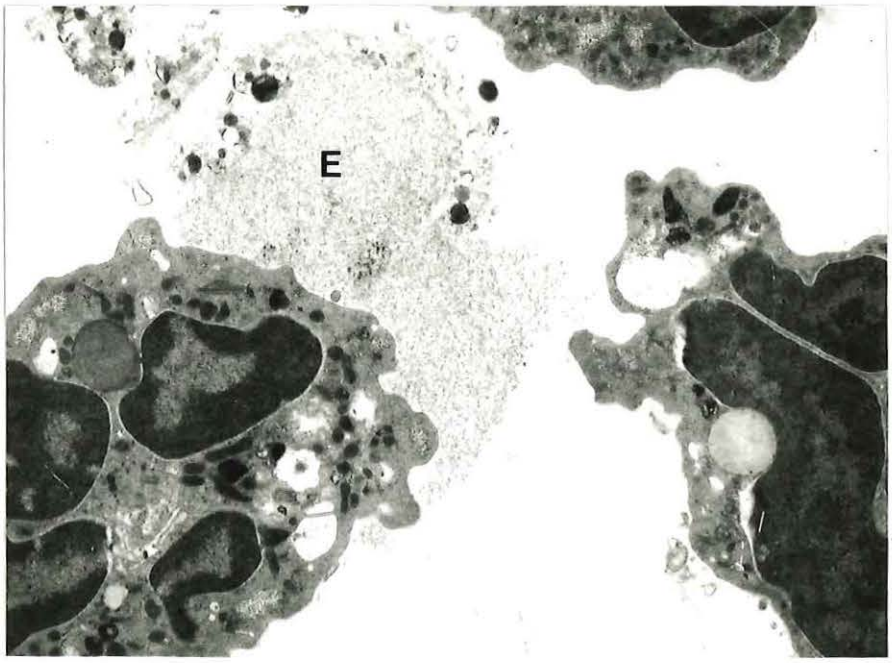
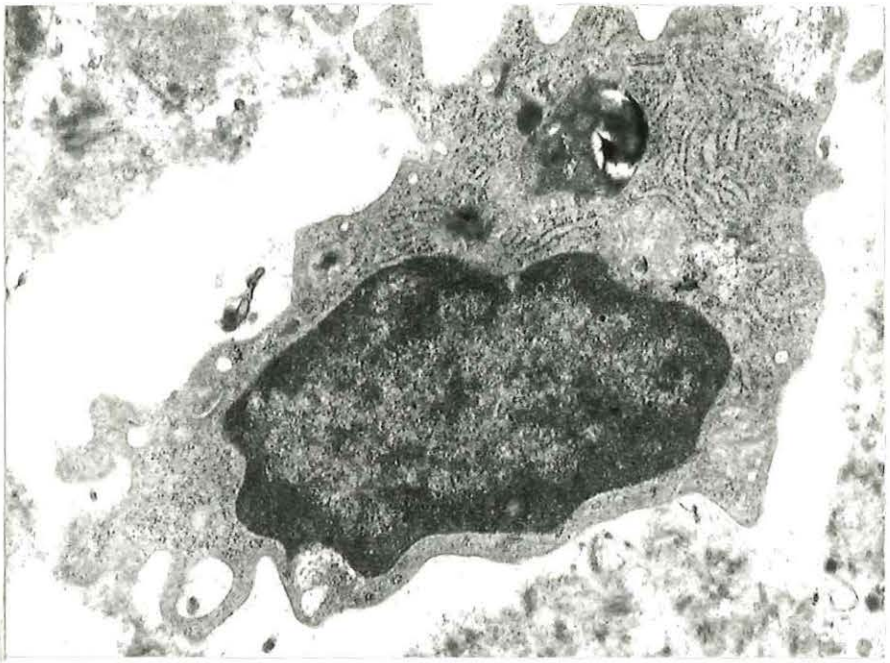

E

FIGURE 82: Surgicel implant T = 18.
Electronmicrograph showing a migrating cell in the fibrillar-granular material within the tube lumen.
(TEM x 15,000).

FIGURE 83: Surgicel implant T = 18.
Electronmicrograph of migrating cell showing phagosome (P) containing amorphous material.
(TEM x 15,000).



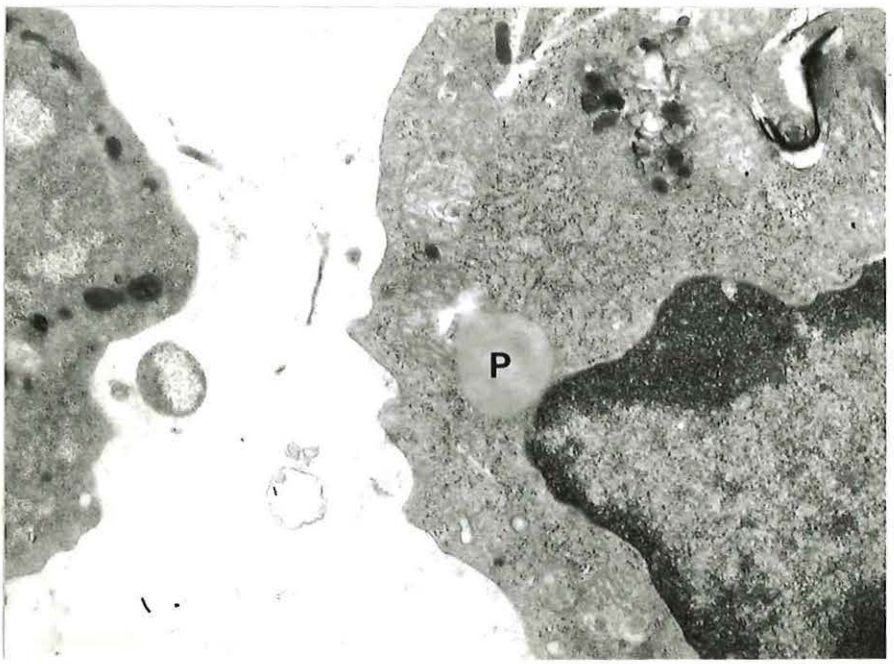
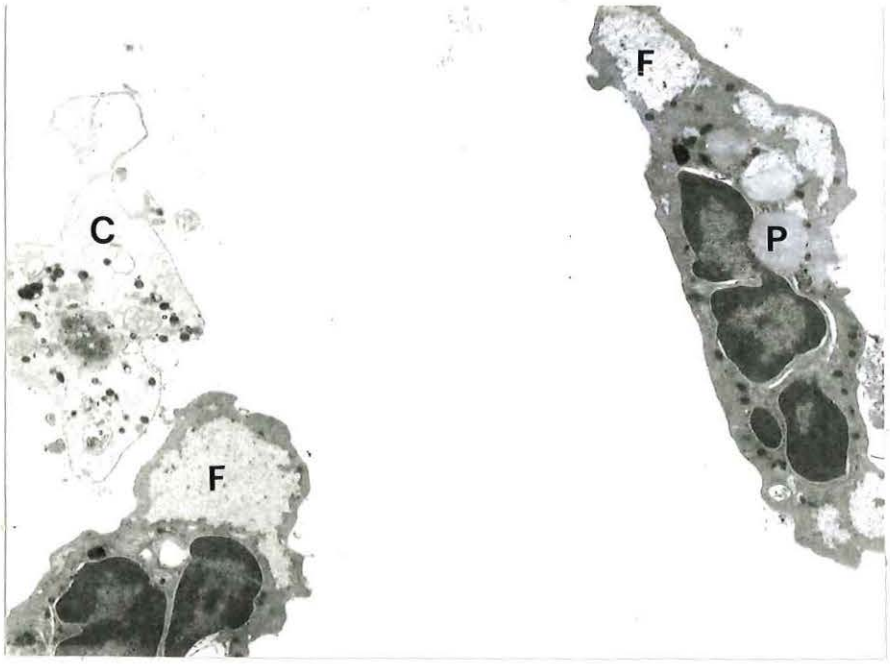

P

FIGURE 84: Surgicel implant T = 18.
Electronmicrograph showing PMNs and cell debris (C). Note phagosomes containing an amorphous material (P), and pale intracellular inclusions of fibrillar material (F) reminiscent of Surgicel.
(TEM x 6,000).

FIGURE 85: Surgicel implant T = 18.
Detail of Figure 84.
(TEM x 25,000).


C
F
F
P

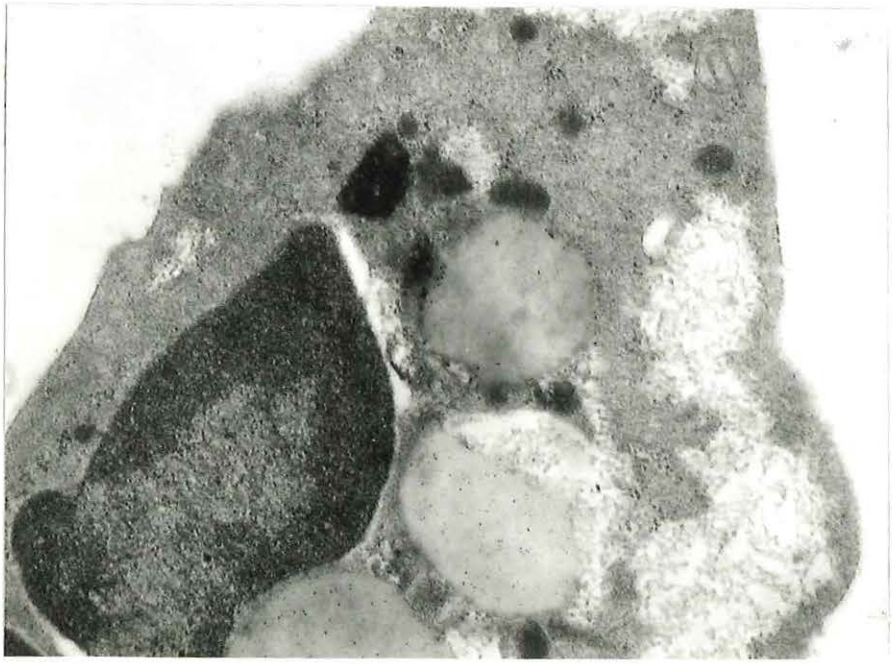
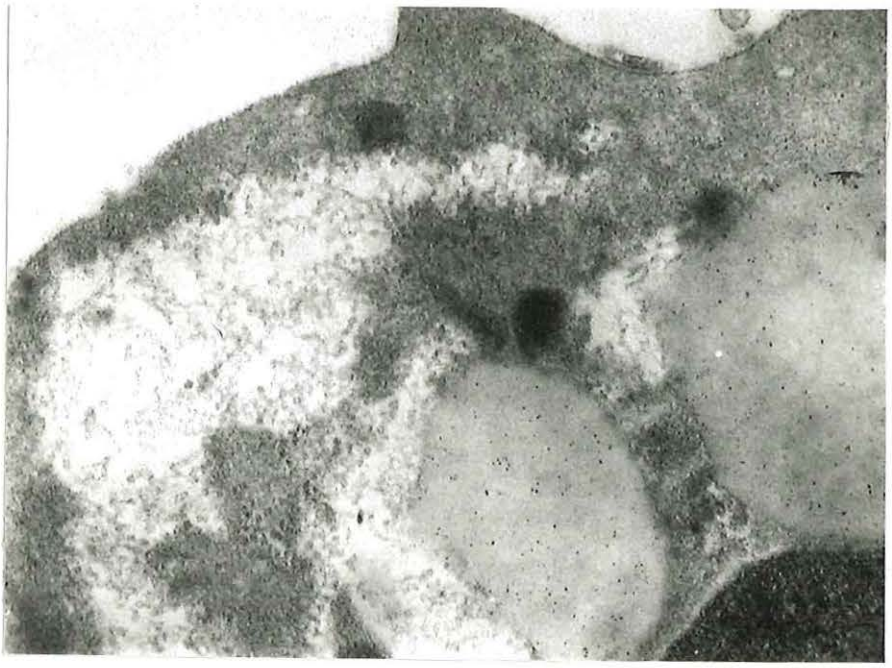


FIGURE 86: Surgicel implant T = 18.
Detail of Figure 84.

FIGURE 87: Surgicel implant T = 24.
Electronmicrograph showing macrophage in tube lumen. Note large phagosomes (P) containing fibrillar-granular material.
(TEM x 8,000).



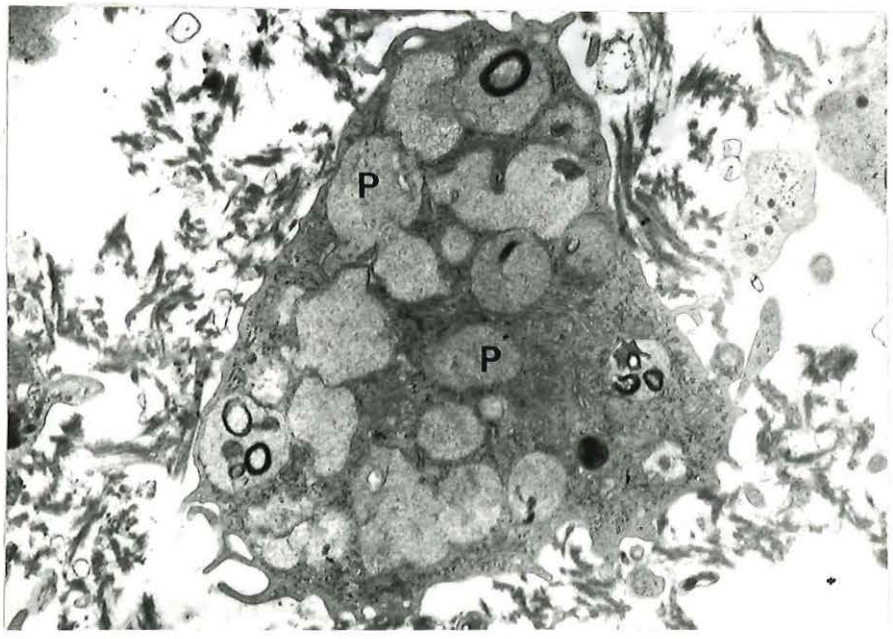
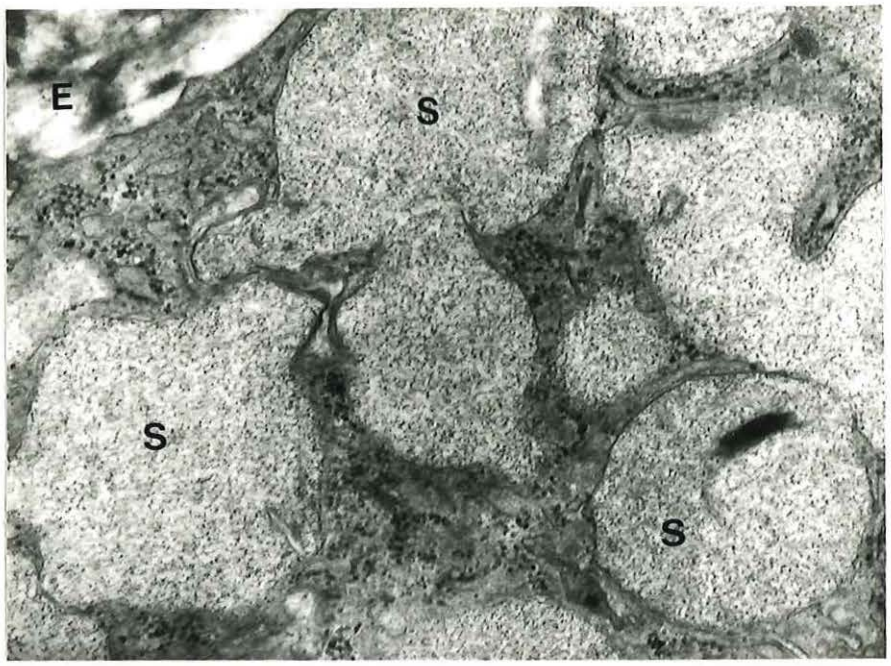

P
P

FIGURE 88: Surgicel implant T = 24.
Detail of Figure 87. Note Surgicel-like material (S) present in phagosomes. E indicates extracellular material.
(TEM x 25,000).

FIGURE 89: Surgicel implant T = 24.
Detail of Figure 87. Phagosome contents at higher magnification.
(TEM x 40,000).


E
S
S
S

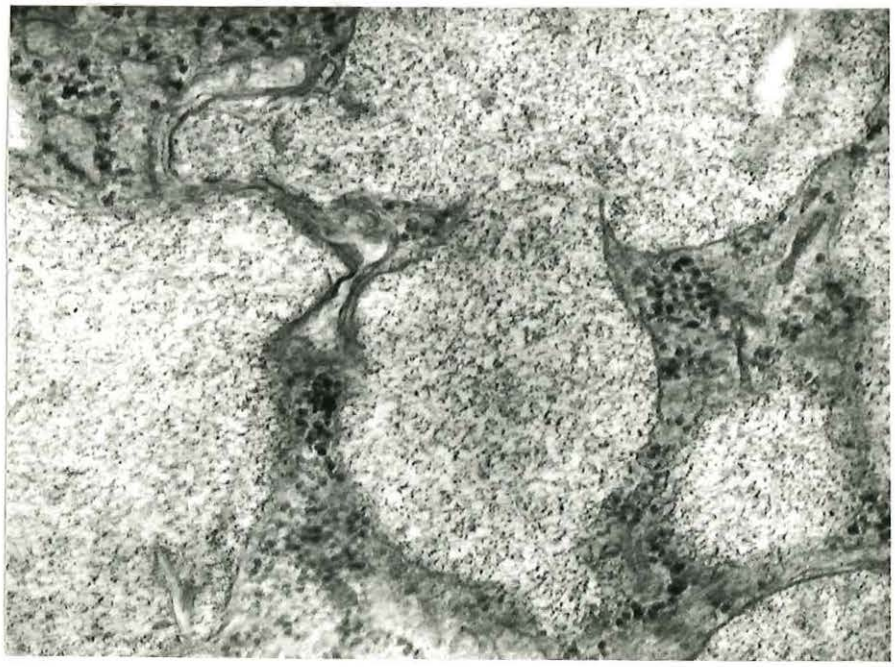
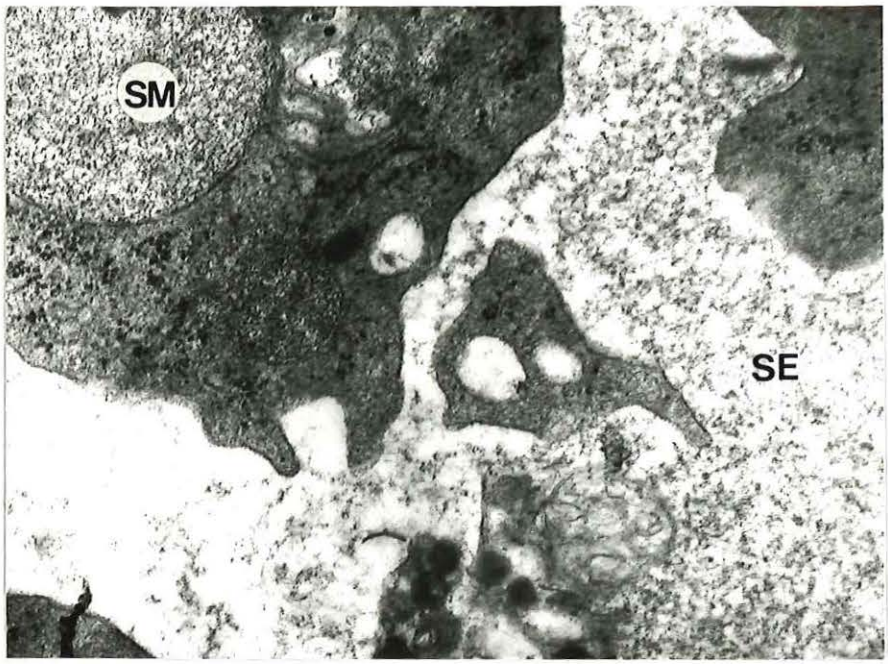


FIGURE 90: Surgicel implant T = 24.
Electronmicrograph showing Surgicel-like material within macrophage phagosome (SM) and extracellularly (SE).
(TEM x 25,000).

FIGURE 91: Surgicel implant T = 48.
Electronmicrograph showing PMNs and a macrophage (M) in tube lumen. The extracellular matrix (F) appears fibrinous. Note large phagosomes (P) in the macrophage.
(TEM x 6,000).


SM
SE

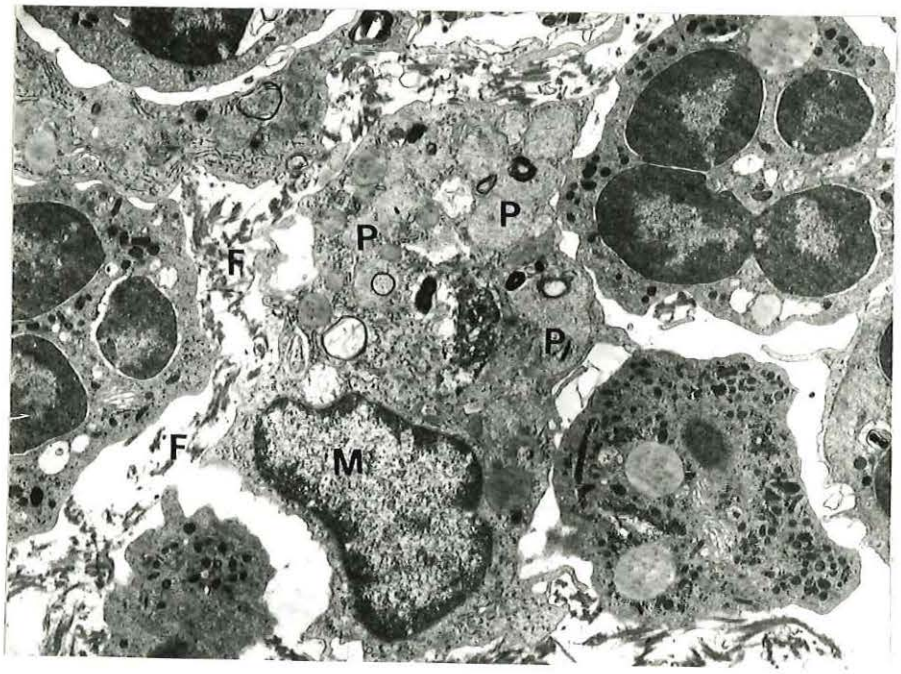
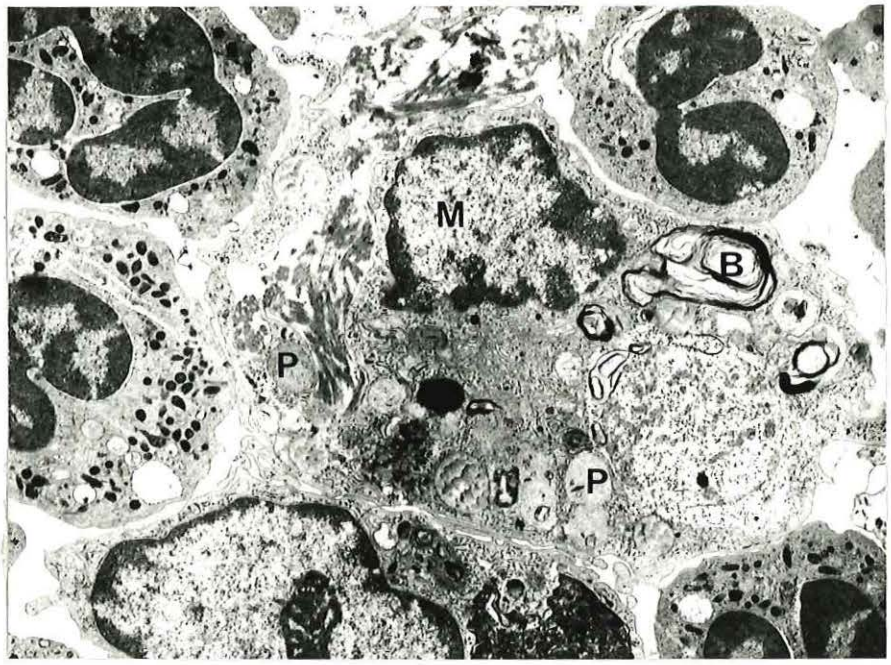

P
P
P
F
F
M

FIGURE 92: Surgicel implant T = 48.
Electronmicrograph showing macrophages (M) and PMNs within tube lumen. Note large macrophage phagosomes (P) and myelin bodies (B).
(TEM x 6,000).

FIGURE 93: Surgicel implant T = 48.
Electronmicrograph showing macrophage phagosomes containing Surgicel-like material and myelin bodies (M).
(TEM x 8,000).


M
B
P
P

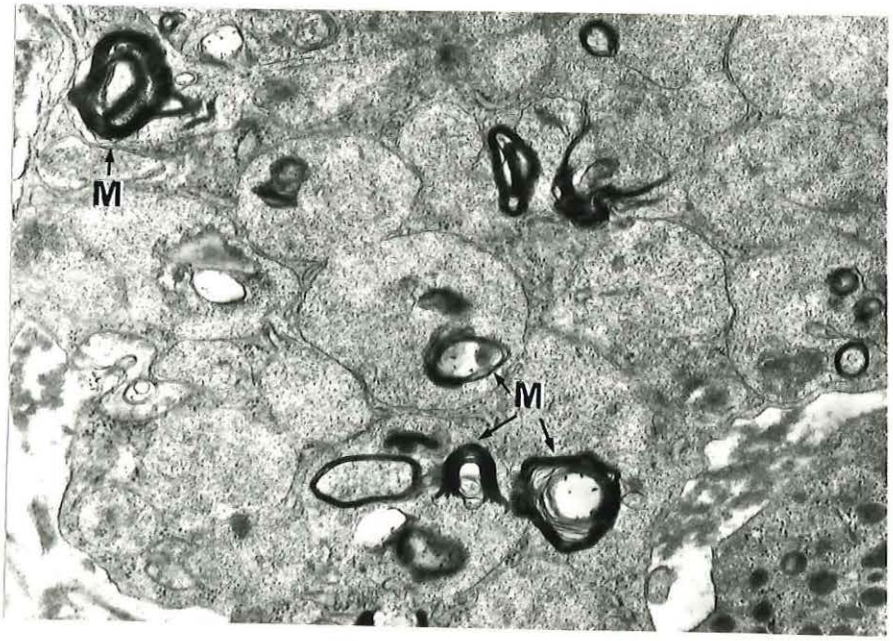
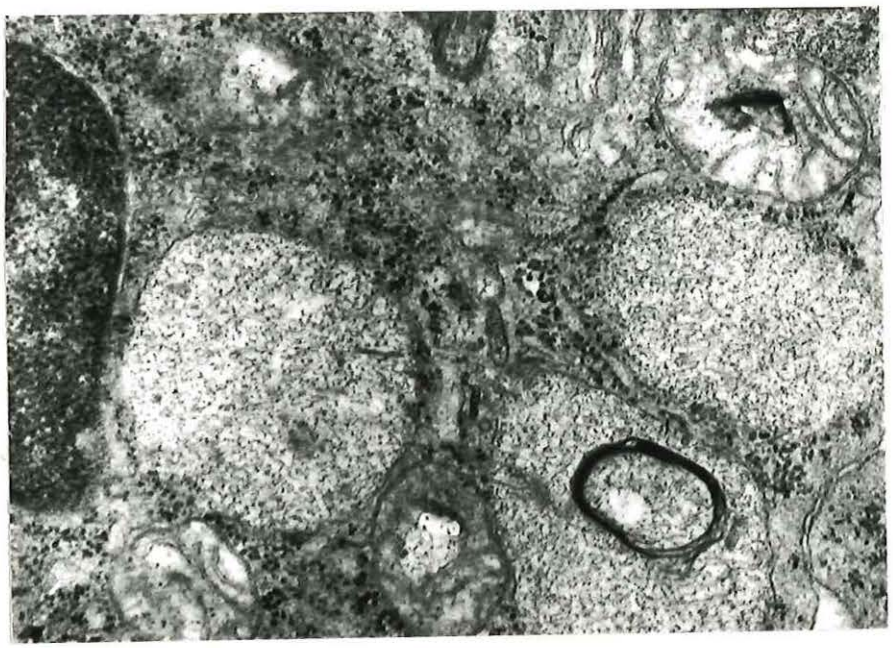

M
M

FIGURE 94: Surgicel implant T = 48.
Electronmicrograph showing macrophage phagosome contents at higher magnification.
(TEM x 25,000).

FIGURE 95: Surgicel implant T = 48.
Electronmicrograph showing macrophage phagosome contents at higher magnification.
(TEM x 30,000).



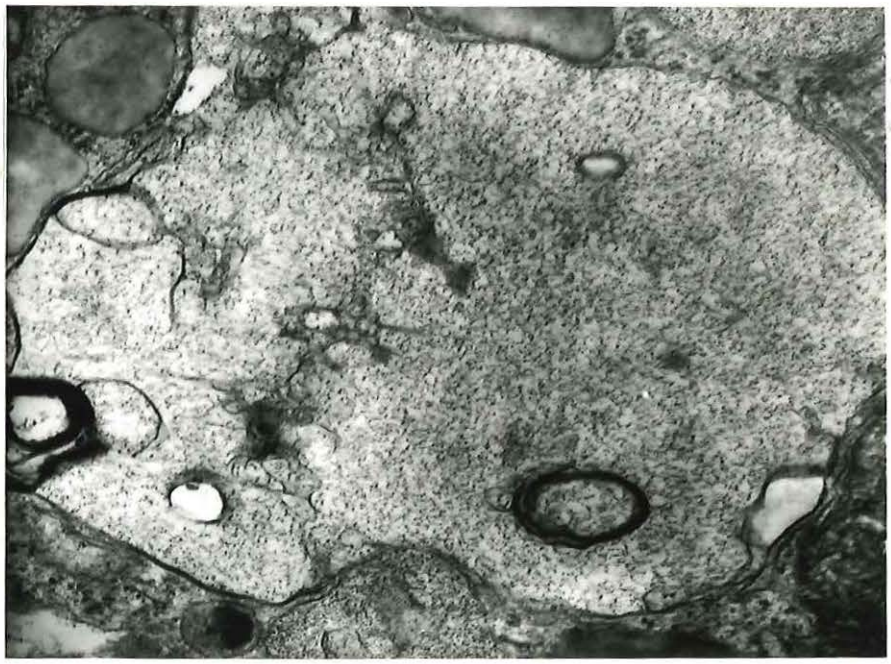
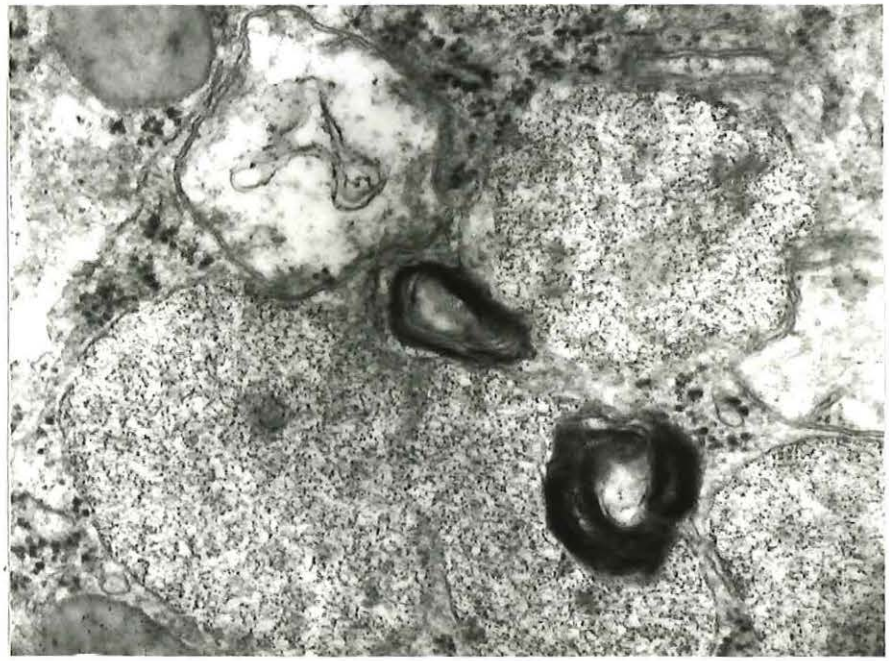


FIGURE 96: Surgicel implant T = 48.
Electronmicrograph showing macrophage phagosome contents at higher magnification.
(TEM x 40,000).



4. HISTOLOGY : FIBROUS RESIDUE

4.1 Optical Microscopy

4.1.1 Haematoxylin and Eosin

CONTROL SECTIONS

0 Hours : Examination of sections obtained from an empty tube, extracted in 4M Gu.HCl and processed for optical microscopy, revealed that no material was demonstrable in the tube lumen when stained with haematoxylin and eosin.

FIBROUS RESIDUE SECTIONS

0 Hours : A basophilic, fibrous material was evident in the tube lumen at 0 hours (Figure 97). Its structure appeared finer than that of Surgicel stained with haematoxylin and eosin at T=6 (see Figure 48).

6 Hours : At this recovery time, no fibrillar material or cells were demonstrable in the tube lumen (Figure 98). However, at both ends of the tube, an oedematous, fibrinous network was apparent, containing an occasional macrophage or PMN. Small amounts of basophilic fibrillar material were also noted in and around this fibrinous coagulum (Figure 99).

18 Hours : The appearance of a fibrous residue implant at 18 hours is demonstrated in Figure 100. Fine remnants of a basophilic coagulum, encompassed by a dense eosinophilic material, were observed near the

tube end at this time period (Figure 101). The tube walls were surrounded by an eosinophilic oedematous fibrinous network containing many plasma cells. Macrophages and PMNs were also observed.

24 Hours : Features noted at 24 hours were essentially similar to those observed at 18 hours, with the exception that foamy macrophages were apparent at the ends of the tube and there appeared to be an early ingress of inflammatory cells into the tube lumen (Figures 102 and 103).

48 Hours : Examination of 48 hour specimens revealed that the tube lumen appeared empty (Figure 104). A cellular infiltrate, comprising macrophages and plasma cells, was observed around the tube (Figure 105). Near one end of the tube, a dense basophilic mass, consisting of cells and fine fibrous material, was observed. The latter material resembled the fibrous residue of Surgicel at T=0.

4.1.2 Alcian Blue

No darkly staining material was evident within any of the tube lumina at 6, 18, 24 or 48 hours post-implantation. Figure 106 demonstrates the appearance of the 48-hour specimen.

4.2 Transmission Electron Microscopy

FIBROUS RESIDUE

0 Hours : The ultrastructure of the fibrous residue at 0 hours revealed a material similar in appearance to that of Surgicel at the same time period (Figures 107-109). A lobulated morphology was apparent

but the density of the material appeared more heterogeneous than that of Surgicel.

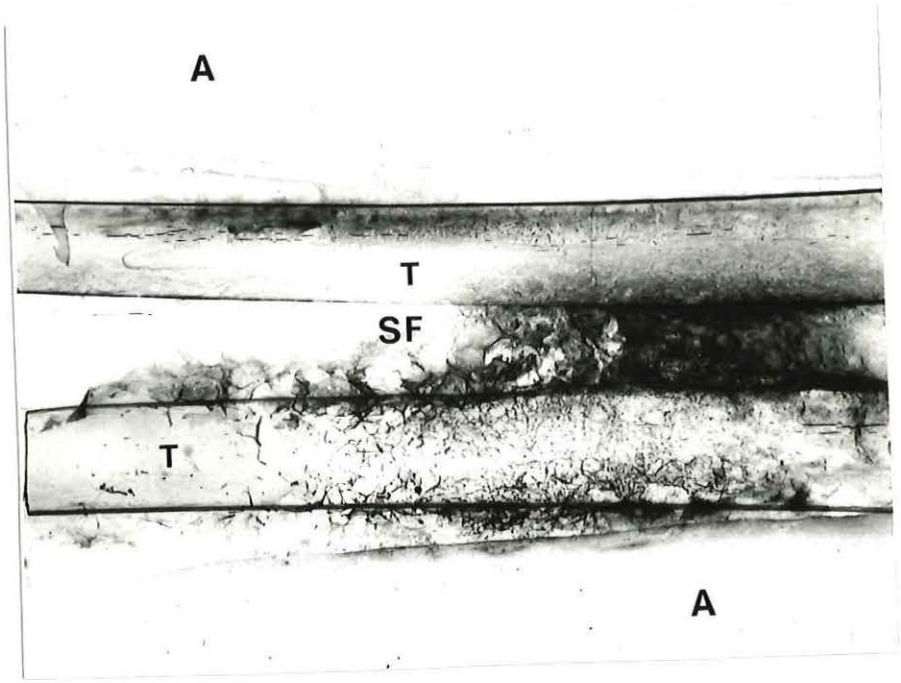
6 Hours : Examination of specimens revealed dense, peripherally lobulated bodies (Figure 110). At high magnifications, the surface appeared "moth-eaten" (Figures 111 and 112). Small fragments were evident peripheral to the main structures.

24 Hours : The tube lumen appeared to be occupied by a fibro-cellular infiltrate and a fibrinous matrix at this time period. Macrophages were the predominant cell type observed but an occasional PMN was evident. Macrophages contained large phagolysosomes enclosing materials similar in appearance to the fibrous residue observed at 0 and 6 hours (Figures 113-115). Similar structures were not observed in PMNs, nor were myelin bodies which were noted in many macrophage phagolysosomes.

48 Hours : Features observed were similar to those noted at 24 hours. Large phagolysosomes, containing material similar to the fibrous residue at 0 and 6 hours, were consistently found in macrophages (Figure 116). None were evident in PMNs. Myelin bodies were also observed in macrophages. An additional feature noted at 48 hours was the presence of free phagolysosomes, containing Surgicel-like material, in the extracellular matrix. These appeared to be associated with degenerating macrophages (Figures 117-119).

FIGURE 97: Fibrous residue T = 0.
Photomicrograph demonstrating a fine fibrillar basophilic material (SF) present in the tube (T) lumen. A represents Agar embedding medium. Sectioning artefact has caused partial loss of the fibrous residue from the tube lumen.
(H & E x 36).

FIGURE 98 : Fibrous residue implant T = 6.
Photomicrograph showing tube (T) contained within muscle. The tube lumen appears empty. Teflon tubing was lost during staining.
(H & E x 30).


A
T
SF
T
A

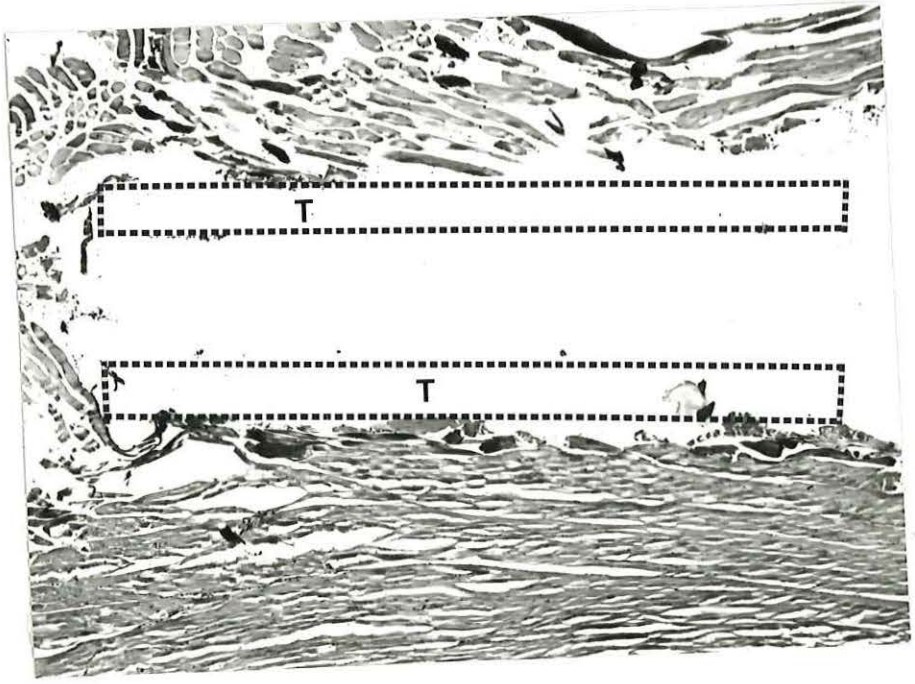
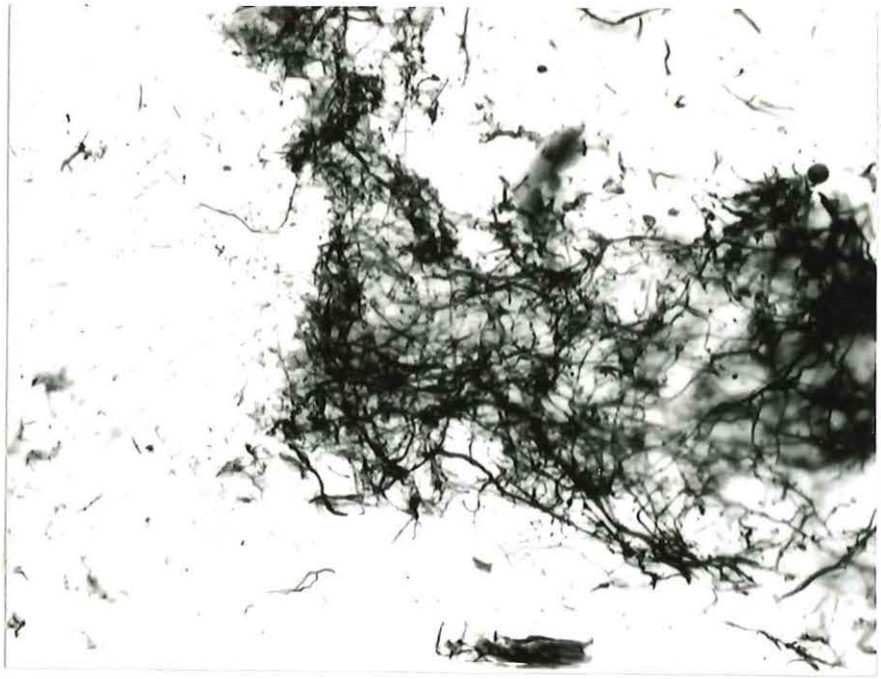

T
T

FIGURE 99 : Fibrous residue implant T = 6.
Photomicrograph demonstrating basophilic fibrillar material detected in the fibrinous coagulum around one end of the tube.
(H & E x 500).

FIGURE 100: Fibrous residue implant T = 18.
Photomicrograph demonstrating tube positioned in muscle. Fibrinous material was detected in the lumen. Note dense structure (S) shown in greater detail in Figure 101.
(H & E x 31).



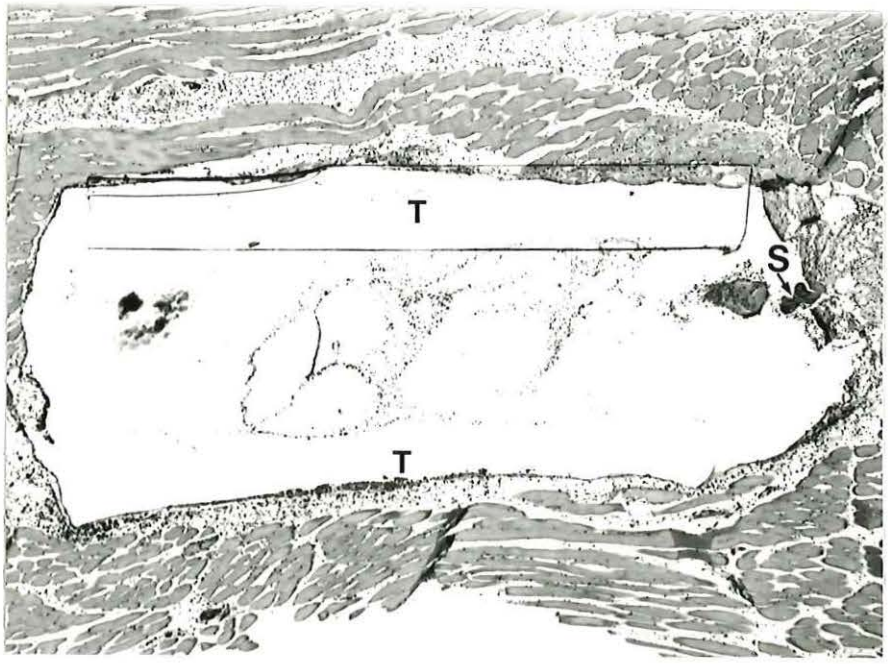

T
S
T

FIGURE 101: Fibrous residue implant T = 18.
Detail of Figure 104 showing an eosinophilic coagulum (E) incorporating a fine basophilic material (F). PMNs and plasma cells surround the structure.
(H & E x 352).

FIGURE 102: Fibrous residue implant T = 24.
Photomicrograph showing early ingress of inflammatory cells (I) into tube lumen (shown in greater detail in Figure 103).
(H & E x 64).


E
F
F
E

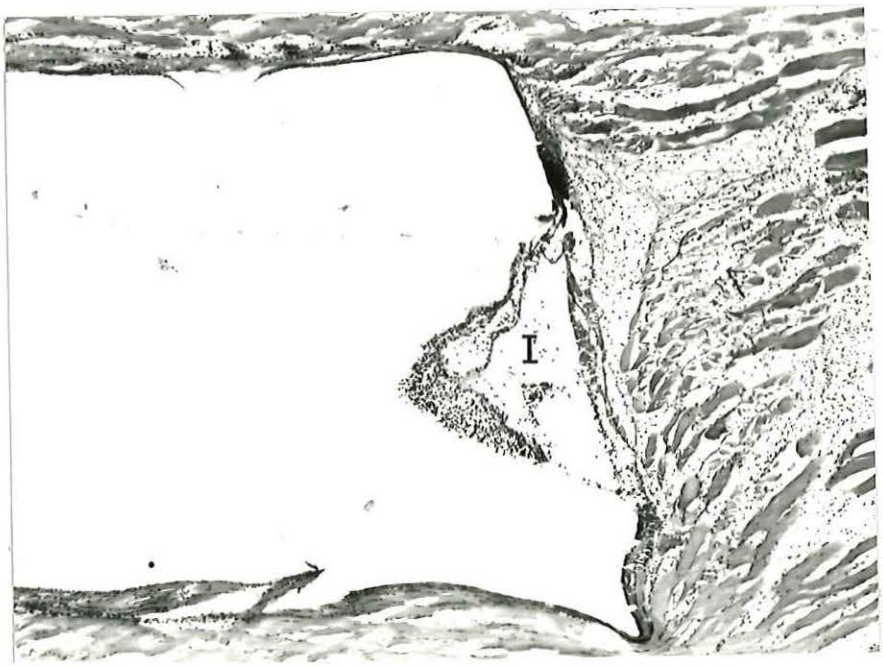
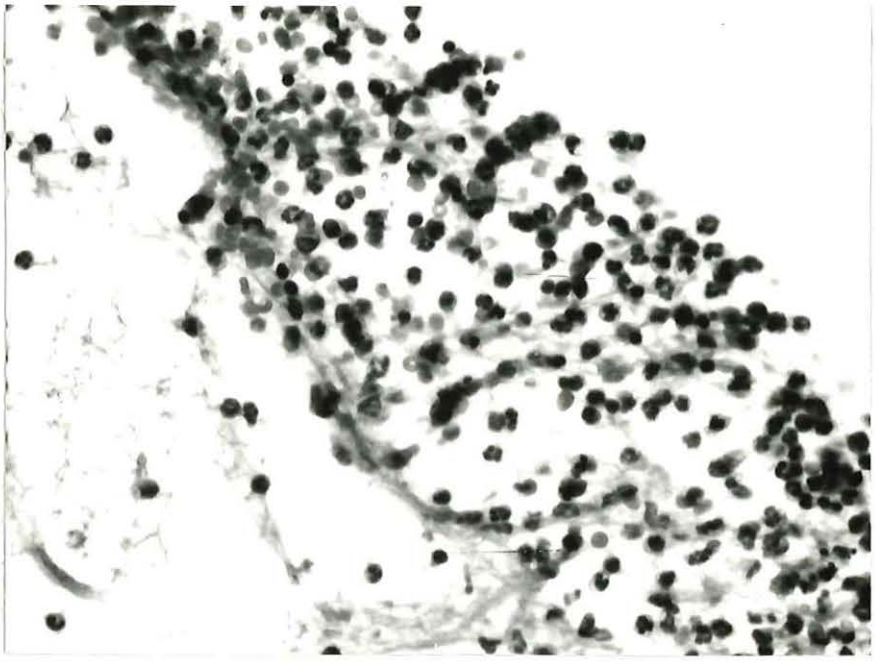

I

FIGURE 103: Fibrous residue implant T = 24.
Detail of Figure 102 showing inflammatory cell infiltrate consisting predominantly of PMNs and plasma cells.
(H & E x 412).

FIGURE 104: Fibrous residue implant T = 48.
Photomicrograph showing tube (T) located in muscle and surrounded by an oedematous cellular matrix (M) shown in greater detail in Figure 105.
(H & E x 28).



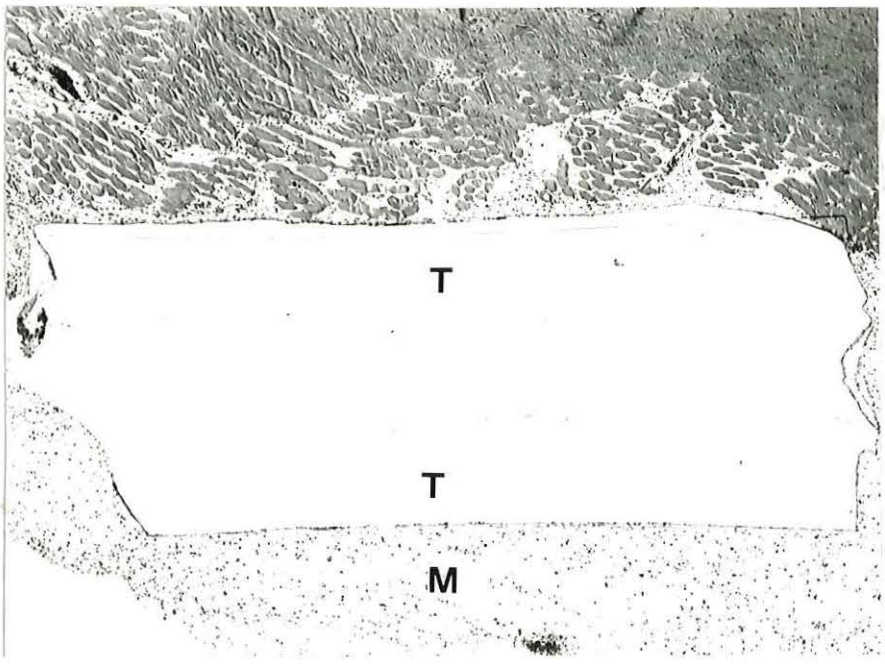

T
T
M

FIGURE 105: Fibrous residue implant T = 48.
Detail of Figure 104 showing foamy macrophages predominating within an oedematous matrix at the ends of the tube.
(H & E x 440).

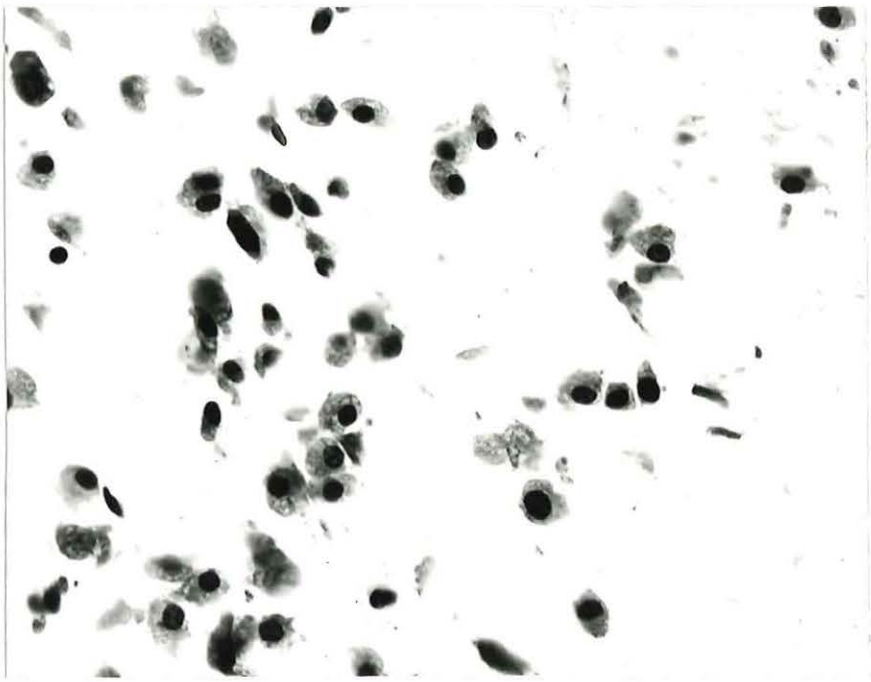


FIGURE 106: Fibrous residue implant T = 48.
No darkly staining material is apparent.
(Alcian Blue (0.15M $MgCl_2$) x 30).

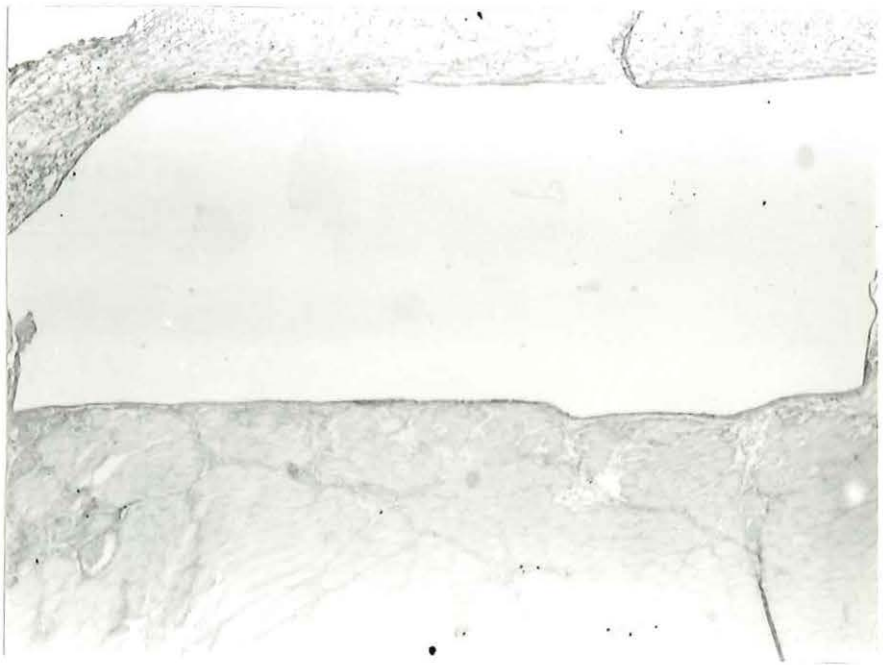
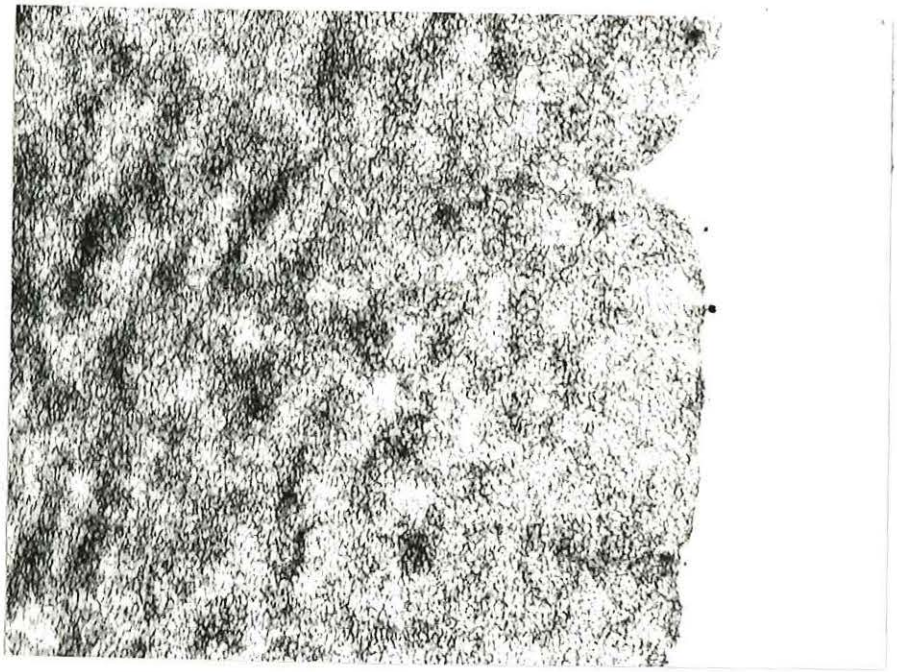


FIGURE 107: Fibrous residue T = 0.
Electronmicrograph showing ultrastructure of unimplanted fibrous residue of Surgicel.
(TEM x 20,000).

FIGURE 108: Fibrous residue T = 0.
Electronmicrograph showing ultrastructure of fibrous residue at higher magnification.
(TEM x 25,000).



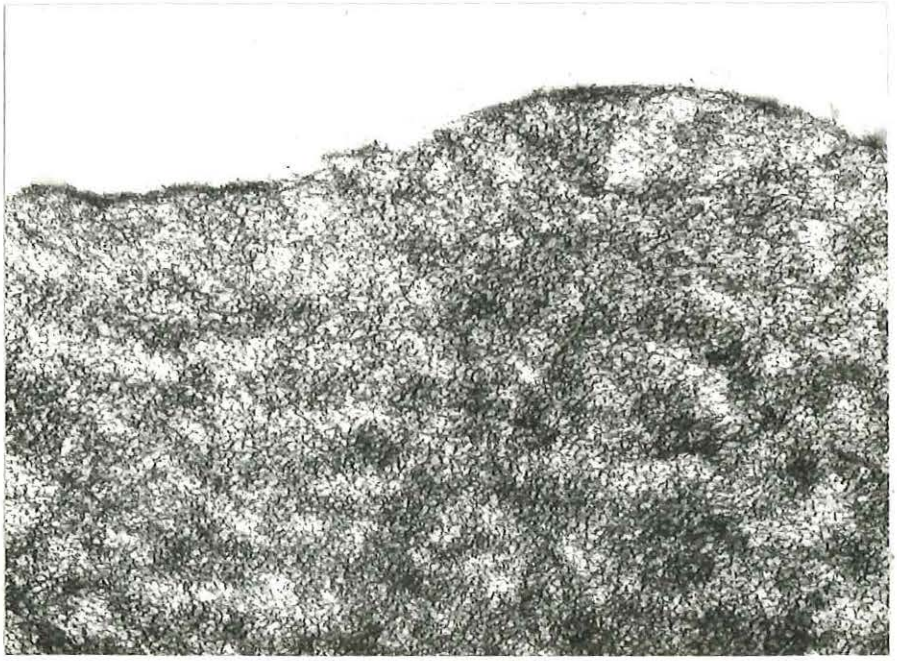
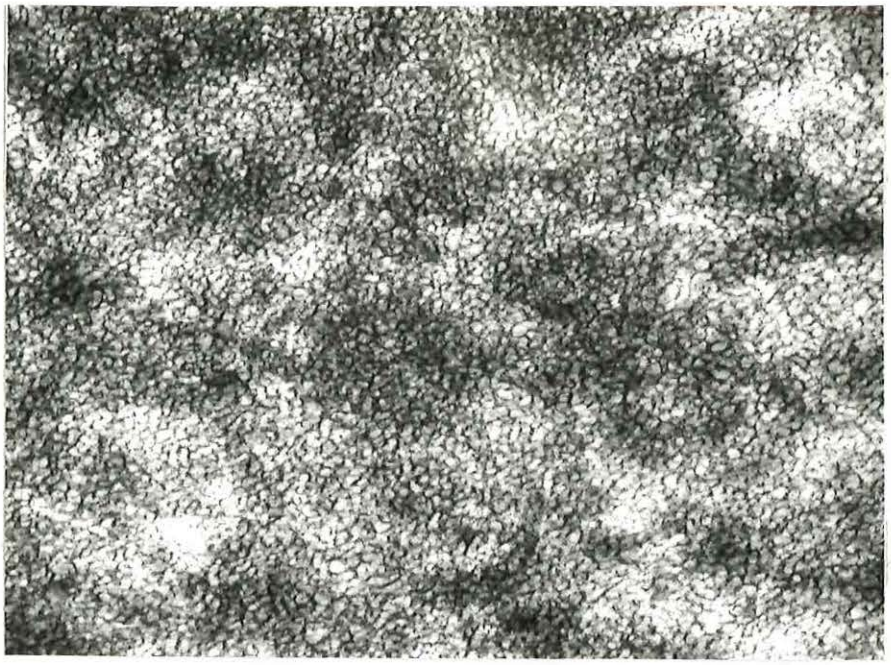


FIGURE 109: Fibrous residue T = 0.
Electronmicrograph showing ultrastructure of fibrous residue at higher magnification.
(TEM x 40,000).

FIGURE 110: Fibrous residue T = 6.
Electronmicrograph showing ultrastructure of implanted fibrous residue.
(TEM x 6,000).



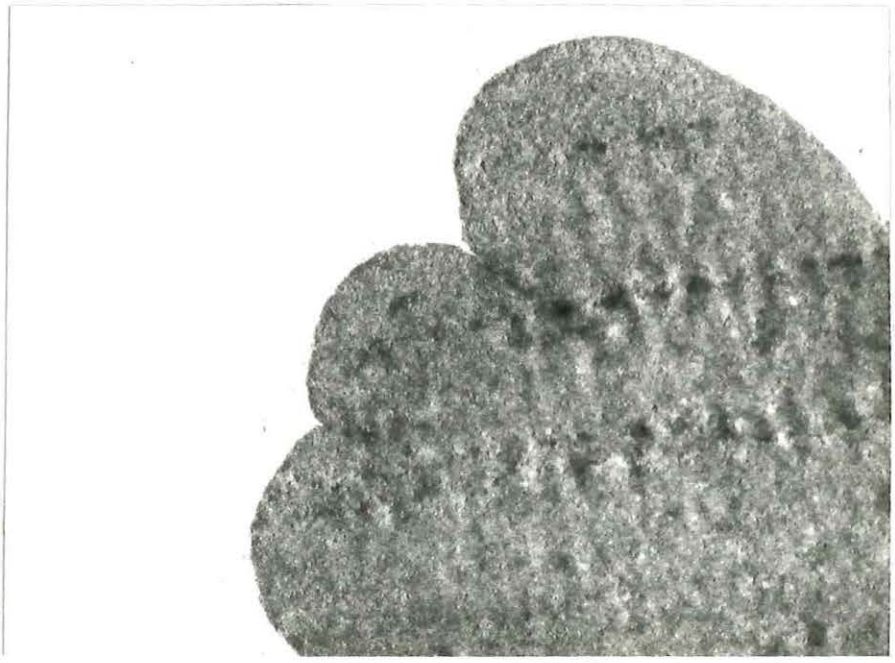
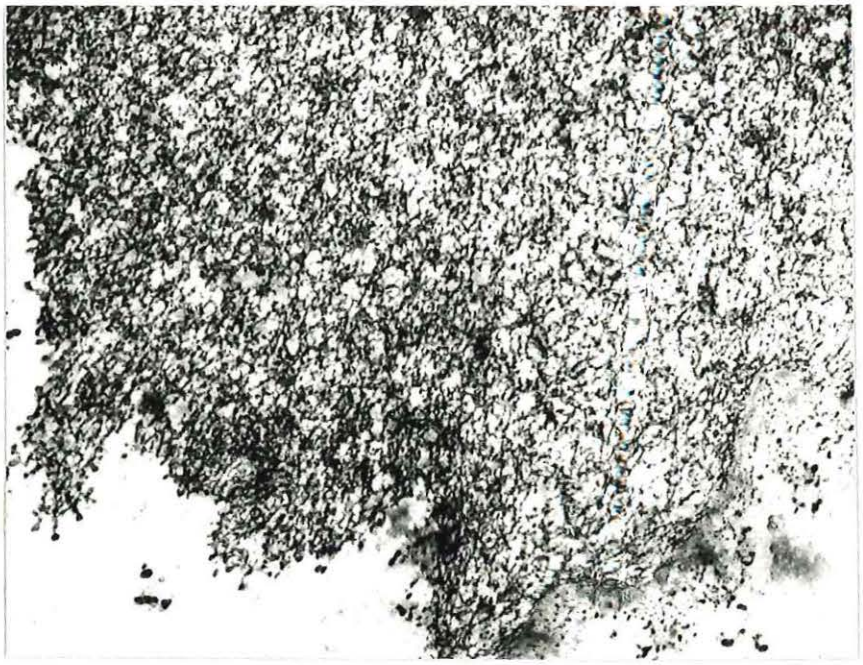


FIGURE 111: Fibrous residue implant T = 6.
Electronmicrograph showing "moth-eaten" appearance of implanted fibrous residue.
(TEM x 25,000).

FIGURE 112: Fibrous residue implant T = 6.
Electronmicrograph showing ultrastructure of implanted fibrous residue.
(TEM x 25,000).



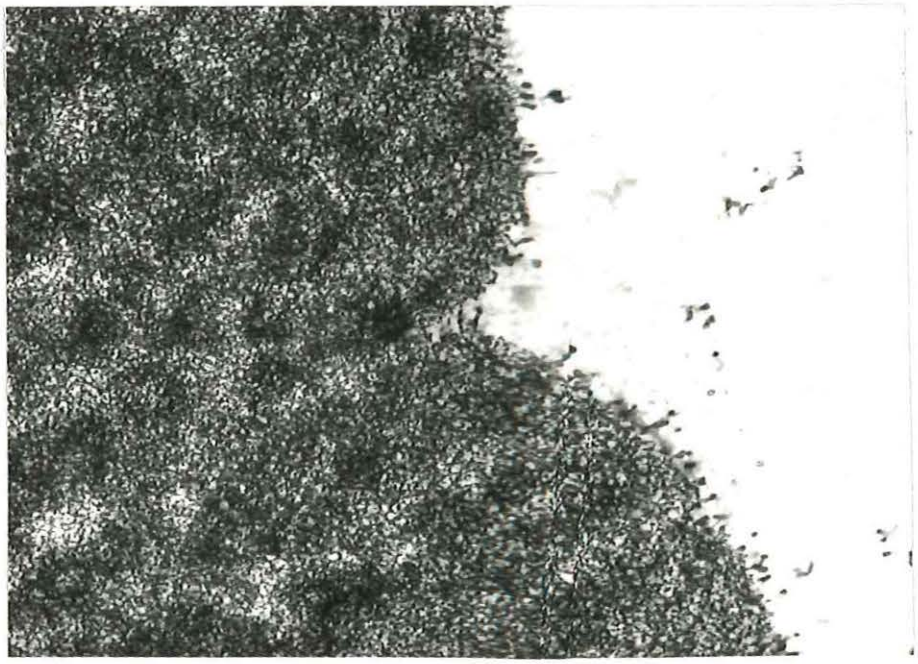
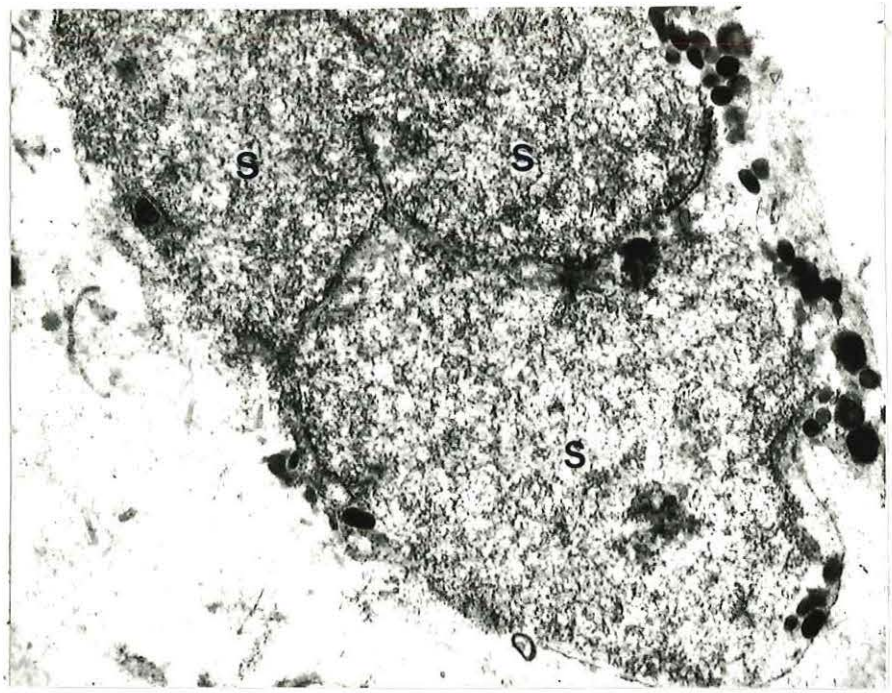


FIGURE 113: Fibrous residue implant T = 24.
Electronmicrograph showing large macrophage phagosomes containing Surgicel-like material (S).
(TEM x 15,000).

FIGURE 114: Fibrous residue implant T = 24.
Electronmicrograph showing phagosome contents at higher magnification.
(TEM x 25,000).


S
S
S
D

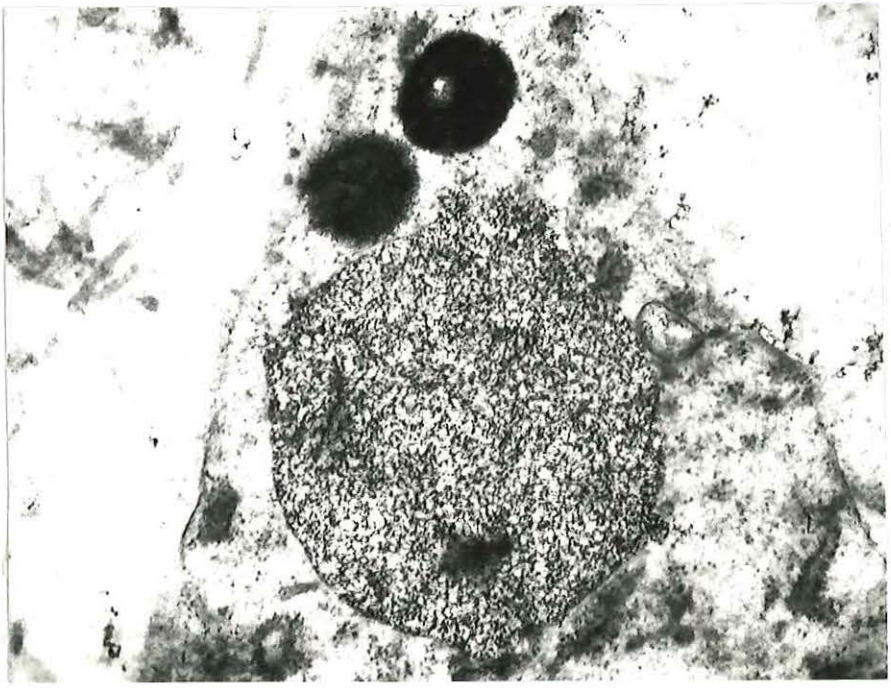


FIGURE 115: Fibrous residue implant T = 24.
Electronmicrograph showing macrophage phagosome contents at higher magnification.
(TEM x 40,000).

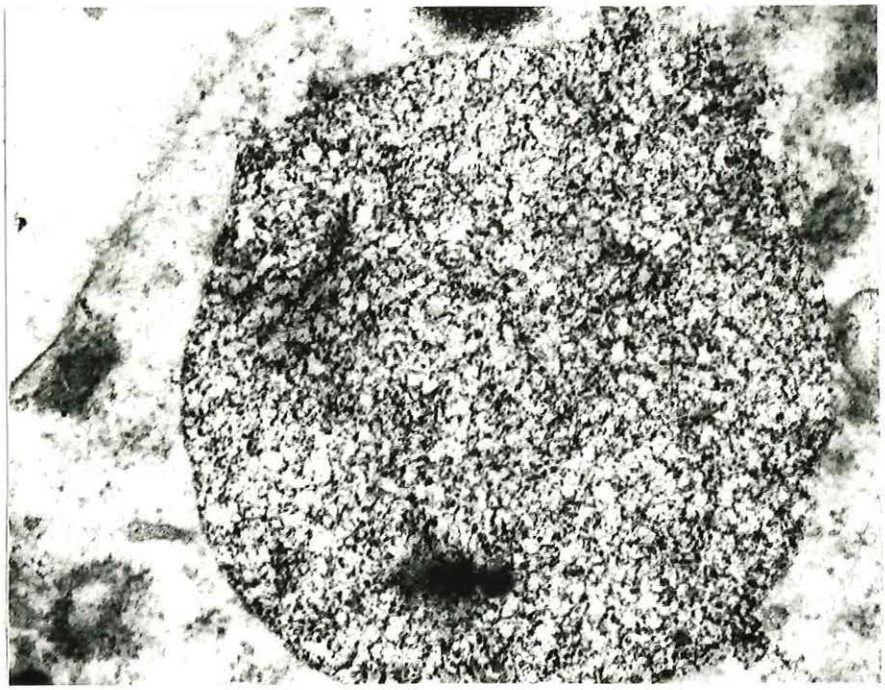
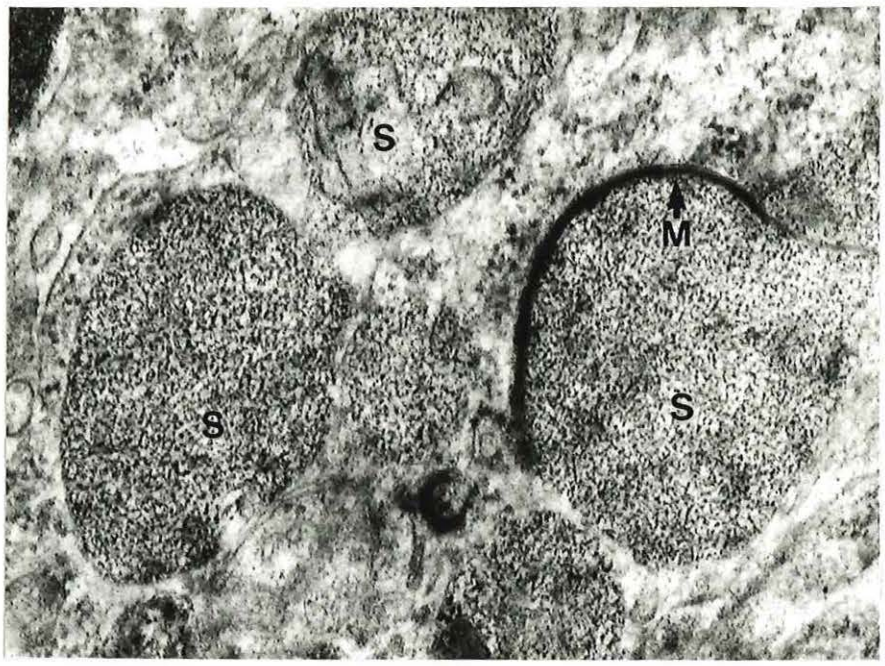


FIGURE 116: Fibrous residue implant T = 48.
Electronmicrograph showing macrophage phagosomes containing Surgicel-like material (S). M indicates myelin body.
(TEM x 40,000).

FIGURE 117: Fibrous residue implant T = 48.
Electronmicrograph showing macrophage (M) discharging its cytoplasmic contents into the extracellular compartment.
(TEM x 8,000).


S
M
S
S

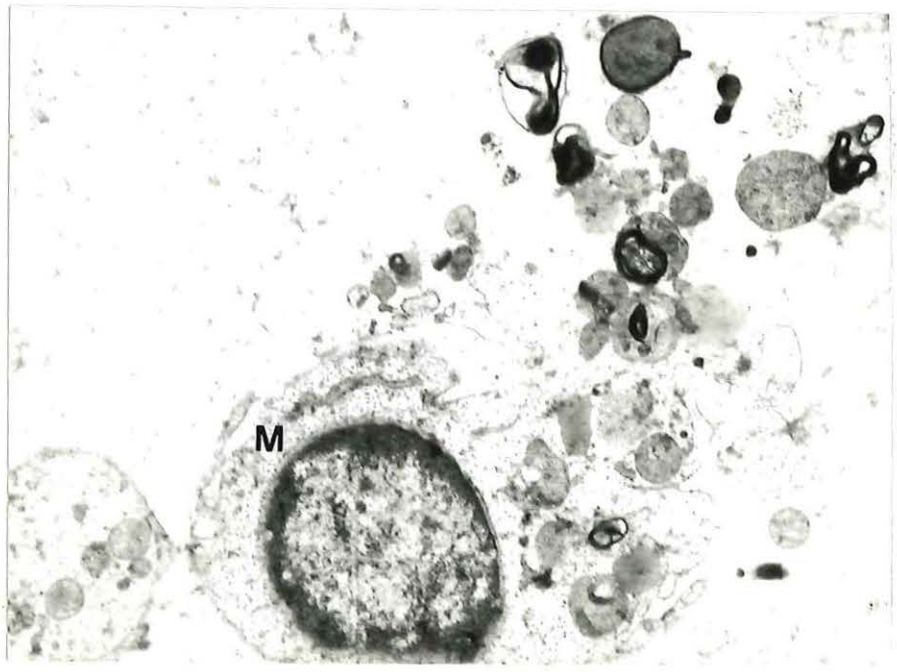
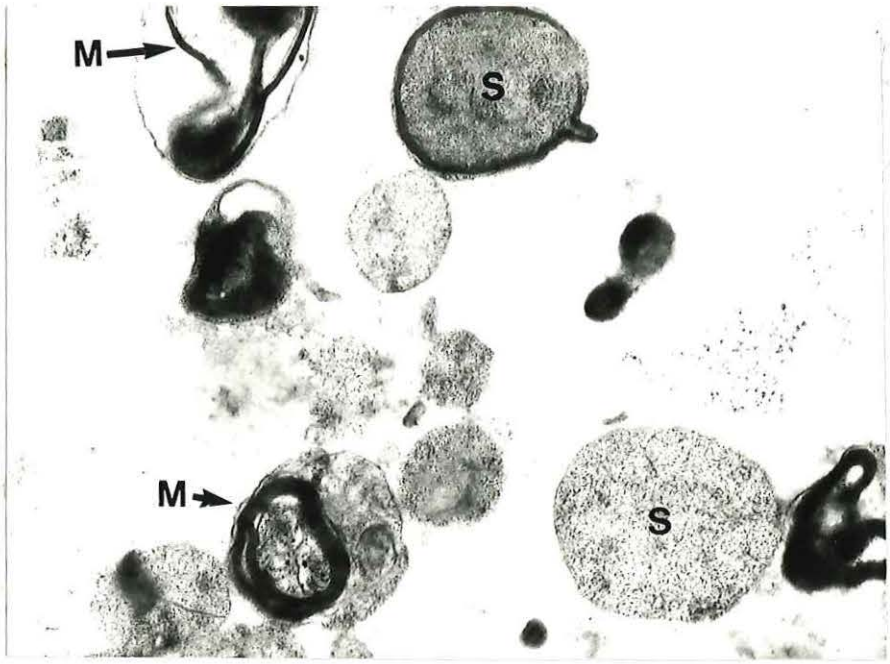
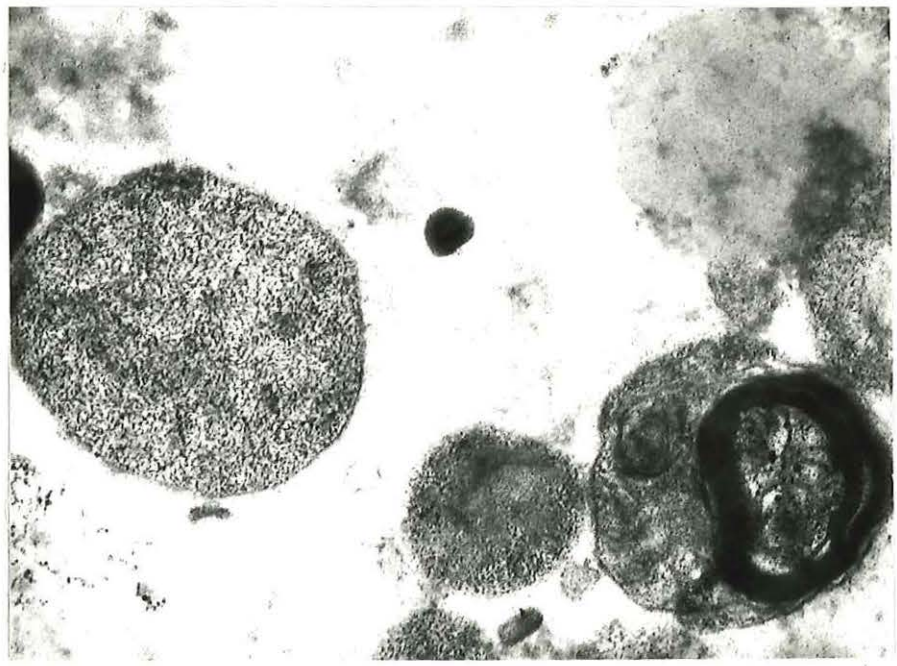

M

FIGURE 118: Fibrous residue implant T = 48.
Detail of Figure 117 showing free phagosomes containing Surgicel-like material (S) and Myelin bodies (M).
(TEM x 25,000).

FIGURE 119: Fibrous residue implant T = 48.
Detail of Figure 117 showing phagosome contents at higher magnification.
(TEM x 40,000).


M
S
M
S



CHAPTER FIVE

DISCUSSION

CHAPTER 5 : DISCUSSION

CHAPTER 5

DISCUSSION

1. GENERAL

Local haemostasis has been an integral part of both major and minor surgical procedures since the father of modern surgery, John Hunter, made his first incision. Methods developed to control local bleeding are reviewed in Table 11.

Table 11: Methods developed to control local bleeding (Adapted from Garach, 1982).

1. Pressure (Lucas, 1966 ; Evans, 1977)
2. Cold Packs (Lucas, 1966 ; Evans, 1977)
3. Cautery (Lucas, 1966 ; Shephard, 1976 ; Evans, 1977)
4. Vasoconstrictors, e.g. adrenalin (Lucas, 1966 ; Evans, 1977)
5. Caustics, e.g. silver nitrate (Lucas, 1966 ; Evans, 1977)
6. Adhesives, e.g. butyl cyanoacrylate (Bhaskar, Frish, Cutright and Margetis, 1967)
7. Biological Agents, e.g. topical thrombin (Tidrick et al., 1943)
8. Implantable local haemostatic agents:
   (a) Gelfoam^{R} (Correl and Wise, 1945)
   (b) Microfibrillar collagen - Avitene^{R} (Benoit and Hunt, 1976)
   (c) Oxidized cellulose (Frantz, 1943)
   (d) Starch sponge (Lattes and Frantz, 1945)
   (e) Absele^{R} (Harris and Capperauld, 1978)
   (f) Bone wax^{R} (Horsley, 1892)
   (g) Surgicel^{R}

The properties of an ideal topical haemostatic material have been enumerated by Chvapil, Owen and DeYoung (1983): such a material should have ample haemostatic action, minimal tissue reactivity and should not delay healing. It should be of low cost, biodegradable in vivo and non-antigenic. The material should simulate tissue structure and be of uniform thickness with small interstices. Furthermore, it should be easy to sterilize and to tailor to specific needs, and, ideally, possess an antibacterial action.

Studies have already shown Surgicel to be a highly biocompatible material (Lebendiger et al., 1959 ; Hurwitt et al., 1960 ; Jantet and Rob, 1960 ; Miller et al., 1961b ; Degenshein et al., 1963 ; Huggins, 1969 ; Thilander and Stenstrom, 1969 ; Garach, 1962). Several authors have commented upon its ease of handling (Lebendiger et al., 1959 ; Hurwitt et al., 1960 ; Jantet and Rob, 1960 ; Georgiade et al., 1961 ; Miller et al., 1961b ; Degenshein et al., 1963 ; Tibbels, 1963 ; Tierney, 1954). It can be sterilized, is inexpensive to produce, and Dineen (1976, 1977a, 1977b) has shown it to possess significant anti-bacterial properties. Although the specific mechanisms by which Surgicel promotes haemostasis are unknown, universal agreement exists regarding its efficacy as a local haemostatic agent. Furthermore, Surgicel is absorbable in situ. Not only does this property negate the need to re-enter operative sites for pack removal, but it also prevents further bleeding such as may occur when non-absorbable material is removed from raw surfaces.

There is a paucity a data relating to the mechanisms of absorption of oxidized regenerated cellulose by body tissues. Blau et al. (1960) suggested Surgicel was hydrolyzed by body fluid enzymes, and Hurwitt et

al. (1960) proposed that these were members of the carbohydrase system. Miller et al. (1961a) showed that thrombin aided solubilisation of Surgicel *in vitro* and suggested that the slightly alkaline medium of the blood aided such a process.

Furthermore, although many *in vivo* trials have been performed with oxidized regenerated cellulose, there are minimal data concerning the time required for its absorption in muscle, the establishment of such being a necessary prerequisite for investigations into absorption mechanisms. The few experiments that have been executed to this end have been hampered by difficulties in microscopic identification of Surgicel.

Garach (1982) observed that an eosinophilic fibrillar coagulum, interpreted as being altered Surgicel due to its absence in control sections, was present at 2 and 7 days, but not at later time periods, in intramuscular implants. It is of some interest to compare these results with those of Jantet and Rob (1960) who also implanted Surgicel intramuscularly in rats. These authors reported that the implants were still "recognizable" at 1 day post-operatively but were "almost fully" absorbed by 5-7 days. Garach did not find the material "recognizable" at any stage following implantation in muscle, even at the earliest period of 2 days.

In consideration of the above, it was apparent that further studies were necessary to investigate the mechanisms of Surgicel absorption by animal tissues.

Criteria used in the present study as indicative of absorption of Surgicel in intramuscular implants included:

(1) Loss of assayable uronic acid (the component saccharide of Surgicel) from the implant system, indicative of either an alteration in chemical structure of the material, or its intracellular inclusion.

(2) Absence of the material in histologic sections after staining with a technique suitable for positive identification.

(3) Identification of extracellular breakdown products or intracellular inclusions of the material.

Once the time required for absorption to occur had been established, further ultrastructural studies were then pursued in order to detect the fate of the material.

The intramuscular implantation model used in the present study was the same as that used by Garach (1982). It was based on the concept of inserting test materials into tubes prior to implantation in animal sites (Torneck, 1966 ; Browne and Friend, 1968 ; Spångberg, 1968 ; Friend and Browne, 1969 ; Spångberg, 1969 ; Langeland and Cotton, 1977 ; Haugen and Mjor, 1978 ; Wenger, Tsaknis, del Rio and Ayer, 1978 ; Olsson, Sliwkowski and Langeland, 1981a, 1981b).

The carrier for the test material, Teflon, evokes a minimal tissue reaction by itself (Harrison, Swanson and Lincoln, 1957) and is inert to almost all chemicals (Bragt, Bonta and Adolfs, 1980). Furthermore, it can be autoclaved (leVeen and Barberio, 1949) and thus forms a most suitable material for a carrier of implant materials. Restriction of the test material to the confines of a tube facilitated quantitation of data

as the implant was easily retrievable from the operative site. Another advantage was the easy identification of implantation sites, both clinically and microscopically, which was mandatory in the present study, considering the absorbability of Surgicel.

Muscles of the anterior chest wall were chosen as the implantation sites as they were easily accessible. Furthermore, according to Coleman, King and Andrade (1974), muscle is a suitable site for implantation studies because it provides a homogeneous environment.

2. URONIC ACID ASSAY OF IMPLANTS

2.1 Validation of experimental approach

Determination of the time required for Surgicel absorption involved the detection of a quantitated relative loss of uronic acid from the implant system. Surgicel, being a straight-chained polymer of glucuronic acid residues, is similar in structure to the glycosaminoglycans (GAGS), polyanions which exist in nature as protein-bound moieties referred to as proteoglycan subunits (PGS). These form the ground substance in the extracellular matrix of connective tissues and consist of a protein core to which many GAGS are covalently bound (White et al., 1978b).

It has been conclusively demonstrated that, in cartilage, some PGS are bound to hyaluronic acid (a GAG) with link glycoproteins and thus form a proteoglycan aggregate (PGA) which has a characteristic "bottle brush" structure (Hascall and Sajdera, 1969 ; Hardingham and Muir, 1974 ; Rosenberg, Hellmann and Kleinschmidt, 1975). [Somewhat hasty extrapolation from this data has led to a popular, but erroneous,

assumption that all PGS are generally aggregated in other tissues, besides cartilage, in a similar manner (O. Wiebkin, personal communication)].

Connective tissue proteoglycans are distinguished from other mammalian carbohydrate-protein complexes (glycoproteins) by the presence of these relatively large GAG chains containing repeating disaccharide units as their most characteristic feature (Rodén, Baker, Cifonelli and Mathews, 1972). These units are usually composed of a uronic acid and a hexosamine. Table 12 shows the composition of the disaccharide units of GAGS which have been recognized thus far. Two uronic acids, D-glucuronic acid and L-iduronic acid, and two hexosamines, D-glucosamine and D-galactosamine, are the constituent sugars of these polysaccharides.

In contrast, Surgicel is a repeating polymer of α- and β-glucuronic acid units, in which only 72-84% of residues have been oxidized to glucuronic acid.

Conventional assay techniques of GAGS involve quantitative analysis of the separate disaccharide components. For example, quantitative analysis of hyaluronic acid entails two separate assays, one for glucuronic acid and the other for N-acetyl-glucosamine. In the present study the quantitative assay of Surgicel was based on the determination of glucuronic acid levels, as reflected in uronic acid concentrations in specimens.

Table 12 Composition of connective tissue glycosaminoglycans (Rodén et al., 1972).

GAG	Components of repeating disaccharide units
Hyaluronic Acid	D-glucosamine and D-glucuronic acid
Chondroitin	D-galactosamine and D-glucuronic acid
Chondroitin-4-sulphate	D-galactosamine and D-glucuronic acid
Chondroitin-6-sulphate	D-galactosamine and D-glucuronic acid
Dermatan sulphate	D-galactosamine and L-iduronic acid or D-glucuronic acid
Heparan sulphate	D-glucosamine and D-glucuronic acid or L-iduronic acid
Heparin	D-glucosamine and D-glucuronic acid or L-iduronic acid
Corneal keratan sulphate	D-glucosamine and D-galactose
Skeletal keratan sulphate	D-glucosamine and D-galactose

Hascall and Sajdera (1969) demonstrated that isolation of bovine nasal cartilage PGS could be effected by dissociative extraction in 4M guanidinium hydrochloride (4M Gu.HCl), and that such a technique yielded up to 95% of the uronic acid. It is likely that the residual macromolecular uronate was tightly associated with insoluble elements such as collagen (Scott, 1980).

GAGS may subsequently be further isolated from the protein core of the PGS by proteolytic digestion with papain and then recovered by ethanol precipitation (Rodén et al., 1972).

In this study, 4M Gu.HCl was used as the dissociative extractant for the glucuronic acid polymer. Initially, the implant was also transferred to papain proceeding the 4M Gu.HCl extraction in order to liberate any polyuronic acid (polyuronate) that may have become protein bound subsequent to implantation. However, the majority of the polyuronates involved in this system appeared to be Gu.HCl soluble, yielding only negligible papain residues. Although initially investigated, these residues were not addressed in the later stages of the present study. However, some relevant experimental attempts to pursue any papain residues will be briefly discussed below.

Subsequent to isolation of the implanted Surgicel by 4 M Gu.HCl extraction, all samples were subjected to uronic acid assay.

The meta-hydroxydiphenyl (mHDP) reaction method for quantitative determination of uronic acids (Blumenkrantz and Asboe-Hansen, 1973) was the assay technique of choice in the present study as it represented a more specific detection of uronic acid levels than the classical methods of either Brown (1946) or Dische (1947) in which pentoses and hexoses, respectively, were found to interfere with the analyses. Furthermore, the mHDP method suffers less from interference by salts and other biological contaminants (Rosenthal, Bentley and Albin, 1979).

In the present study, recovery of implants for uronic acid assay from the block of muscle tissue proved difficult in the first

experiment. Although a nylon suture aided localization, two problems arose : firstly, the "hidden" tube was often subjected to a significant amount of movement as muscle tissue was dissected away from it and this could have resulted in loss of tube contents; secondly, Surgicel, present in gel form, appeared to be protruding from either end of the tube at 6 hours post-implantation.

The subsequent use of transillumination (using a light box) facilitated localization of the implant in the muscle block. This enabled immobilisation of the tube with tweezers while the surrounding tissue was carefully removed with a sharp scalpel.

In order to overcome the problem of Surgicel extrusion from the ends of the implanted tubes and the consequent risk of partial loss of tube contents, Surgicel tubes were exhaustively hydrated overnight in Hanks' B.S.S./Bovine albumin. Excess hydrated and expanded material protruding from either end of the tube (refer to Fig. 17) was subsequently removed with a scalpel prior to implantation, and samples of the hydrating solution were assayed for the presence of uronic acid, in the fourth assay. Insignificant amounts were detected. In all subsequent uronic acid assay experiments, "pre-swelled" Surgicel was employed.

In addition to the physiological reasons for hydrating Surgicel prior to implantation, initial quantitation of the material to be implanted could be more accurately standardized than when initial dry weight estimates were calculated. It was hypothesized that the weight of hydrated Surgicel within the Teflon tube might be extrapolated from the tube lengths following recovery at the end of implantation experiments.

Indeed, a linear correlation between Surgicel weight and tube length was confirmed, and a consistent density of Surgicel within the tube could be assumed (refer to Table 13).

Table 13 Correlation between Surgicel weight and tube lengths for 3 quantitation trials (refer Figs. 32-36).

	Sample	Number (N)	Associated Parameters	Correlation Coefficient(r)*
1.	Dry Surgicel, conventionally fed into tubing	34	Tube length vs. Surgicel weight	0.469
2.	Dry Surgicel, tightly twisted before feeding into tube	40	Tube length vs. Surgicel weight	0.791
3.	Hydrated Surgicel in tube	19	Tube length vs. weight of tube containing Surgicel	0.989

* There was a significant difference between all r values at $p < 0.05$, demonstrated using the Z transformation test (Sokal and Rohlf, 1969).

The initial pre-implantation dry weight of Surgicel could be calculated from in vitro degree of hydration studies. Subsequently, a conversion factor (4.21) was derived from a series of Surgicel samples which were weighed pre- and post-hydration (Appendix 4). This enabled extrapolation of the dry weight of Surgicel from the wet weight calculated from the relationship between tube length and weight of tube containing hydrated Surgicel. Accordingly, data obtained in the fifth

uronic acid assay was standardized for an average 10 mg dry weight sample of Surgicel (Appendix 4).

Inconsistency in values obtained for papain-digested Surgicel in early assays prompted the speculation that 4M Gu.HCl was not "wetting" the relatively intact Surgicel gauze in the centre of the Teflon tubing recovered at T = 6 hours. In order to test this theory, 8 Surgicel tubes were placed in 4M Gu.HCl and observed with the naked eye after 8 hours. The middle third did not appear wet. Consequently, in the fourth assay experiment, sequential extraction of non-implanted Surgicel tubes (Group A) in 4M Gu.HCl was undertaken over a period of 72 hours in order to determine whether complete extraction had occurred by this time.

Results revealed that uronic acid was still being extracted at 24 hours and persisted to 72 hours, although levels at this time were substantially lower. (Control tubes (Group B) showed minimal uronic acid levels.) Further investigation of 4M Gu.HCl extraction included assaying samples of Surgicel pushed out of tubes (Group C) and extracted for 24 hours in 4M Gu.HCl. Values were compared with those of Group A tubes. It was assumed that if 4M Gu.HCl extraction had proceeded to equilibrium, summation of the 24, 48 and 72 hour values for Group A tubes would yield a figure similar to that obtained for Group C tubes at 24 hours in which the tube did not form a "wetting" barrier. Results, although not quantitated, revealed that this was indeed the case.

From these observations, it was concluded that future assay experiments would incorporate sequential extraction of all tubes in 4M Gu.HCl for 72 hours after recovery.

2.2 Assay results

Absorbances obtained for all pure glucuronic acid standards were proportional to their uronic acid content, according to Blumenkrantz and Asboe-Hansen (1973).

In initial experiments, uronic acid absorbances recorded for Surgicel tubes appeared low at T = 48 h and T = 24 h, when compared with values obtained for uronic acid analysis of Surgicel at T = 0 h.

From data obtained from samples at T = 48 h and T = 24 h in subsequent experiments, it was concluded that Surgicel absorbance values at T = 24 h appeared, in general, slightly higher than those at T = 48 h. However, values were still low compared with those recorded for Surgicel at T = 0 h. In order to explain this observation, it was hypothesized that Surgicel was being absorbed before T = 24 h. Initial absorbance values obtained for all Surgicel implants at T = 18 h and T = 6 h were significantly higher than those recorded at later recovery times.

The presence of small amounts of uronic acid in some of the control tubes may be explained by the presence of endogenous proteoglycan and hyaluronic acid from muscle tissue.

Comparison of quantitated glucuronic acid levels at T = 6 h with T = 0 h in the final assay reflected a substantial loss or chemical transformation of the polyuronic acid from Surgicel tubes within this time period (Fig. 38). Furthermore, it appeared that the uronic acid was almost fully lost or chemically transformed in implants by 18 hours.

Loss of the polyuronic acid was interpreted as being representative of the absorption process.

3. HISTOLOGY : SURGICEL IMPLANTS

Some difficulties were initially encountered in obtaining sections for light microscopic examination due to the Teflon tubing. It did not adhere well to the surrounding muscle and, consequently, the tube easily slipped out of its implantation site during sectioning. This problem was especially noticeable at recovery times of 24 hours or less and was later overcome to some extent by the use of double-embedding techniques (Appendix 5).

A further problem encountered was incorrect alignment of the tube with respect to the plane of sectioning of the specimens. Reblocking, after thick sections were removed for localization, resulted in more accurate alignment.

3.1 Haematoxylin and Eosin Stained Material

Conventional staining of paraffin sections with haematoxylin and eosin was undertaken as part of the overall investigations into the morphological aspects of Surgicel absorption.

It is pertinent to examine the observations of Jantet and Rob (1960) and Garach (1982) regarding the microscopic presence of Surgicel after intramuscular implantation, and compare then with those of the present study.

Jantet and Rob stated that total resorption of Surgicel from intramuscular implants occurred between 2-5 days but omitted to describe the microscopic appearance of Surgicel stained with haematoxylin and eosin. Garach described the presence of an "eosinophilic fibrillar coagulum, containing cells" in his 2 and 7 day experimental specimens and interpreted this as being altered Surgicel due both to its absence in control sections and its structural dissimilarity to fibrin. At 14 days, this material was no longer observed, but a coarse eosinophilic material resembling fibrin was present in both experimental and control sections. In the present study, Surgicel was clearly identifiable as a basophilic fibrillar structure at 6 hours in experimental sections. Although fine traces of basophilic fibrillar material were evident at the tube ends and intraluminally at later recovery times, any eosinophilic material observed in experimental sections was indistinguishable from fibrin or comparable material in control sections.

It was the equivocality of Surgicel and fibrin staining after 6 hours post-implantation which presented the greatest difficulties in interpretation of haematoxylin and eosin-stained sections. It is felt that interpretation of the results of previous investigators who used conventional staining techniques to investigate Surgicel absorption must be made with some caution. Garach, (1982), cognizant of the equivocality of H & E staining, attempted to stain Surgicel selectively with safranin-aniline blue (a cellulose stain), but with little success. Interestingly, he further investigated the effects of fixation on the staining properties of Surgicel and found that sections of unfixed material stained positively with safranin-aniline blue, but formalin-fixed material did not stain. Garach concluded that

conventional fixation possibly altered the chemical structure of Surgicel.

In the present study, the nature of the inflammatory infiltrate at all recovery times was similar in experimental and control sections, and consistent with surgical trauma. The presence of vacuolated (foamy) macrophages was noted at, and subsequent to, 18 hours post-implantation, a feature which was also noted in control sections. This was also observed by Garach in experimental sections at his earliest recovery period of 2 days. Furthermore, Lebendiger et al. (1959) and Hurwitt et al. (1980) described the presence of "large macrophages" in relation to Surgicel 3 days subsequent to subcutaneous implantation. Jantet and Rob (1960) reported the presence of macrophages in subcutaneous, intramuscular and intraperitoneal Surgicel implants and further stated that small particles of Surgicel could be "seen distinctly" in such cells. Jantet and Rob suggested that macrophages were responsible for phagocytosis of Surgicel. However, no details of identification criteria were given by these authors.

The concept of macrophage phagocytosis of absorbable therapeutic implant materials is not a new one in the medical literature. It is well accepted, for example, that absorption of the suture material catgut is mediated by macrophage phagocytosis (Salthouse, 1980 ; Yu and Cavaliere, 1983).

Macrophages play an important role in wound healing, both through ingestion of foreign material and tissue debris, and through induction of fibroblastic proliferation (Diegelmann, Cohen and Kaplan, 1981).

Phagocytic activity may result in enlargement and vacuolisation of the macrophage (Piller and Clodius, 1979).

The large vacuolated macrophages noted in relation to Surgicel in the optical microscopy component of the present study and by Lebendiger et al. (1959), Hurwitt et al. (1960), Jantet and Rob (1960) and Garach (1982) possibly indicated phagocytosis of Surgicel by these cells. However, in the present study it is felt that conclusions regarding phagocytosis of Surgicel by macrophages, based on examination of haematoxylin and eosin stained paraffin sections, must be made with extreme caution due to the inability to unequivocally identify the material within the cells. Furthermore, vacuolated macrophages were also observed in control sections and, consequently, a dual role of wound debridement must not be overlooked for the macrophage in this system.

The presence of an ingrowth of granulation tissue into the tube lumen of experimental and control specimens at 48 hours in this study was a feature also noted by Garach (1982) in both his control and experimental 7-day specimens, but not in his 2-day specimens. The 7-day sections in Garach's study were stained with Van Gieson's stain which demonstrated the presence of collagen in the tissue wedge. The appearance of the tissue ingrowth in the present study was similar to that noted by Garach and was consistent with that of granulation tissue.

The methods by which the granulation tissue ingrowth could have proceeded were reviewed by Garach (1982). Fibrin meshwork in the serous fluid filling the tubes provides a scaffolding along which fibroblasts and blood vessels can extend (Torneck, 1966). An alternative mechanism, offered by the same author, related to the direct extension of blood

vessel endothelial cells and fibroblasts into the lumina of the Teflon tubes. According to Walter and Israel (1979), endothelial cells are capable of forming pseudopodia which anastomose with their neighbours to form a series of vascular arcades. These arcades provide active fibroblasts with a rich supply for nutrition, as well as a scaffolding along which they can migrate (du Plessis, 1970 ; Forrester, 1976).

Garach (1982) compared the mean percentage ingrowth of tissue in control sections and experimental sections and found a difference at 7 days (34.66% and 20.96% respectively) but not at later recovery times. He postulated that Surgicel delayed ingrowth of tissue into the tube lumina during the first week post-implantation, the period during which the material was undergoing resorption. The figures, however, when subjected to the student's t-test, did not reveal a significant difference at the 5% level.

Although small numbers of paraffin-embedded haematoxylin and eosin stained sections were examined in the present study, it is of interest to compare observations with those of Garach (1982) with respect to ingrowth of granulation tissue. Not only was such a feature evident at 48 hours in the present study (compared with 7 days, Garach), but it was observed in both experimental and control specimens. If it is possible to extrapolate the effects of Surgicel on healing from the present study, it would appear that the material does not delay healing. This proposal is supported by the observations of Miller et al. (1961b), Shea (1962) and Huggins (1969) in clinical experience with the material, and further by the studies of Skoog (1967) and Thilander and Stenstrom (1969) who concluded that Surgicel had a beneficial effect on bone healing. However, Rosenquist and Finne (1974), Engdahl (1972),

Uddströmer (1978) and Olson et al. (1982) concluded that Surgicel delayed bone healing, especially in the earlier stages.

3.2 Alcian Blue Stained Material

Scott and Dorling (1965) first described the application of the critical electrolyte concentration (CEC) concept to the differentiation of acid glycosaminoglycans. Alcian Blue 8GX (a cationic dye) stoichiometricaly binds polyanions with increasing selectivity as increasing amounts of $MgCl_2$ (the electrolyte) are incorporated into the dye solution.

Glycosaminoglycans characterized thus far are densely negatively charged because of the presence of sulphate ester groups and/or carboxyl groups of uronic acid. In consequence, they bind, and are precipitated by, cationic dyes. Scott and Dorling (1965) showed that specificity of binding could be introduced by preventing combination of dye with one or more members of the polyanionic group and further demonstrated that the binding of dye to carboxylate (COO^-) or phosphate ($PO_4^=$) groups ceased at low electrolyte concentrations (< 0.3M) whereas dye continued to be held by sulphate ester groups at concentrations 5-10 times greater. In practice, all COO^- containing polyanions do not have the same CEC because the molecular weight and the distance between anionic sites (charge density) affects the binding of the neighbouring cations.

The similarity of oxidized regenerated cellulose to hyaluronic acid and other glycosaminoglycans prompted the application of Scott and Dorling's concepts in the present study. Preliminary investigations were

modelled on the *in vitro* filter paper experiments of these authors in order to , firstly, determine the CEC of Surgicel and, secondly, to compare it with those of other GAGS and tissue polyanions in order to establish specificity of staining.

Determination of the CEC of Surgicel on filter papers revealed that staining was most intense using 0.1M $MgCl_2$ and became significantly weaker at 0.2M. Thus, a staining differential existed between 0.1 and 0.2M $MgCl_2$. Comparison with the CEC's of other secreted tissue polyanions confirmed a specificity for Surgicel at this concentration (see Table 14).

In the present study, the results of Alcian Blue staining of Surgicel implants recovered 6 hours post-operatively, using different $MgCl_2$ concentrations, demonstrated a CEC of 0.15 M $MgCl_2$. Staining of control tubes did not reveal any intensely stained material at any of the $MgCl_2$ concentrations employed. It was concluded from these observations that the uronic acid component of Surgicel could be stained specifically and selectively with Alcian Blue 8GX at a critical electrolyte concentration of 0.15M $MgCl_2$.

Staining of Surgicel specimens recovered at 18, 24 and 48 hours post-implantation showed no intensely or selectively stained material at any of the $MgCl_2$ concentrations, nor was any demonstrable in control sections. These results indicated that Surgicel was not present at these recovery times in its polyanhydroglucuronic acid form. Such a conclusion is consistent with the results of the uronic acid analyses which demonstrated a dramatic loss of uronic acid after 6 hours post-implantation in Surgicel implants.

Type of Anionic Group	Polyanion	MOLARITY of $MgCl_2$									
		0.0	0.025	0.05	0.1	0.2	0.3	0.45	0.65	0.8	1.0
$-COO^-$	Hyaluronate	++	++	++	∓	-	-	-	-	-	-
$=PO_4^-$	RNA	∓	+	++	++	∓	-	-	-	-	-
	DNA	∓	++	++	+++	∓	-	-	-	-	-
$-COO^-$ and $-OSO_3^-$	Heparin	∓	++	++	+++	+++	+++	+++	+++	∓	-
$-OSO_3^-$	Keratan sulphate	++	++	++	+++	+++	+++	+++	+++	+++	∓

Table 14 Staining of spots containing polyanions on filter paper in Alcian Blue - pH 5.8 - $MgCl_2$. (Adapted from Scott and Dorling, 1965).

3.3 Transmission Electron Microscopy

Although the use of Teflon tubes aided localization of the implant for ultrastructural examination, it hindered the tethering of ultrathin sections for the following reasons:

(1) Lack of adhesion between tube and embedding resin necessitated the dependence of tube immobilisation on mechanical retention alone. When preparing the block face for sectioning, removal of resin surrounding the tube occasionally led to its dislodgement.

(2) Ultrathin silver sections often proved difficult to obtain due to the elasticity, or "sponginess", of the tube which caused a reverberation of the block as it passed the knife edge.

(3) Inadequate infiltration of embedding resin (due to its high viscosity) into the tube lumen produced further initial difficulties in obtaining ultrathin sections. Not only did the porosity of the resin create difficulties in cutting the block, it produced "chatter" lines on silver sections due to "pitting" of the block face. Such sections were easily split by a low intensity electron beam. In order to overcome this problem, subsequent blocks of tissue, embedded in pre-polymerized resin, were placed in a vacuum chamber until bubbles were no longer detectable in the specimens. Embedded blocks were left to infiltrate for 48 hours prior to polymerization of the resin.

The basic ultrastructural appearance of Surgicel at 6 hours post-implantation was similar to its native appearance at T = 0 (compare Figs

78 and 74), after fixation and processing for transmission electron microscopy. However, in 6 hour specimens, the scalloped edge of each fibre appeared less well-demarcated and the border of the fibres exhibited a "moth-eaten" appearance (Fig. 79), possibly indicative of early biodegradation. No cells were observed at 6 hours post-implantation in association with the Surgicel. This, however, probably reflects inherent sampling limitations.

By 18 hours, polymorphonuclear leucocytes (PMNs) were the predominant cell type present in T.E.M. specimens. This was in accordance with optical microscopic observations. Material exhibiting morphological features consistent with Surgicel was apparent extracellularly. Higher magnification views of phagolysosome contents in PMNs did not reveal the presence of Surgicel-like material. At 18 hours, Surgicel-like material was occasionally noted in PMNs (for example, refer to Figures 84-86). However, this material was not contained within membrane-bound phagosomes. Furthermore, although having a Surgicel-like morphology, it could not be conclusively identified as Surgicel on a morphological basis.

In 24 hour specimens, macrophages were more abundant. Surgicel-like material was still present extracellularly, but analysis of macrophage phagolysosome contents revealed the presence of material similar in morphology to that of Surgicel at 0 hours (for example, compare Figures 87 and 74).

At 48 hours, extracellular Surgicel-like material was not evident, but large, vacuolated macrophages were abundant. Higher magnification views of phagolysosome contents revealed morphologic compatibility

between this material within phagolysosomes and Surgicel at 0 hours. No material resembling Surgicel was present either extracellularly or intracellularly in control sections at any of the recovery times, nor was it evident in PMN phagosomes on examination at higher magnifications.

It was proposed, on the basis of these ultrastructural observations, that early catabolism of Surgicel (i.e. at 6 and 18 hours) involved extracellular breakdown, possibly through the secretion of hydrolytic enzymes by macrophages. Further absorption (at 24 and 48 hours) appeared to be mediated by macrophage phagocytosis and intracellular digestion through the release of lysosomal enzymes into the phagosomes. Such conclusions concerning the fate of Surgicel, based on ultrastructural findings, were further supported by the results of uronic acid assays of implants, and by the Alcian Blue staining of specimens at the 0.15 M $MgCl_2$ level.

The presence of myelin figures is considered by some to be representative of macrophage activation via phagocytosis (Fedorko and Hirsch, 1970). Others, however, have suggested that such structures represent post-gutaraldehyde fixation artefacts (Glauert, 1975 ; Carr, 1981 ; Robinson, 1982). Interestingly, while myelin figures were found in macrophages from Surgicel specimens, no such structures were observed in macrophages from control implant specimens. This observation tends to reinforce the concepts of Fedorko and Hirsch (1970) who regard myelin figures as being indicative of active phagocytosis.

4. HISTOLOGY : FIBROUS RESIDUE OF SURGICEL

The observation that Surgicel was not completely solubilized by 4M Gu.HCl (see Section 4) led to speculation concerning the finer composition of the manufactured product as presented on the market. Hascall and Sajdera (1969) showed 4M Gu.HCl to be an efficient dissociative solvent for isolation of tissue uronates. However, in the present investigations, a fibrous residue, clearly visible to the naked eye, remained after prolonged and repeated attempts at dissolution of Surgicel in 4M Gu.HCl.

Accordingly, this fibrous residue was implanted in rat muscle using the same protocol as that used for implanted Surgicel. Implants were recovered at 6, 18, 24 and 48 hours. Paraffin sections obtained for histology were stained with Alcian Blue at a $MgCl_2$ concentration of 0.15M, or haematoxylin and eosin. Absence of definitive staining of tube contents by Alcian Blue at the CEC for whole Surgicel revealed that no polyanhydroglucuronic acid was present at T = 0 or at any other post-implantation recovery time. Haematoxylin and eosin staining revealed a fine, basophilic fibrillar material in experimental tubes at 0 hours, (but not in corresponding control tubes). This material was also noted in trace amounts near the entrance to the tube lumen at all post-implantation recovery times.

No granulation tissue ingrowth was observed at any post-implantation recovery time, but organization of tissue at either end of the tube had commenced at 48 hours. Vacuolated macrophages were also noted using optical microscopy, especially at 48 hours post-

implantation. It was hypothesized that these cells were involved in both phagocytosis of the fibrous residue and wound debridement.

Further examination of the nature and fate of this fibrous component in vivo was pursued by means of ultrastructural studies. T.E.M. analysis of the fibrous residue at T = 0 revealed a lobulated fibrillar structure, reminiscent of that observed for native Surgicel, but exhibiting greater heterogeneity of density. A comparable morphology was evident at 6 hours post-implantation but, like its Surgicel counterpart at 6 hours, small areas of erosion were evident at the edges of the fibres (see Figures 111 and 112).

At 24 and 48 hours post-implantation, large macrophage phagolysosomes contained fibrous material similar in appearance to that seen in Surgicel specimen macrophages at comparable times. No such material was observed extracellularly at either of these recovery times, nor was it demonstrable in PMN phagolysosomes. Myelin figures were evident in both 24 and 48 hour macrophages.

The findings of this section of the study would appear to support the conclusions drawn from earlier experiments: namely, that the macrophage plays a central role in phagocytosis of Surgicel, and, secondly, that Surgicel is being digested intracellularly by 24 hours. Furthermore, it is proposed that Surgicel consists of at least two potentially reactive components. The readily-soluble uronic acid component is lost from implants and degraded extracellularly or cleared systemically within 18 hours, whilst the fibrous residue requires macrophage phagocytosis for subsequent clearance.

5. THE FATE OF SURGICEL : A PROPOSAL

It has been proposed that the absorption of Surgicel is due to a combination of extracellular breakdown (or systemic clearance) and macrophage phagocytosis. We are, as yet, ignorant of the true chemical and conformational status of Surgicel. However, it is pertinent to speculate on the mechanisms operative at these levels. In this context, a search for an analogous physiological situation is appropriate : do similar polymers exist in any mammalian tissues? Glucuronic acid per se frequently occurs in the body as a detoxifying agent (Walker, Boyd and Asimov, 1957a). However, it is not present as an exclusive polymer, but as a monosaccharide in association with a uridine coenzyme.

Dawson (1978) documented the types of disaccharide units found thus far in complex carbohydrates of animal origin, but no disaccharide unit resembling that of Surgicel has yet been reported. Nevertheless, as mentioned above, there is some similarity between certain GAG polymers (especially hyaluronic acid) and Surgicel. A review of the mechanisms involved in the catabolism of the former may lead to some conjecture regarding Surgicel breakdown.

Eriksson, Fraser, Laurent, Pertoft and Smedsrød (1983) have reviewed the catabolism of hyaluronic acid. It enters the general circulation from the lymph (Laurent and Laurent, 1981) and is rapidly removed from the bloodstream (Fraser, Laurent, Engström-Laurent and Laurent, 1984). Although the main site of uptake is the liver (Fraser, Laurent, Pertoft and Baxter, 1981 ; Fraser, Appelgren and Laurent, 1983), autoradiography indicates small amounts of uptake by the spleen, bone marrow and lymph nodes (Fraser et al., 1983). Using cell culture

techniques, Eriksson et al. (1983) demonstrated that hyaluronic acid was taken up by rat liver endothelial cells where the polysaccharide was degraded into low molecular weight products which were excreted in the urine. Most of these products had the molecular size of monosaccharides. Larger fractions, which formed a very small proportion, were proposed by Fraser et al. (1984) to arise from degradation by either urinary or renal hyaluronidase.

Fraser et al. (1983) demonstrated a high affinity for hyaluronic acid by bone marrow and lymph nodes, as well as liver and spleen, and suggested that this property was shared by cellular elements of the reticulo-endothelial system. Furthermore, the reactions of GAGS with isolated cells in vitro supported the notion of distinct binding sites, or at least of widely varied affinities, depending on the GAG, tissue and type of cell. It has been demonstrated that the stimulation of pinocytosis in mouse peritoneal macrophages is influenced by the charge and molecular weight of anionic polysaccharides. Furthermore, hyaluronic acid aggregates pulmonary alveolar and peritoneal macrophages, depending on species and prior stimulation (Love, Shannon, Myrvik and Lynn, 1979). Fraser et al. (1983) concluded that there was still a need to identify cellular binding sites in tissues other than the liver.

The final metabolic fate of the constituent N-acetyl-glucosamine and glucuronic acid of hyaluronic acid have yet to be determined, but Fraser et al. (1984) suggested that the end products of hyaluronic acid degradation might include any metabolite derived from Acetyl CoA. Furthermore, Revell and Muir (1972) reported that the GAG levels in normal urine in vivo were small and much less than the probable turnover of proteoglycans. These authors suggested that the initial breakdown

products of proteoglycans were taken up by cells of the body and degraded to low molecular weight products no longer identifiable as originating from proteoglycans.

Rodén (1980) reviewed the catabolism of GAGS at a molecular level. Generally, a battery of exoenzymes remove glycosyl and sulphate groups and an additional concert of endoenzymes of different specificities cleave internal sugar linkages. Currently, individual enzymes are being characterized and purified but there is still insufficient information regarding integration of their activities within the cell. It is likely that most polymeric sugars are degraded to oligosaccharides.

In the catabolism of hyaluronic acid, Leaback (1970) and Rodén (1980) suggested that the endoenzyme hyaluronidase, a β-N-acetyl-hexosaminidase, digested hyaluronic acid to oligosaccharides which could be degraded further to monosaccharides by the two exo-enzymes, β-glucuronidase and β-N-acetyl-hexosaminidase. Several β-glucuronidases of varying specificities have been identified : Rodén (1980) suggested that β-glucuronidase was capable of removing glucuronic acid residues from glucuronic acid containing GAGS when such residues were located in non-reducing terminal positions of tetrasaccharides or larger fragments.

In consideration of the above, several hypotheses are offered concerning the catabolism of oxidized regenerated cellulose by body tissues:

(1) The readily soluble component of Surgicel may be systemically cleared from the extracellular compartment, either through lymph or blood circulation. This would explain the rapid loss of uronic acid

from the implant system. Further studies, employing radioactively labelled material and autoradiography (according to the method of Fraser et al. (1983)) would be necessary to determine if this actually occurs, and to delineate the nature of such a clearance mechanism and the pattern of distribution of the material throughout the body.

(2) The readily soluble component of Surgicel may be degraded, either partially or completely, by local extracellular mechanisms. Macrophage-derived secretory products could be responsible for breakdown at the site of implantation. It has been shown that oxygen-derived free radicals, similar to those released by tissue macrophages, are capable of depolymerizing both gingival proteoglycans and hyaluronic acid (Bartold and Wiebkin, 1984). Lysosomal acid hydrolases, secreted by activated macrophages (Davies, 1981) may play a role in the catabolism of Surgicel. However, a depolymerase, specific for the β-1,4 glycosidic link between adjacent glucuronic acid residues in Surgicel remains to be identified. A relatively non-specific β-glucuronidase may be involved in degradation but extracellular release of such an enzyme also remains to be demonstrated.

(3) Intracelluar lysosomal digestion appears to be responsible for breakdown of the more insoluble component of Surgicel after 18 hours post-implantation. Speculation regarding the molecular interactions responsible for this digestion can only be of a limited nature, considering that the chemical conformation of the fibrous component has yet to be studied. Nevertheless,

depolymerization of the polyglucuronate may occur by several mechanisms.

Miller et al. (1961a) reported that digestion of oxidized regenerated cellulose did not occur in the presence of β-glucuronidase in vitro. (β-glucuronidase has been consistently identified in macrophage lysosomes). There may be several explanations for their observations : (a) the polyglucuronate may well be non-susceptible to attack by any β-glucuronidase; (b) many β-glucuronidases have been characterized since 1961 and that used by Miller et al. may not have possessed the appropriate specificity of binding; (c) breakdown of the polyglucuronate could require an initial depolymerization by a hereto unidentified endoenzyme, proceeded by β-glucuronidase (an exoenzyme) hydrolysis. This mechanism is analogous to that responsible for enzymatic degradation of hyaluronic acid in liver endothelial cells. Alternatively, Surgicel catabolism may involve an initial alteration in the conformational status of the sugar residues, followed by enzymatic lysis of the β-1,4 link. (It is well accepted that uronic acids have the property of readily forming cyclic esters called lactones and, accordingly, glucuronic acid easily forms glucuronolactone (Walker et al., 1957b)).

(4) Considering the multi-component nature of the product, and the fact that only 72.84% of sugar residues are claimed to be oxidized, it is concluded that a multi-enzyme system is responsible for degradation of oxidized regenerated cellulose.

CHAPTER SIX

SUMMARY

CHAPTER 6

SUMMARY

In the present investigation, the time required for Surgicel to be absorbed in rat muscles and the mechanisms by which such a process occurs were investigated. Following initial chemical characterization of the material, it was inserted into Teflon tubing for implantation studies. Empty tubes served as controls.

Animals were sacrificed at T = 6, 18, 24, 48 hours and 7 days post-implantation. Selected specimens were processed according to one of the following techniques:

(1) T = 0, 6, 18, 24, 48 hour and 7 day specimens were quantitatively assayed for the presence of uronic acid. A loss of polyuronate from the implant system was assumed as being indicative of Surgicel catabolism (absorption).

(2) T = 6, 18, 24 and 48 hour specimens were processed for histological examination and stained with Alcian Blue 8GX at a magnesium chloride concentration of 0.15 M, in order to selectively demonstrate the presence of Surgicel microscopically.

(3) T = 0, 6, 18, 24 and 48 hour specimens were processed for optical microscopy and stained with haematoxylin and eosin. Examination criteria included the identification of Surgicel in conventionally-stained sections, the presence of phagocytes at the implantation site and the general biologic response to the material.

(4) T = 0, 6, 18, 24 and 48 hour specimens were processed for transmission electron microscopy. Criteria used in examination of sections included identification of Surgicel at an ultrastructural level and localization of the material and/or its breakdown products intra- and extracellularly.

A fibrous residue, which remained after prolonged dissociative salt extraction of Surgicel, was also implanted. After recovery at T = 0, 6, 18, 24 and 48 hours, these specimens were processed for both optical and transmission electron microscopy. Sections obtained for optical microscopy were stained with Alcian Blue 8GX at a magnesium chloride concentration of 0.15 M, or haematoxylin and eosin. Criteria used in examination of fibrous residue specimens were similar to those described for Surgicel implants.

The conclusions drawn from this study were:

(1) Surgicel is an absorbable local haemostatic agent. This finding is in accord with the observations of Hurwitt et al. (1960), Jantet and Rob (1960), Georgiade et al. (1961), Miller and Thomas (1961), Skoog (1967), Thilander and Stenström (1969), Larsson et al. (1978) and Garach (1982).

(2) Surgicel consists of at least two active components. These are (a) a soluble uronic acid component which is absorbed after 6 hours post-implantation and (b) a fibrous component which persists. The latter material resembles Surgicel in the electron microscope and is still evident at the implantation site 48 hours post-implantation. Moreover, Surgicel can be characterized <u>in vitro</u> into

at least two components according to its solubility under dissociative salt conditions (4 M guanidinium chloride). A residual fibrous material could then by hydrolyzed by 0.3 N sodium hydroxide.

(3) Surgicel, an analogue of tissue polyanions such as hyaluronic acid and chondroitin sulphate, can be selectively stained using Alcian Blue 8GX at a magnesium chloride concentration of 0.15 M (The Critical Electrolyte Concentration).

(4) It appears that absorption of the salt-soluble uronate in vivo is effected by early extracellular degradation and/or systemic clearance whilst removal of the fibrous material requires macrophage phagocytosis. Future investigations such as enzyme histochemical assays and whole body autoradiography would be required to further delineate these absorption mechanisms.

APPENDICES

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APPENDIX 1

1. HOUSING OF EXPERIMENTAL ANIMALS

All experimental animals were housed in RB 3 polypropylene cages with stainless steel tops. The dimensions of the cages were 45cm x 28cm x 22cm, and they were manufactured by North Kent Plastic Cages Ltd., England. No more than three rats were placed in each cage.

2. DIET OF EXPERIMENTAL ANIMALS

All experimental animals were fed on Charlick's Feeds Mouse Ration, manufactured by Wm. Charlick Ltd., South Australia.

Contents:	Minimum Crude Protein	21.0%
	Minimum Crude Fat	3.5%
	Maximum Crude Fibre	6.0%
	Minimum Vitamin A Content	10000.0 I.U./kg
	Minimum Vitamin D Content	2000.0 I.U./kg
	Maximum Added Salt	0.5%
	Ethoxyquin	100 p.p.m.

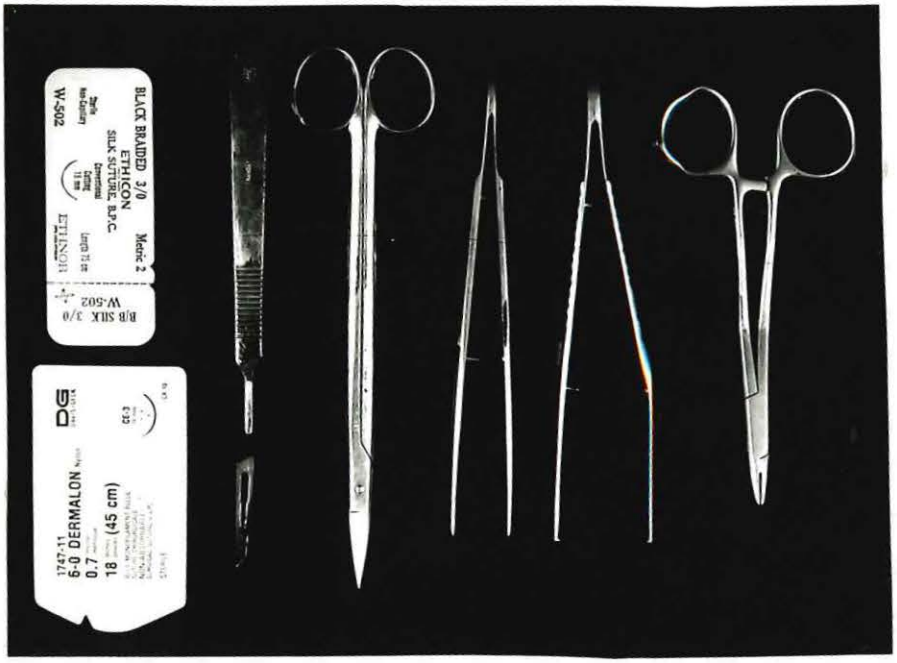
Ingredients

Wheat, Barley, Bran, Pollard, Lucerne, Meat Meal, Soyabean Meal, Yeast, Cottonseed Meal, Fish Meal, Salt, Dicalcium Phopshate, Vitamins, Minerals and Antioxidant.

APPENDIX 2

INSTRUMENTS USED IN THE OPERATIVE PROCEDURE

(1) Mayo Needle Holder
(2) Gillies Tissue Forceps
(3) College Tweezers
(4) Kelly Scissors
(5) No. 3 Scalpel Handle
(6) No. 15 Scalpel Blade
(7) 3/0 Black Silk Sutures
(8) 6/0 Nylon Sutures



APPENDIX 3

Method of Uronic Acid Assay - (Blumenkrantz and Asboe-Hansen, 1975)

REAGENTS

- Metahydroxydiphenyl (m HDP) : 0.15% solution of mHDP in 0.5% NaOH. Kept at -4°C and covered with aluminimum foil for no longer than 1 month.

- Sulphuric Acid/sodium tetraborate solution : 0.0125 M solution of tetraborate in H_2SO_4 conc.

METHOD

To a 0.2 ml sample containing 0.5 - 20 g uronic acid add 1.2 ml of H_2SO_4/tetraborate solution and refrigerate in crushed ice. Shake on vortex and heat in boiling water bath for exactly 5 minutes. After cooling in water-ice bath, add 20 l mHDP. Read the test-tube solutions at 520 nm on a spectrophotometer after at least 5 minutes against a blank of 0.5 M sodium acetate. Before assaying test samples, prepare a standard curve between 1 and 100 g/ml of glucuronic acid.

Note: Subsequent to the addition of mHDP in the final stages of the second assay experiment, all samples for spectrophotometry assumed a yellow tinge, which appeared to be superimposed upon any pink colour present in the test tubes. Assuming that this was due to some form of

contamination, the assay was repeated using freshly prepared reagents. The yellow colour persisted and absorbances were measured against a yellow blank at 520 nm. Those of pure standards were directly proportional to uronic acid concentration.

The subsequent utilization of a glass rod instead of the magnetic flea used formerly in stirring assay reagents resulted in elimination of the yellow contamination. Evidently, iron ions were being leached from the Teflon-covered flea by concentrated sulphuric acid.

APPENDIX 4

QUANTITATION OF ASSAY DATA

1. Calculation of Dry Weight from Wet Weight of Hydrated Surgicel

8 randomly cut samples of Surgicel were hydrated in Hank's B.S.S./Bovine Albumin solution for 12 hours. Weights were measured pre- and post- hydration and a hydration factor calculated as follows:

Dry Weight (mg)	Wet Weight (mg)
32	127
27	122
28	131
22	76
35	86
31	144
25	142
26	124
Average Weight (mg) 28.25	119

HYDRATION FACTOR = 4.212

2. Standardization of data from fifth uronic acid assay

1. Surgicel Implants

T (hours)	6	18	24	48
A. Average tube length (mm)	3.326	3.377	3.362	2.969
B. Weight of tube containing Surgicel (mg) (from Fig. 35)	11.53	11.8	11.7	10.34
C. Weight of tube (mg) (from Fig. 33)	8.92	9.05	9.0	8.0
D. Wet weight of Surgicel (B-C) (mg)	2.61	2.75	2.7	2.34
E. Total wet weight of Surgicel in 8 tubes (mg) (D x 8)	20.88	22.0	21.6	18.72
F. Dry weight of Surgicel (mg) (E ÷ 4.212, the hydration factor)	4.96	5.23	5.13	4.45
G. Absorbance (nm)	2.263	0.014	0.015	0.005
H. µg Glucuronic Acid/ 2ml sample	332.8	2	2	0.6
I. µg Glucuronic Acid/ 10mg Surgicel (dry weight) implanted $\frac{(H \times 10)}{F}$	670.96	3.82	3.89	1.348

2. Control Implants

T (hours)	6	18	24	48
A	3.712	3.748	3.718	3.732
B	13.62			
C	10.0			
D	3.62			
E	28.96			
F	6.88			
G	0.136	0	0	0
H	16.0			
I	23.25	0	0	0

APPENDIX 5

HISTOLOGICAL AND HISTOCHEMICAL TECHNIQUES

1. OPTICAL MICROSCOPY

1.1 Tissue Fixation

Specimens for optical microscopy were fixed in neutral buffered formalin.

Recipe:	Formalin	500ml
	Tap Water	4500ml
	Acid Sodium Phosphate Monohydrate	20g
	Anhydrous Disodium Phosphate	32g

1.2 Embedding Procedure

All specimens were processed in the following reagents:

(1)	Alcohol 70%	Overnight
(2)	Absolute Alcohol	2 Hours
(3)	Absolute Alcohol	2 Hours
(4)	Absolute Alcohol	2 Hours
(5)	Absolute Alcohol	2 Hours
(6)	Methyl Salicylate and Absolute Alcohol (50:50)	Overnight
(7)	Methyl Salicylate	8 Hours
(8)	Methyl Salicylate	8 Hours

(9)	Methyl Salicylate and Celloidin 1%	2 Days
(10)	Methyl Salicylate and Wax (50:50)	1 Hour
(11)	Wax 100%	2 Hours
(12)	Wax 100%	2 Hours
(13)	Wax 100%	2 Hours

All specimens were placed in a vacuum chamber for 1 hour for the evacuation of air bubbles, and subsequently blocked in Paraplast +. 7µ sections were mounted from warm water on gelatin-coated slides and dried at 37°C.

1.3 Staining Procedures

1.3.1 Haematoxylin and Eosin Stain

METHOD

(1) Xylol 2 to 5 minutes
(2) Xylol 2 to 5 minutes
(3) Absolute alcohol 2 to 5 minutes
(4) Absolute alcohol 2 to 5 minutes
(5) Dip in tap water
(6) Harris' Haematoxylin 3 to 5 minutes
(7) Tap water 5 minutes
(8) Differentiate in 0.5 to 1.0% hydrochloric acid in 70% alcohol for 30 seconds
(9) Immerse in running tap water for 10 minutes
(10) Eosin 45 seconds
(11) Absolute alcohol 1 minute

(12) Absolute alcohol 1 minute

(13) Xylol 1 minute

(14) Xylol 1 minute

(15) Mount in Depex

RESULTS

Nuclei	: Blue to blue black
Karyosomes	: Dark blue
Cartilage	: Pink or light blue
Cement lines of bone	: Blue
Calcium and calcified bone	: Purplish blue
Basophil cytoplasm	: Purplish
Red blood cells, eosinophil granules, zymogen granules	: Bright orange red

1.3.2 Alcian Blue : Filter Papers (Scott and Dorling, 1965)

REAGENTS:

- Polyanion (Surgicel) : 1% w/v solution
- Alcian Blue 8GX : 0.05% w/v in 0.05M sodium acetate buffer at pH 5.8
- Magnesium chloride ($MgCl_2$) : 1.5M stock solution

Solutions were prepared by mixing dye, buffer, 1.5M $MgCl_2$ and distilled water to give 50 ml of solution containing the required concentration of $MgCl_2$.

METHOD

Circles of Whatman No. 1 filter paper were spotted with 2 µl samples of polyanion solution and dried in air. They were placed in beakers each containing 50 ml Alcian Blue/sodium acetate solution containing $MgCl_2$ at the following concentrations : 0 M ; 0.05 M ; 0.1 M ; 0.2 M ; 0.3 M ; 0.45 M ; 0.65 M and 0.8 M. After 1 hour, circles were removed, washed rapidly in a large volume of distilled water and dried in air.

1.3.3 Alcian Blue : Histologic Sections (Scott and Dorling, 1965).

REAGENTS:

- 0.05% Alcian Blue 8GX in pH 5.8 0.025 M sodium acetate buffer
- Magnesium chloride : 1.5 M solution.
  Solutions were prepared by mixing dye, buffer, 1.5 M $MgCl_2$ and distilled water to give 50 ml of solution containing the required concentration of $MgCl_2$.

METHODS

1. Xylene for 4 minutes
2. Xylene for 4 minutes
3. Absolute alcohol for 2 minutes
4. 90% alcohol for 2 minutes
5. 70% alcohol for 2 minutes
6. 50 ml of Alcian Blue solution (containing magnesium chloride at the following concentrations : 0 M ; 0.1 M ; 0.15 M ; 0.2 M ; 0.3 M ; 0.55 M ; 1.0 M and 1.25 M) overnight at room temperature in upright Coplin jars.
7. Rinse individually in a stream of distilled water

8. Distilled water bath
9. 70% alcohol for 2 minutes
10. 90% alcohol for 2 minutes
11. Absolute alcohol for 2 minutes
12. Xylene for 4 minutes
13. Xylene for 4 minutes
14. Mount in Depex

2. TRANSMISSION ELECTRON MICROSCOPY

2.1 Tissue Fixation

2.5% Glutaraldehyde solution

REAGENTS:

- Sorenson's Buffer (0.2 M Na_2HPO_4 and 0.2 M NaH_2PO_4)
- Glutaraldehyde solution 25%
- Glucose

METHOD

To 100 ml of 0.1 M Sorenson's buffer at pH 7.2 add 20 ml Glutaraldehyde solution and make up to 200 ml in distilled water. Add glucose at the rate of 6 g%.

2.2 Post-fixation and Embedding Procedures

REAGENTS

- 0.1 M Sorenson's buffer containing 6 g% glucose
- 2% osmium tetroxide (OsO_4) : add 1 g OsO_4 to 25 ml 0.1 M Sorenson's buffer and make up to 50 ml in distilled water.
- TAAB epoxy resin : 50 ml TAAB embedding resin
  45 ml DDSA
  5 ml MNA
  2 ml DMP-30
- Proplylene oxide

METHOD

For a trimmed sample of 3mm x 3mm:

1.	Sorenson's buffer for	15 minutes
2.	Sorenson's buffer for	15 minutes
3.	Osmium tetroxide for	2 hours
4.	Sorenson's buffer for	15 minutes
5.	Sorenson's buffer for	15 minutes
6.	50% alcohol for	30 minutes
7.	70% alcohol for	30 minutes
8.	90% alcohol for	30 minutes
9.	100% alcohol over $CuSO_4$ for	30 minutes
10.	100% alcohol over $CuSO_4$ for	30 minutes
11.	Propylene oxide for	15 minutes
12.	Propylene oxide for	15 minutes
13.	Propylene Oxide : TAAB 2:1 for	30 minutes

14.	Propylene Oxide : TAAB 1:2 for	30 minutes
15.	TAAB	Overnight
16.	TAAB for	48 hours
17.	Place in 60°C oven to polymerize TAAB for	48 hours

2.3 Staining Procedures

Toluidine Blue for 1 sections:

1. Flatten sections with chloroform
2. Dry sections on a hotplate at 90°C for 1 minute
3. Add toluidine blue in 1% aqueous borax solution and dry on hotplate for 1–2 minutes
4. Flood sections with millipored water
5. Differentiate with 50% alcohol
6. Dry on hotplate
7. Clear in xylene
8. Mount in Depex.

Uranyl acetate/lead citrate for ultrathin sections:

REAGENTS:

1. Uranyl acetate : 5% solution at 37°C
2. Reynold's lead citrate stain :

Add 1.33 g lead nitrate and 1.76 g of sodium citrate to 30 ml distilled water and shake vigorously for 1 minute. A heavy white precipitate will form. Shake the solution every 5 minutes over a 30

minute period to facilitate conversion of lead nitrate to lead citrate. Add 8 ml 1N solution of sodium hydroxide (carbonate free) with agitation and dilute to 50 ml with distilled water. The white precipitate of lead citrate will dissolve at this step. Store solution in the dark at -4°C.

STAINING METHODS:

Cover 2 petri dishes with plastic film and place one in a box containing wet tissue paper and keep on a hotplate at 37°C. Place the other dish in a tightly-sealed box containing potassium hydroxide pellets and keep at room temperature. Filter both stains through millipore filters and place drops of uranyl acetate on to the plastic film in the container kept at 37°C. Likewise, place drops of lead on to the plastic film in the other box. Float the grid, with sections facing downwards, on a uranyl acetate drop for 1.5 - 2 minutes. Rinse in 2 changes of millipored distilled water at 37°C. Allow the grid to dry on filter paper. Float the dry grid on a lead drop for at least 30 seconds, making sure that the box remains tightly sealed to avoid stain precipitation. Rinse in 2 changes of millipored distilled water at room temperature. Leave to dry for a few minutes. Sections are now ready for viewing.

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