Studies of the Inflammatory Potential of Hydroxyapatite

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SUMMARY

Apatite deposition disease, in which hydroxyapatite crystals (HAC) of submicroscopic size are found in the synovial fluids of some patients with osteoarthritis has been recently defined as a clinical syndrome. It is now established as a crystal-related arthropathy alongside gout and pseudo-gout. This study was undertaken to assess the role of HAC in destructive joint disease, primarily as an effect of any phlogistic potential. The investigation was divided into four main sections, involving a wide field of in vivo and in vitro methodologies.

1. A semi-quantitative assay of the inflammatory potential of HAC was developed using subcutaneously implanted polyurethane sponges in rats. The sponges had been impregnated with commercially obtained HAC prior to implantation. Plain sponges were implanted as controls. A method for the quantitation of sponge dry weight gain and extent of ingrowth of granulation tissue was developed. Light microscopy and transmission electron microscopy (TEM) were extensively used. Implantations in which the amount of HAC per sponge, the physical characteristics of HAC and the time of implantation were varied were carried out. The effect of antiinflammatory and antirheumatic drugs on the tissue reaction to HAC was investigated. A variety of microcrystalline materials were used in other implantations.

2. The tissue reaction to biologically deposited HAC was studied using the phenomenon of simple calcergy. Again, polyurethane sponges implanted in rats were used as a matrix within which the calcergic reaction was induced (a) by lead acetate injection into sponges into which granulation tissue had grown for 14 days (subsequently studied for up to 21 days) and (b) by the implantation of sponges which had been soaked in a

lead acetate solution prior to implantation and subsequently studied for up to 20 weeks.

Sponge dry weight gain and extent of granulation tissue ingrowth were quantified. Light microscopy and TEM were also used to study these unique models of simple calcergy. Deposits of HAC were found in granulation tissue, in addition to the central zones of the sponges which were unoccupied by tissue.

3. Morphological studies of the interaction of HAC and other microcrystals with cultured murine macrophages and bovine synovial cells in vitro were undertaken. Light microscopy, scanning electron microscopy (SEM) and TEM were used to assess cell response to HAC at different times after addition to the cultures.

4. The effect of intraarticular injection of HAC and other microcrystals was studied in rat knees. Sequential smears of synovial fluids were taken and undecalcified sections through knee joints were prepared to observe the effects of HAC on synovial cells in vivo .

Sponges impregnated with synthetic microcrystalline HAC induced a transitory acute inflammation when implanted into two rat strains. In Dark Agouti rats, HAC accelerated the ingrowth of granulation tissue into the sponges and this stimulation was modified by some of the drugs tested. In general, HAC elicited a macrophage and multinucleated giant cell (MNGC) response with increased density of lymphocytes compared with control sponges. The HAC deposited as part of the calcergic reactions elicited a similar response.

HAC were avidly endocytosed by murine macrophages which took up

large quantities of crystals as early as 4 minutes after the material came into contact with the cells. The HAC did not appear to be toxic to the cells, despite the large amount of material engulfed.

Introduction of HAC into rat knees resulted in a transitory increase in PMN, followed by a rise in the synovial fluid mononuclear cell population. HAC were taken up by synovial and mononuclear cells and synovial thickening was observed around the HAC.

This series of experiments indicated that hydroxyapatite crystals elicited a transient acute inflammation which was followed by a chronic inflammatory response in which macrophages and MNGC were engaged in phagocytosis of the HAC. The response did not appear to be immunologically mediated. The avidity with which cells endocytosed HAC in vitro and its non-toxic nature could implicate the by-products of cellular metabolism during phagocytosis in the induction of altered tissue metabolism viz. the inductive effect of HAC on granulation tissue.

The results of these investigations indicate that HAC can act as a low grade, chronic inflammatory stimulus and as such be relevant to the pathogenesis of osteoarthritis in which synovial thickening is a consistent feature.

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DECLARATION

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

The work described herein has been the subject of the following publications:

HIRSCH, R.S., McCLURE, J. and VERNON-ROBERTS, B. 1983
Induction and Characterisation of Experimental Calcification in Rats.
Journal of Dental Research 62: 679

HIRSCH, R.S. and VERNON-ROBERTS, B. 1983
Anti-inflammatory Drugs in a Model of Chronic Inflammation
Clinical and Experimental Pharmacology and Physiology 10: 675

I hereby give consent for this thesis to be made available for photocopying and loan.

Signed

Robert S. Hirsch
December, 1983

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LIST OF ABBREVIATIONS

ACP	amorphous calcium phosphate
Cl_2MDP	dichloromethylene diphosphonate
CPPD	calcium pyrophosphate dihydrate
DA	Dark Agouti (rats)
DF	degrees of freedom
DNA	deoxyribose nucleic acid
DPA	D-penicillamine
EHDP	disodium ethane-hydroxy-1, 1-diphosphonate
EDAX	energy dispersive analysis of Xrays
EM	electron microscopy
ESR	electron spin resonance
F	F- ratios
h	hours
HAC	(calcium) hydroxyapatite
HE	haematoxylin and eosin
IAP	intraarticular pressure
IgG	immunoglobin G
IgM	immunoglobin M
m	metre(s)
ml	millilitre(s)
mm	millimetre(s)
MC	mast cell(s)
MNGC	multinucleated giant cell(s)
MS	mean squares
MSB	Martius Scarlet Blue
MSU	monosodium urate
nm	nanometre(s)

OD	optical density
OA	osteoarthritis
p	F probabilities
PbAc	lead acetate
PMN	polymorphonuclear leucocytes
PBS	phosphate buffered saline
RA	rheumatoid arthritis
SATM	sodium aurothiomalate
SEM	scanning electron microscopy
SS	sums of squares
TBC	Mycobacterium tuberculosis
TEM	transmission electron microscopy
VK	von Kossa
µ	micron(s)
µg	microgram(s)
µl	microlitre(s)

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Chapter One

Introduction

CHAPTER ONE
INTRODUCTION

Calcium hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, is the natural mineral of bone and teeth. It is also the main constituent of nearly all extraarticular pathological calcifications (Gatter and McCarty, 1967). Hydroxyapatite crystals (HAC) may also be present in the articular cartilage and closely associated synovial, tendinous or ligamentous structures in patients with pathological calcification, and in patients with crystal-induced pseudogout due to calcium pyrophosphate dihydrate (CPPD) (McCarty et al, 1966). Less frequently, tendinous origins of adductor muscles, Achilles tendon insertion sites, and areas adjacent to the intervertebral discs are subject to deposition of HAC (Faure and Daculsi, 1983). Mineral deposits in aortic plaques and costal cartilage also consist of HAC (McCarty et al, 1966).

The ubiquitous formation of calculus by the calcification of dental plaque is another instance of ectopic deposition of HAC. Calculus plays an integral part in the pathogenesis of periodontal disease, which destroys the supporting structures of the teeth. The tenacious attachment of calculus to teeth is largely accomplished by a melding of the calculus matrix to the cementum surface (Canis et al, 1979), since the mineral phase of both structures is calcium hydroxyapatite.

Large deposits of bony or amorphous calcium hydroxyapatite are known to occur in some osteoarthritic soft tissues (Doyle et al, 1979b, Huskisson and Willoughby, 1979), and HAC have been found in the midzone as well as being embedded in the surface of articular cartilage (Ali, 1977, 1980). While the pathological deposition of HAC in cartilage does not appear to be removed if the underlying cause is rect-

ified, such as following parathyroidectomy in hyperparathyroidism associated with chondrocalcinosis (O'Duffy, 1976), it is evident that HAC may be removed during the resorption of calcified cartilage of the tidemark zone during recurrent capillary invasion from the bone marrow in osteoarthritis (OA) (Lane et al, 1975). HAC deposition can be promoted by products of proteoglycan degradation which occurs during the development of fissures in osteoarthritic cartilage (Meachim, 1973). Furthermore, inorganic pyrophosphate is a strong inhibitor of HAC nucleation and the inhibition of HAC deposition in normal articular cartilage may be one of its important physiological roles (Fleisch et al, 1966; Francis, 1969). In OA, metabolic and morphological changes in articular cartilage occur which are predisposing to the formation of matrix vesicles and the deposition of small clumps of HAC (Ali, 1977).

There is growing awareness of the importance of arthropathies associated with HAC deposition, judging by the increasing frequency of reports of clinical and laboratory investigations in the last five years. Recently, two important additions to the literature have appeared, one being the publication of the book "Crystals and Joint Disease" by Dieppe and Calvert (1983) in which all known interactions of crystals and joints have been documented and discussed. The second is the supplement to Volume 42, Annals of the Rheumatic Diseases (1983) entitled "Crystal-Related Arthropathies" in which the clinical syndromes associated with crystal depostion are reviewed and new research data presented.

Although HAC have been associated with bursitis and calcific periarthritis, the significance of HAC in synovial effusions from some patients with arthritis (Dieppe et al, 1976; Schumacher et al, 1977; Halverson and McCarty, 1979; Dieppe et al, 1979) remains unclear. HAC are

more likely to be present in synovial fluids from patients with advanced OA, although they may be occasionally observed in some patients in whom OA is not clinically evident or in whom otherwise unexplained acute joint effusions have occurred (Schumacher et al, 1977; Dieppe et al, 1979). HAC have also been detected in patients with rheumatoid arthritis (Reginato et al, 1982b).

The total or differential cell count in synovial effusions is not consistent with acute inflammation, and it appears that the HAC are preferentially taken up by monocytes than by PMN (Schumacher et al, 1983). The concentrations of HAC in synovial fluids from patients with the "Milwaukee Shoulder Syndrome" have been shown to be remarkably constant over many months, suggesting a homeostatic control mechanism (Halverson et al, 1981), even though effective mechanisms exist for the clearance of crystals from joints (McCarty et al, 1979a,b). Both HAC and CPPD are endocytosed by synovial cells in vitro and uptake is associated with the production of collagenase and neutral protease by the cells (Cheung et al, 1981a). Articular chondrocytes also phagocytose HAC and subsequently release the same battery of enzymes (Cheung et al, 1983). Applied to the in vivo situation, these enzymes could destroy cartilage and other articular tissues and cause the release of more HAC from cartilage or periarticular sites of calcification into the joint space, thereby closing the cycle and perpetuating destruction of joint tissues.

The identification of HAC in synovial fluids and cells poses difficulties because, unlike monosodium urate (MSU) and CPPD, the crystals are usually too small to be seen by light microscopy and are not birefringent (Dieppe et al, 1976; 1979). They may be identified with certainty using analytical electron microscopy. The HAC observed in

synovial fluids take on different morphological forms (Dieppe and Calvert, 1983b), including small crystals (0.01-1μ); small nodular clumps of needle-shaped crystals (0.1-1μ); or larger ovoid, smooth surfaced bodies (0.1-5μ) or particles showing varying degree of crystallinity, size and shape.

The injection of HAC intradermally into normal and arthritic human volunteers (Dieppe et al, 1982), intradermally and intrapleurally into rats (Denko and Whitehouse, 1976; Glatt et al, 1979) and intraarticularly into dogs (Schumacher et al, 1977) induces features of acute inflammation.

Elucidation of the pathogenic pathways in experimental crystal reactions have been concerned with MSU and CPPD. Activation of Hageman factor by MSU or CPPD has been demonstrated in vivo (Kellermayer, 1968 which suggests the subsequent generation or activation of various mediators of acute inflammation. However, the question as to whether such pathways are essential to crystal-induced inflammation has not been answered. Moreover, different crystals have been shown to be membranolytic for PMN (Wallingford and McCarty, 1971), and the ensuing release of inflammatory and destructive enzymes could amplify the inflammatory response. Recently, Doherty et al (1983) showed that HAC could split complement C3 via the alternative pathway, which suggested that specific antibody was not required to trigger an inflammatory response. Plasma proteins, particularly IgG, are readily adsorbed to the surface and other crystals (Glueckauf and Patterson, 1974; Kozin and McCarty, 1976). This provides a mechanism whereby the Fc end of the IgG molecule may combine with Fc receptors of phagocytes, thus facilitating crystal phagocytosis. Once the crystal is contained within the phagosome, the protein coating would have to be removed enzymatically

prior to any interaction of the crystal with the lysosomal membrane.

While circumstantial evidence suggests a pathological role for HAC, little is known of the phlogistic properties of HAC compared wth MSU and CPPD. In particular, the chronic inflammatory potential of HAC has received scant attention.

Based on the hypothesis that the pathological deposition of calcium hydroxyapatite is an underlying cause of some forms of articular and periarticular disease and plays a role in pathology of OA, this project was undertaken to ascertain whether HAC could induce acute and chronic inflammation in articular and extraarticular tissues and to study aspects of the cellular events in cell and tissue reactions to HAC.

Specifically, the aims of the project were to study:

a) the ability of synthetic and biologically deposited HAC to induce chronic inflammation,

b) the ability of antiinflammatory and antirheumatic drugs to modulate HAC-induced inflammation,

c) the effects of HAC on articular tissues in vivo ,

d) the interaction of HAC with macrophages and synoviocytes in vitro .

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Chapter Two

Review of the Literature

CHAPTER TWO
REVIEW OF THE LITERATURE

1 OSTEOARTHRITIS-A PERTINENT REVIEW

Osteoarthritis, osteoarthrosis or degenerative joint disease has been defined by Sokoloff (1979) as:

> "a noninflammatory disorder of movable joints characterized by deterioration and abrasion of articular cartilage, and also by formation of new bone at the joint surface."

The disease is widespread in adult populations but knowledge of its etiology and pathogenesis is still far from complete. According to Pritzker (1980), degenerative joint disease refers to a heterogenous group of diseases which includes osteoarthritis and the crystal-related arthropathies as two major and distinct categories. Although OA is thought to be a degenerative rather than an inflammatory process, focal areas of secondary chronic synovitis (comprising small infiltrates of lymphocytes and macrophages) are usually seen in advanced cases. Villous hypertrophy and fibrosis of the synovium is also common.

It is not known whether the primary changes in OA occur in bone or cartilage. Growth may not cease completely at the articular ends of bones in the adult and bone remodelling probably occurs in response to changes in functional demand, quite independently of changes occurring in cartilage. When the rate of remodelling exceeds that of cartilage repair, OA could develop. Alternatively, early articular cartilage changes (focal chondromucoid softening and focal loss of cartilage substrate) may compromise its energy absorbing function and initiate secondary bone remodelling in response to changes in the pattern of

mechanical forces. It is also possible that a combination of these factors operate in OA pathogenesis.

One of the most notable features of OA is its relationship to ageing. Old joints may have been subjected to wear and tear over a long period and also to time-dependent chemical alterations in the cartilage (Sokoloff, 1979). Systemic factors (eg. obesity, genetic, hormonal) have not been able to be specifically implicated in OA pathogenesis.

1.1 Inflammation in Osteoarthritis

The potential role of HAC will be considered in the context of this brief review of possible disease mechanisms in OA. If HAC is involved in OA, its action could be through:

(a) direct irritation via its inflammagenic properties,

(b) indirect action via endocytosis by PMN or macrophages and subsequent production of lysosomal enzymes and prostaglandins and the secondary release of other inflammatory mediators,

(c) elaboration of an immunological response (HAC in synovial fluid may be recognized as "non-self" by the immune system),

(d) interference with metabolism of the joint tissues.

Alternatively, HAC may be a by-product of metabolic joint disorders occurring as a consequence of OA and have no important bearing on its pathogenesis.

Concepts of the role of inflammation in OA have changed over the last decade and the relevant arguments will now be considered. Inflammatory osteoarthritis can have an abrupt and painful onset according to Ehrlich (1972). Nodose forms of arthritis (eg. Heberden's nodes) may begin abruptly and painfully, with redness and warmth overlying the involved

joints. These inflammatory symptoms gradually subside over a period of several months to five years and the resulting joint deformities are identical to "benign" OA. Ehrlich (1975) followed up his earlier observation with a five year clinical study of 170 patients (164 were women) who presented with acute onset interphalangeal OA. In 15% of patients a second acute inflammatory episode occurred 12 years on average after the first and these patients subsequently developed rheumatoid arthritis.

In a review entitled "New Knowledge of Osteoarthritis", Ali (1978) considered osteoarthritis to be an age-related heterogenous disease with a complex etiology. Excessive wear and tear related to athletic activity was discounted as an underlying cause of primary degeneration of cartilage, as were joint surface changes, joint geometry and congruity and changed compliance of cartilage and subchondral bone. These were considered to be secondary predisposing factors to biochemical changes in the collagen, proteoglycan and mineral components of joints. No early warning diagnostic test was available to predict the onset of clinically apparent, irreversible osteoarthritis.

Dieppe (1978) reviewed the systemic and inflammatory features of polyarticular disease. A common type of OA occurred in women and began with inflammation. Histopathological studies of synovium in cases of OA showed inflammatory involvement. The possible causes of inflammation in OA were listed as follows:

traumatic synovitis
crystal-induced synovitis
immunological reaction to altered cartlilage
reaction to chemical components of cartilage
foreign body reaction to cartilage and bone fragments

Prior cartilage alteration followed by inflammation is implied, although clinical studies have indicated the early appearance of

inflammation in some forms of OA. The beneficial action of antiinflammatory rather than analgesic drugs in OA (reported by Lee et al, 1974) also points to an inflammatory component.

In an extensive review of bone and joint disease, Teitelbaum and Bullough (1979) claimed that some OA patients tended to improve spontaneously. Breakdown of joint components was usually followed by a repair phase and if a balance was reached between the destructive and reparative processes, the disease stabilized. The primary event in OA was thought to be loss of proteoglycan from articular cartilage through ageing, direct trauma or enzymatic digestion of collagen and proteoglycans. Disuse was also an important factor since decreased mechanical stress may predispose to a reduction in proteoglycan output by chondrocytes. No mention of the potential role of HAC in OA was made.

The traditional view of mechanical wear and tear causing degenerative joint disease was thought to be "inappropriate" by Huskisson et al (1979). Up to date morphological and biochemical evidence pointed to differences in osteoarthritic and aged cartilage. Evidence of inflammation included morning stiffness, redness of distal interphalangeal joints, warmth and greater numbers of inflammatory cells than normal in synovial effusions from the knees. Middle-aged women were predominantly affected and the disease was mostly polyarticular, the knees being most frequently involved. Radiological evidence of calcification was frequently seen in the distal interphalangeal joints of the hands and in knees. It was speculated that HAC could account for the inflammatory component in OA but could not explain the characteristic loss of cartilage.

Dieppe et al (1980) pursued the hypothesis that inflammation is a

fundamental part of the pathogenesis of OA, with the HAC deposits in articular cartilage and soft tissues acting as a primary inflammagen. One hundred patients with OA were screened for signs of inflammatory joint involvement. Of 74 patients with knee involvement, 73% had effusions and 26% of these were warm. Radiographic signs were consistent with OA and abnormal areas of calcification within and around joints were found. Of the 34 synovial effusions studied, intra- and extracellular deposits of CPPD were detected in six and HAC in nine. The patients were referred to the rheumatology clinic and therefore may not have been representative of the population at large suffering from osteoarthritis.

Dieppe (1981) presented more evidence for an inflammatory component of OA. Joint thermography showed that patients with OA had significantly "hotter" joints than those of a normal control group. Some "hot" areas were radiologically normal but there was a significant correlation between positive scans and Xray grading of disease. In addition, the response of OA sufferers to antiinflammatory drugs was investigated using placebo controlled trials of intraarticular steroids and comparative studies of analgesics and a non-steroidal antiinflammatory agent (Ketoprofen). The inflammatory component of OA was reversed by conventional antiinflammatory treatment. Both the steroid and Ketoprofen caused a significant reduction of joint tenderness and of the thermographic index. Dieppe suggested that crystal deposition was predisposed by local tissue damage. Crystals may accelerate destructive changes by induction of inflammation.

Acute attacks of crystal-related arthritis are known to be self-limiting even if active treatment is given (Dieppe and Doherty, 1982). Crystal phagocytosis by synovial fluid cells and synovium or

crystal dissolution due to factors altering crystal solubility (eg. changes in pH, temperature or calcium concentration) may modify the inflammatory response. Components of the inflammatory process itself may render the crystals less inflammagenic by altering their protein coating or by the local production of inactivators of chemotactic activity (Wright and Gallin, 1975).

2 APATITE DEPOSITION DISEASE

An early report linking heterotopic hydroxyapatite deposition and recurrent episodes of acute articular inflammation came from McCarty and Gatter(1966). Clinical data from four patients were thoroughly documented; joint fluids were white and contained "shiny coin" bodies, 3-65μ in diameter. These structures had interplanar spacings characteristic of HAC when examined by Xray diffraction. Microscopically, granular areas stained intensely for phosphate and calcium.

Dieppe et al (1976) coined the term "apatite deposition disease" to describe the association of calcium hydroxyapatite particles and certain cases of osteoarthritis. Samples of synovial fluids taken from selected patients with osteoarthritis contained HAC (0.15-0.8μ), identified as such by energy dispersive analysis. Five of the six patients examined had calcification in or around their joints visible radiographically. Synovial effusions from joints affected by rheumatoid arthritis did not contain HAC.

Dieppe (1977) investigated synovial fluids from 20 osteoarthritic patients and found them to contain nodules of HAC. Occasional nodules in membrane-bound vesicles were seen inside synovial fluid cells. The

mean cell count of synovial fluid cells was 83% mononuclear and 17% PMN. Serum calcium and phosphate levels were normal. Dieppe hypothesised the following scenario for the participation of HAC in the pathogenesis of osteoarthritis. Trauma, genetic and local factors may influence chondrocyte function, resulting in altered cartilage metabolism, release of free calcium from proteoglycans and the production of abnormally high amounts of alkaline phosphatase, thereby favouring HAC deposition. The HAC may cause direct cartilage damage by alteration of its structure and biomechanical properties. Alternatively, HAC may evoke an inflammatory response, indirectly initiating tissue destruction by release of proteolytic enzymes from synovium and synovial fluid cells.

Schumacher (1977) reviewed the information on apatite deposition disease available to that time. Clumps and individual crystals of HAC, surrounded by an unidentified granular material, may be found in synovial fluid. The origin of the HAC was unknown and Schumacher concluded:

> "Too little is yet known to fully characterize the effects of hydroxyapatite crystals in joint fluids and whether they will often be seen in asymptomatic joints as with urates and pyrophosphates."

The granular material observed by TEM around the crystals or their clumps were thought to an important part of their potentially inflammatory nature. Uric acid crystals in gout and CPPD crystals in pseudogout have been found to be coated with immunoglobins, complement, albumin and phospholipids. Adsorbed immunoglobin may bind to cell membrane receptors, activate complement and stimulate phagocytosis. In addition, the presence of a critical number of crystals may be

necessary to trigger an inflammatory response.

Schumacher et al (1977) found clumps of needle-shaped HAC (75-250Å) within and outside synovial fluid mononuclear and PMN cell vacuoles in cases of acute undiagnosed arthritis and in exacerbations of OA. More than 200 synovial fluids taken from patients with diseases such as gout and pseudogout, rheumatoid arthritis and scleroderma were negative for HAC when examined by electron probe analysis, Xray diffraction and TEM. Wet preparations of joint fluids containing HAC showed characteristic shiny round cellular inclusions. HAC in joint fluids were possibly responsible for previously unexplained inflammation seen in "erosive osteoarthritis" or inflammatory OA where HAC could be released from bone.

The source of HAC in synovial fluids was studied by Ali (1977, 1980) who described extracellular matrix vesicles capable of inducing calcification in human osteoarthritic articular cartilage. Matrix vesicles, 100nm in diameter, are thought to be associated with the mineralization of growth cartilage, medullary bone and dentine. In growth cartilage, the matrix vesicles are derived from chondrocyte cell processes and contain most of the enzymic activity associated with cartilage. Calcium is taken up by matrix vesicles against a concentration gradient. Phosphorus is then absorbed and crystals of apatite grow to form mineral nodes, then calcified septa. Osteoarthritic human articular cartilage was found to be biochemically similar to growth cartilage and contained matrix vesicles at all levels. The amount of alkaline phosphatase was 30 times higher than that measured in healthy articular cartilage obtained from subjects of the same age group. TEM and biochemical evidence indicated an abnormality of the calcification mechanism in osteoarthritic cartilage. Increased amounts of matrix

vesicles and mineral nodules, especially in the middle cartilage zone, may lead to ectopic cartilage calcification. Greater pressure may then be applied to remaining HAC-free cartilage by normal "wear and tear" , resulting in increased susceptibility to degradative changes. Abrasive wear due to the prescence of mineral nodules may also contribute to excessive cartilage destruction. The size of the nodules of mineralization associated with matrix vesicles was similar to that of the clumps of HAC found by Dieppe et al (1976) in synovial fluids. Vertical clefting or deep fibrillation of the articular cartilage, a feature of advanced OA, may give the mineralized nodules access to the joint cavity. The case for the existence of HAC associated arthropathy was reinforced by Ali's findings.

Evidence of HAC and CPPD crystal deposition occurring in the same joint was found by Dieppe et al (1978) who obtained biopsy samples of synovial fluid, synovial membrane and cartilage from 6 patients with OA. Although CPPD formed in conditions unfavourable to HAC formation according to Howell et al (1975). local variations in alkaline phosphatase may account for the presence of both calcium phosphates. No obvious clinical differences could be found from cases of OA associated with mixed crystal deposition, CPPD or HAC crystals alone or no crystals. Dieppe et al concluded:

> "calcification appears to be intrinsic to the evolution of many cases of osteoarthritis".

Dieppe et al (1979) systematically studied the frequency of occurrence and the disease association of HAC in unselected samples of synovial fluids using polarized light microscopy and analytical electron microscopy. The results were correlated with the clinical diagnosis and synovial fluid cell counts. HAC were commonly present in

synovial fluids in chronic arthritis. Only a weak correlation existed between the presence of smaller crystals and the clinical diagnosis of acute arthritis. SEM showed that the HAC had acquired a protein coating.

A summary of the clinical syndromes associated with HAC was prepared by McCarty (1979). The conditions include calcifying tendinitis, "calcinosis" associated with polymyositis or scleroderma, vitamin D excess and OA. HAC may gain access to the joint space by rupture from a synovial or cartilaginous deposit or may provoke inflammation at an extraarticular site. McCarty suggested three possibilities which could explain the relationship between crystal deposition and OA:

(a) Crystal deposition and cartilage degeneration being the result of a common underlying metabolic abnormality,

(b) Crystal deposition following cartilage degeneration,

(c) Crystal deposition preceding cartilage degeneration (as in gout).

Most information regarding the mechanism of crystal-induced inflammation has been obtained from the study of the interaction of MSU and CPPD crystals with inflammatory cells. In general, the number of crystals in aspirated joint fluid does not correlate with the severity of inflammation. In some cases of acute gout or pseudogout, no crystals have been detected in joint fluids (Schumacher et al, 1975), a finding explained by their submicroscopic size, or dissolution during preparation for microscopy. Alternatively, the acute inflammatory response may be located in adjacent tissues with oedema fluid finding its way into the joint space.

Dissolution of macrophage phagolysosomes containing endocytosed

protein-coated crystals may result by hydrogen bond mediated membr-anolysis. Hydrolytic enzymes released into the cytoplasm result in cell autolysis and release of mediators of inflammation into the environment. It is not known whether similar cellular events initiated by HAC are involved in the pathogenesis of joint destruction.

Hydroxyapatite deposition in and around joints was studied radiographically by Bonavita et al (1980) who found a spectrum of abnormalities ranging from periarticular calcification with periarthritis to gross joint destruction. Dalinka et al (1982) reviewed the roentgenological features of calcium deposition disease. The radiologic finding of acute calcific periarthritis was frequently suggestive of the diagnosis of apatite deposition disease, although crystal identification could provide the only definitive diagnosis. Large aggregates of HAC deposited around joints can also be associated with metabolic disease, such as hyperparathyroidism.

Bjelle et al (1980) recognized the importance of the diagnosis of HAC in synovial fluids in painful joints because of the effectiveness of antiphlogistic therapy in relieving symptoms. The clinical forms of apatite associated arthropathy were claimed to be:

Bursitis calcarea
"Milwaukee Shoulder"
Erosive apatite arthropathy
Multiple tendon calcification disease
Subacute and chronic inflammation in OA
Periarthritis in patients on chronic dialysis

Apatite deposition seemed to occur in the mid-zonal and lower layers of the articular cartilage. No further light on the cause-effect relation between HAC and OA was shed.

Howell (1980) collated evidence in the literature that supported a hypothesis of HAC deposition in articular cartilage and its sporadic leakage into synovial fluid. Low concentrations of CPPD may be incorporated in cartilage as a controller or inhibitor of mineralization. Enzymatic hydrolysis of the pyrophosphate by altered metabolism of articular cartilage chondrocytes may favour apatite crystal growth. Fleisch et al (1966) suggested that pyrophosphates were physiological regulators of calcification by binding to HAC and blocking crystal growth centres.

A syndrome in which HAC microspheroids are deposited in shoulder joints was termed "Milwaukee Shoulder" by McCarty et al (1981 a,b). Radiographic changes involved a complete tear of the fibrous rotator cuff in seven out of the eight shoulders examined. Most patients had weakness, loss of mobility and chronic discomfort particularly during and immediately after joint movement. Microspheroids of HAC were identified by the EHDP binding assay and energy dispersive analysis in 10 of 11 joint fluids examined. Collagenase and neutral protease activities were found in joint fluids from three patients. The source of these enzymes was presumed to have been synovial since few inflammatory cells were identified in joint fluids. An hypothesis for the involvement of HAC and the "Milwaukee Shoulder" syndrome was made. The source of HAC was the heterotopic deposits in altered capsular synovial tissue or degenerative articular cartilage. The HAC was released into the joint spaces together with their tissue matrix, engulfed by macrophage-like synovial cells, thereby inducing collagenase and neutral protease release. The enzymes attacked the periarticular tissues, including the rotator cuff, causing the release of additional HAC into the synovial fluid. This effect was termed enzymatic "strip mining". The degenerative tissue change may then

become more extensive, resulting in joint instability and recurrent cycles of mineral deposition and shedding.

The microspheroids containing HAC ranged from 1.9 to 15.6 microns in diameter and were thought to have entered the joint space through denuded areas of synovial lining. Particulate native collagen (Types I, II and III) were also found in shoulder joint fluids. Halverson et al (1981) postulated the existence of an "intraarticular crystal traffic" for HAC based on the finding of stable levels of HAC in samples of synovial fluid taken from the same patients over an 11 month period.

Morphological and biochemical studies on synovium excised from a patient with "Milwaukee Shoulder" syndrome were made by Garancis et al (1981). The pathology noted at arthrotomy included completed absence of the rotator cuff, severe osteoarthritis of the glenohumeral joint, loose osteochondral bodies in the synovial space (suggesting chondromatosis) and a markedly thickened multi-pedunculated synovium. Calcific masses entered the joint space through synovium denuded of surface lining cells.

Further evidence of enzymatic "strip mining" of HAC was provided by Halverson et al (1982). Synovial tissue obtained at surgery from a patient with the "Milwaukee Shoulder" syndrome released HAC microspheroids when incubated with mammalian collagenase in vitro. The microspheroids were the same size as those found in the synovial fluid of the same patient. Crystal shedding into the joint space may depend on the joint fluid levels of calcium and phosphate as well as enzymes released by phagocytic cells.

Schumacher et al (1981) postulated that HAC were low grade inflammagens. Both HAC and CPPD were found frequently, even in "non-inflammatory" osteoarthritic effusions. High percentages of patients with CPPD also had HAC. More sophisticted methods for rapid identification of crystals in synovial fluid were introduced.

Dieppe (1981a) reviewed the crystallization process in relation to crystal-induced arthropathies. Crystal formation is dependent on ions of optimum activity being present at the appropriate site in the presence of a promotor of nucleation or a relative lack of nucleation inhibitor. Crystal growth and dissolution are also dynamic processes, dependent on the available surface on which the crystals are forming (eg connective tissue components) and on a continuous supply of ions. HAC may form from inorganic orthophosphate via octacalcium phosphate and dicalcium phosphate dihydrate crystals. The site of formation may be intra- or extracellular, or via matrix vesicles extruded from cellular tubules (Ali, 1977).

Speculations regarding the mechanisms of HAC-induced joint damage were made. They involved factors associated with (a) the crystals and (b), those of their environment.

(a) Properties of the crystals

Crystal related properties that may dictate their ability to cause tissue damage are their size and surface characteristics. The size of individual crystals or aggregates of crystals may affect crystal phagocytosis and the extent of mechanical damage. In general smaller crystals are thought to be most inflammagenic since they are readily phagocytosed and phagocytosis appears to be an important factor in

crystal-induced acute inflammation (Phelps and McCarty, 1966). A size limit below which the crystals have minimal effect on cells may also occur. To date, the lack of correlation between the numbers of crystals in synovial fluid and the degree of inflammation may be explained by their submicroscopic size.

The negatively charged surface of the crystals would encourage protein adsorption and SEM studies of HAC in synovial fluids (Schumacher, 1977; Dieppe et al, 1979) have confirmed this phenomenon. Binding of immunoglobin to crystal surfaces may be important in initiating phagocytosis in vivo according to Kozin and McCarty (1976).

(b) Properties of the crystal environment

Crystals may be exposed to phagocytic cells in the synovial fluid. Mediators of inflammation may be released from cells following crystal phagocytosis. Alternatively, HAC may be phagocytosed by Type A synovial lining cells and elicit a giant cell reaction. Frequently no obvious cellular response is seen. Crystal deposition may also occur below intact cartilage surfaces, modifying its physical characteristics and altering its biochemical behaviour.

Whether HAC are etiological agents of pathological change in OA, are deposited as a result of OA or are present because of a combination of the two, remained unresolved.

Reginato et al (1982a) saw crystal-induced arthritis as "one of the best understood inflammatory diseases in humans" which gave a unique opportunity to study the efficacy of antiinflammatory agents. Although crystal deposits were usually associated with acute articular

inflammation and temporary disability, they may lead to secondary joint disease with more permanent disability. HAC may cause acute synovitis or play a role in the low grade inflammation and progressive articular cartilage deterioration seen in OA. Rapid control of HAC related inflammation could be achieved using nonsteroidal antiinflammatory drugs. Diphosphonates, which inhibit the formation and dissolution of calcium phosphate crystals, may also prove of use.

The frequency and site of tissue calcification, the type of crystal deposited and the relationship of crystalline deposits to tissue inflammation was investigated by Doyle (1982). The articular tissues of 28 patients (22 female, mean age 45-87 years) who had clinical and radiological evidence of polyarticular OA were studied. The tissues from 16 hip and 12 knee joints were obtained at operations for replacement arthroplasty. Synovium, capsular tissues and cartilage were examined using a variety of histochemical techniques. TEM was used to examine specimens of fresh tissue and sections from paraffin blocks containing calcific deposits. All patients had calcification of either cartilage, synovium or capsule. Early calcification was characteristically found by TEM as aggregates of HAC between collagen bundles around the chondrocytes in the deepest third of the cartilage. HAC in the superficial cartilage regions were found in 36% of patients while CPPD crystal aggregates occurred in middle and superficial cartilage regions in 11% of cases. Synovial calcification (identified as HAC) was seen in 68% of specimens which all exhibited patchy lining cell hyperplasia. The calcified material had a varied morphology; some resembled bone fragments, others were aggregates of HAC. None of the calcified material was associated with an acute inflammatory reaction in the surrounding tissues. Giant cells were seen both around and in the absence of calcified particles but frequently no inflammatory

reaction directed towards the calcified material occurred. However, perivascular aggregates of lymphocytes and plasma cells were seen in both superficial and deep areas of the joint capsule and around areas of advanced cartilage destruction. Giant cells were present in 54% of synovial specimens and lymphocyte/plasma cell aggregates in 61%.

No control material was obtained because "of the difficulty of finding a matched control population without radiological evidence of OA". Doyle commented that the deposits of HAC in articular cartilage may be influential in the degenerative process seen in OA by altering the normal physical properties of cartilage and by impairing its function. The source of HAC in synovium was thought to be crystals in the synovial fluid which had been endocytosed by Type A synovial cells.

The chronic inflammatory features observed in the synovium were "the result of the osteoarthritic process, though they cannot be attributed to crystal deposition alone." A progressively destructive process involving deposition of crystals was envisaged: lysosomal enzyme release, induced by crystal or bone/cartilage fragment phagocytosis could release antigenic proteoglycan from cartilage, thereby eliciting an immune response, potentiating inflammation and further raising lysosomal enzyme levels. Doyle concluded that synovitis was an integral part of the pathology of the osteoarthritic joint and that the pathogenic role of calcification in OA was limited, despite its high prevalence.

Resnick and Resnick (1983) reviewed the clinical and radiological features of apatite deposition disease. The symptoms included pain, tenderness to palpation, local swelling, retricted motion and occasionally, fever. The characteristic radiological features were those

associated with calcifying tendinitis and periarthritis.

3 HYDROXYAPATITE DEPOSITION IN HETEROTOPIC CALCIFICATIONS

Gatter and McCarty (1967) examined 86 autopsy and 25 surgical specimens of human tissues displaying pathological calcification. The tissues included arterial atheroma, heart valves, pineal glands and rib cartilage. Xray diffraction showed that 96% of the calcified material consisted of hydroxyapatite. No evidence of CPPD deposition was found. Whitlockite was present in 3.6% of specimens. Local factors were thought to be more important than systemic factors in determining the type of mineral deposited in heterotopic calcifications.

3.1 Calcifying Tendinitis

Calcifying tendinitis of the shoulder occurs by calcification of living tissue rather than by dystrophic calcification (Uhthoff, 1975). The calcification was multifocal and it resembled the first stages of endochondral ossification. The main difference was the lack of blood vessels in proximity to the calcifying tissue, resulting in its degeneration into an "amorphous mass". The etiology was unknown but both calciphylaxis and calcergy were not considered to be involved.

Uhthoff et al (1976) reported that there was no inflammatory reaction around the calcific deposits which had been identified as being hydroxyapatite by Xray diffraction. A new theory of the formation and fate of calcified deposits in calcifying tendinitis was put forward. Mechanical injury to the shoulder could compromise the already precarious blood supply to the supraspinatus tendon, triggering the

transformation of tendon into fibro-cartilage and its subsequent calcification at multiple foci. With time, giant cells accumulate around individual foci and vessels proliferate, restoring normal perfusion and oxygen tension. It is possible that the tendon regains its original structure after resorption of the calcified material although it is most likely that some deposits are being formed while others are being resorbed in the same specimen.

McKendry et al (1982) studied calcifying tendinitis of the shoulder in 57 patients before and after surgical removal of the calcified material. Acute pre-operative pain was consistently related to active resorption of the calcific deposits (seen histologically). The distinction between this syndrome and "Milwaukee Shoulder" lay in the intra- tendinous location of the calcified material and the absence of clinical or radiological evidence of a rotator cuff tear. Deposition of hydroxyapatite is common to both clinical entities. Faure and Daculsi (1983) thoroughly reviewed the clinical features, diagnosis and treatment of this syndrome. The role of the macrophage in the removal of the calcific deposits was thought to be central to the understanding of the disease.

4 CALCIFICATION MECHANISMS

A brief consideration of some aspects of the calcification reaction is relevant to this dissertation in terms of the possible mechanisms of extra-skeletal HAC deposition and calcergy. However, despite continuing extensive investigations, the mechanisms initiating and controlling calcium salt deposition in connective tissues are still poorly understood. Two reviews of the calcification process have

appeared recently (Fleisch, 1980; Boskey, 1981) and their findings are now summarized.

4.1 The Nature of the Calcified Mineral

While there is general agreement that the major constituent of biological and pathological calcifications is hydroxyapatite, it is evident that it is not in a pure form. Bone mineral is a poorly crystalline, carbonate containing analog of hydroxyapatite which exhibits improved crystallinity with aging. The size and degree of perfection of HAC in various calcified tissues differ in ways that may reflect both the mineralization process and the function of the tissues.

4.2 The Calcification Process

Biologic calcification is thought to depend on the interaction of cells, extracellular macromolecules, ions (such as magnesium, carbonate, pyrophosphate), hormones and nonmatrix proteins. Plasma, although supersaturated with respect to hydroxyapatite, is metastable because of the presence of calcification inhibitors.

a) Cells and Calcification

Cells (eg. osteoblasts, chondroblasts) may be the primary agents controlling calcification by removing or inactivating calcification inhibitors and by storing calcium and phosphate in mictochondria, thereby creating favourable microenvironments for mineralization. Matrix vesicles of cellular origin are generally considered to be extracellular sites of initial mineral deposition especially in

cartilage and pathologic calcifications more so than in bone. However, controversy exists as to whether all matrix vesicles are formed specifically by the cell or whether they are a result of cellular degeneration.

Matrix vesicles are thought to promote calcification by concentrating or transporting calcium and phosphate while removing or excluding calcification inhibitors. Matrix vesicles are capable of promoting calcification by themselves *in vitro* .

b) Extracellular Macromolecules Macromolecules of the extracellular matrix may control calcification by:

1. providing sites for HAC growth,
2. acting as nucleators for HAC,
3. stabilizing HAC precursors and/or
4. regulating the size and orientation of the mineral deposited.

Native collagen promotes mineralization by virtue of the periodic arrangement of collagen fibrils but its action is not now considered that of HAC nucleator *in vivo* . The major role of collagen appears to be in providing an oriented support for newly formed crystals and in regulating their size.

The phosphoproteins (peptides containing phosphorylated amino acids) may play a structural and/or regulatory role in calcifying matrices by providing sites of nucleation for HAC. The heterogenous nature of phosphoprotein populations would suggest a multiplicity of roles in HAC formation. Proteoglycans, the major non-fibrous extracellular macromolecules also play complicated roles in calcification as

determined by *in vivo* and *in vitro* studies. However, their function in control of calcification is not known. Some proteolipids (membrane components of most cells) are thought to be involved in the initiation of calcification - their concentrations peak in epiphyseal plate cartilage as it begins to calcify. Boskey concluded the review by stating:

> "If one were to seek a unifying factor in all calcification processes, it would have to be the cell, for the cell may unify all the nucleators and inhibitors in such a way that they can act in concert to produce a calcifiable matrix."

4.3 Early Events in Calcification

The precipitation of calcium phosphate solids from aqueous solutions at physiological pH is a complex dynamic phenomenon. *In vitro,* the first phase of mineralization formed under conditions of high and low supersaturation is an amorphous calcium phosphate, ACP (Eanes et al, 1973). ACP is highly hydrated, inherently unstable in solution and is converted through several stages of lower hydration to a spheroidal state and then rapidly to HAC (Eanes and Posner, 1965). The first crystals of HAC appear in contact with the surfaces of ACP particles, the initial nucleation site. In TEM micrographs, ACP coated with a variety of biological polymers was seen as dense compacted clusters around which needle-shaped HAC had formed (Eanes et al, 1973).

Uncertainty also exists regarding the chronological development of the crystalline phases deposited. *In vitro* they are dependent on the supersaturation and pH of the solution. Brushite is formed preferentially at low pH and high supersaturation and octocalcium phosphate under physiological conditions, according to Fleisch (1980). Electron

dense nucleation centres for HAC have been shown *in vivo* . Anderson (1967) used a technique of inducing heterotopic bone deposition by the injection of human amniotic cells into thigh muscles of cortisone-conditioned mice. Within 12 days, the discrete cell colonies became invested with bone through a process resembling endochondral ossification. TEM of early stages of calcification showed rounded dense centres around which needle-shaped crystals of HAC were clustered.

Gay (1977) used ultracryotomy and ultramicroincineration to demonstrate the ultrastructure of embryonic chick femur. This technique avoided artefactual loss, dislocation or dehydration of mineral constituents. Amorphous mineral was present in large amounts. It existed as 15-30nm diameter spheres and as a "structure-free haze" both similar in appearance to the *in vitro* findings of Eanes et al (1973). The ACP was almost always associated wih HAC and it appeared that ACP was rapidly converted to HAC *in vivo* . ACP was associated with non-collagenous matrix components. In well-calcified areas, little amorphous mineral was found and hydroxyapatite crystals were oriented along collagen fibres.

In summary, there is abundant evidence from *in vitro* and *in vivo* studies that ACP is always deposited initially at sites of calcification under physiological conditions. Subsequently, biological HAC grows through heterogenous nucleation and epitaxy by adsorbing lattice ions (inorganic phosphate, calcium and hydroxyl ions) from the surrounding fluids.

5 EXPERIMENTAL CALCIFICATION

5.1 Simple Calcergy

Simple calcergy is the calcification of subcutaneous injection sites after the injection of a variety of metallic compounds and was first described by Selye (1962). The trichlorides of lanthanum and cerium were the calcergens used at first, with lead acetate being introduced as a calcergen by Selye et al (1964). Intravenous administration of lead acetate in rats resulted in calcification at mechanically or chemically traumatised sites. Gabbiani et al (1966) discovered that the trichlorides of all the rare earth elements could produce dose dependent calcification at the site of injection in rats. These salts also initiated precipitation when added to normal Tyrode solution.

Extremely low levels of lead normally present in biological fluids such as blood, plasma and urine were shown to have an activating effect on calcium phosphate crystal formation *in vitro* under physiological conditions (Fleisch et al, 1965). The (Ca)x(P) products at which crystals were formed in the presence of lead were as low as those previously obtained with the most active nucleating collagens. The authors were "tempted to speculate that the local concentration of lead might play some role in the regulation of calcification."

Gabbiani et al (1970) used TEM to study the mechanism of calcergy. Five minutes after injection of lead chloride, electron dense clusters of lead triphosphate, approximately 100nm in diameter, were observed around collagen bundles and contacting collagen fibrils. Granules of lead were also found within macrophages, fibroblasts and mast cells, which appeared to become necrotic three hours after injection. PMN

infiltration and capillary endothelial damage were also seen at this time. Five hours after injection, thin rod-shaped crystals appeared in contact with the lead triphosphate clusters while after 12 hours, the number of crystals increased. Electron diffraction patterns resembled those of poorly-crystallized hydroxyapatite. Calcification of collagen fibrils became more prominent five days after initial contact with the lead. The crystals were randomly orientated.

Bridges and McClure (1972) induced calcergic reactions in mice, guinea-pigs, hamsters and even in the domestic fowl. Mast cell degranulation occurred at the sites of simple calcergy in rodents but the participation of mast cells in calcergy was thought to be non-specific since conventional mast cells are not found in the domestic fowl. In the mouse, von Kossa positive material was deposited around dilated blood vessels five hours after lead acetate injection.

Quantitative studies of simple calcergy in the rat, again induced by lead acetate were reported by Takimoto in 1973. Up to 32 days after injection, the total dry weight of minerals of the calcified site increased in a biphasic fashion. Five times more calcium and phosphorus was deposited in the first four days than during the subsequent 28 days. It was suggested that the calcergic reaction had growing and maturation phases. Xray diffraction showed an increasing degree of crystallinity with time. The amount of lead in the calcified tissues rapidly decreased after eight days to a third of the level at the time of injection. However, the amount of lead was still 40 times higher than in control skin after 32 days.

McClure and Gardner (1976) showed that the acetate, chloride and nitrate salts of lead caused calcification in the connective tissues of

the dorsal fascia in mice. The reaction was elicited with very small doses of lead salts (100µg/0.2 ml). Potasssium permanganate caused cutaneous necrosis and calcification in the panniculus carnosus muscle. Even high concentrations of zinc chloride, copper sulphate and cobalt chloride failed to produce calcification; necrosis and acute inflammation of connective tissues was observed. The results indicated that calcification depended on the presence of lead, since variation of the anion associated with the lead caused identical reactions. This type of calcification was:

> "accompanied by a minimum reaction and mimics closely the calcification seen in physiological situations."

This study was followed by detailed investigations of possible calcergens in mice, starting with those found by Selye (1962), compounds in Group IV of the periodic table and salts of silver and barium (McClure, 1980). The reactions were studied histochemically and by Xray microprobe analysis of frozen dehydrated sections. Of the 11 compounds tested, lanthanum and cerium trichlorides caused calcification of the dorsal fascia. The positive reactions studied were interpreted as representing "models of the calcification process" which were suitable for the investigation of agents that could influence calcification. In fact, McClure (1979) used the simple calcergy phenomenon to compare the inhibitory effects of disodium pyrophosphate and EHDP on the calcification process. Lead acetate was the calcergen and the mouse the experimental animal. A circular, white, radio-opaque plaque, 10mm in diameter had formed at seven days after PbAc injection. Histochemically, the plaque was both von Kossa positive (phosphate ions) and chloranilic acid positive (calcium ions). Both chemicals tested were effective inhibitors of simple calcergy. However, whereas EHDP was an

effective inhibitor when given both systemically or locally before the calcergen, disodium pyrophosphate was only effective when given at the same site as lead acetate.

Biological deposition of hydroxyapatite crystals by potassium permanganate induced calcergy was used by Doyle et al (1979) to study the inflammatory nature of the crystals and the effect of drugs on their formation. A 1 in 40 dilution in saline (0.2ml) of a saturated solution of $KMnO_4$ was injected subcutaneously in rats. The size of the calcified plaque, its histology and TEM appearance were studied, along with Xray energy and infra-red spectroscopy. The effects of the drugs EHDP, Cl_2MDP, indomethacin and dexamethasone on the development and clearance of the calcified plaque were also evaluated.

The initial response to $KMnO_4$ injection was an acute inflammatory reaction. Calcification of collagen fibres was demonstrable by TEM after 48 hours and histologically after 60 hours. The calcified plaque reached its maximum size by the seventh day and by the sixth week the calcificied area had been almost entirely resorbed. Histologically, there was no evidence of acute inflammation attributable to the calcified material; multinucleated giant cells predominated, actively removing calcified collagen bundles and persisting after their removal. The crystalline material was hydroxyapatite. Neither antiinflammatory drug tested inhibited the formation of calcified plaques indicating the calcergic response to be independent of inflammation.

Predosage with EHDP inhibited local calcergy while Cl_2MDP (given after $KMnO_4$) inhibited resorbtion of calcified material. Relating their findings to OA the authors concluded:

> "The results suggest that EHDP could inhibit crystal deposition in the osteoarthritic joint and that Cl_2 MDP might have a role in slowing apatite crystal shedding from osteoarthritic cartilage and so reduce the synovitis seen in osteoarthritis."

The reproducibility of the lead acetate induced calcergy model in mice was also used by McClure in 1982 to test the mode of action of anticalcific drugs and the effect of antirheumatic and antiinflammatory agents. EHDP was confirmed to have an inhibitory effect when given for 7 days after lead acetate injection. Prior administration of EHDP had no effect on the calcergic reaction. Cl_2 MDP, another diphosphonate, had no effect on the development of calcergy but decreased the rate of resorption of the calcified plaque when given daily for up to 7 weeks. Prednisolone was without effect on the development of the calcergic response, indicating acute inflammation was not central to the pathogenesis of lead acetate induced calcergy in the mouse. Sodium aurothiomalate was also ineffective in inhibiting the calcergic reaction.

The progression of potassium permanganate induced local calcergy in rats was studied by Tochon-Danguy et al (1983) using Xray diffraction, infra-red spectrometry and electron spin resonance (ESR) techniques. TEM showed continuous development of mineralization for 20 days, followed by the appearance of newly formed, unmineralized collagen fibrils. Calcification persisted after 100 days. High levels of iron, magnesium, phosphorous and calcium were detected shortly after injection of the calcergen. Xray diffraction revealed an increase in crystal size during the first 40 days. Infra-red spectrometry showed that early transformation of calcium phosphate into apatite occurred and ESR confirmed that the proportion of hydroxyapatite in the mineral deposit remained the same, even while it was undergoing resorption.

5.2 Calciphylaxis and Synovial Calcification

Calciphylaxis (Selye, 1962) is a calcification reaction occurring in response to the subcutaneous injection of a challenging agent whose administration is preceded by systemic sensitisation of the experimental animal. The original calciphylactic sensitizers were vitamins D2, D3 and parathormone and the challengers included mechanical trauma, albumin and metallic salts.

Wolfe (1967) used histochemical techniques to study the development of calciphylactic heterotopic calcifications in rats induced by feeding them Dihydrotachysterol for one day followed by mechanical injury to the skin. The calcification reaction was labeled using systemically administered tetracycline, free rhodamine and fluorochrome labeled homologous serum protein containing albumin, transferrin and haptoglobin. Calcification was first demonstrable histochemically 24 hours after mechanical injury and the calcified zones increased in size up to the fifth day. Selective binding of the labeled proteins to the sites of calcification was particularly evident during the growth phase of the calcified areas. Labeled protein given two weeks after mechanical injury was not bound to the "old" mineral deposits. Tetracycline also produced specific fluorescence of calcifed sites similar to that seen in the osteoid phase of bone.

The findings of specific uptake of proteins by the calcifying sites emphasized the importance of serum proteins in the mineralization process. Apparently, 30-50% of blood calcium is protein-bound, indicating that the proteins carry calcium to sites of mineralization. The exact change thought to render a particular site "calcifiable" was not

known.

The first detailed report of biological deposition of HAC within knee synovial tissue by calciphylaxis was made by Reginato et al (1982). Large doses of dehydrotachysterol (the sensitizing agent) were given by gastric tube to New Zealand white rabbits, followed one day later by intraarticular injection of 1mg of ferrous chloride (the challenger).

Macroscopically, 5 days after ferrous chloride injection, intense, diffuse synovial membrane congestion and necrosis was seen. A progressive, firm, noninflammatory swelling of the joints developed over 45 days, when irregular calcified synovial deposits were noted. Ferrous chloride injection alone (control) caused slight and transient swelling of the knee and a mild diffuse proliferation and brown pigmentation of the synovium after 45 days. Radiographically, no areas of calcification were seen until 2 weeks after active treatment. Histologically, very small calcified deposits were detected after 5 days in the superficial connective tissue of the synovial membrane. After 45 days, there was proliferation of synovium and giant cell formation. Iron deposits were found inside the superficial synovial cells. Calcification occurred in the most superficial regions of articular cartilage. No calcification was demonstrable in control knees injected with ferrous chloride or saline.

TEM showed that needle-shaped crystals were arranged on collagen fibres and masked their periodicity; other deposits were seen within synovial cell processes and vacuoles. Some of the electron dense material was amorphous.

Long term (90 day) dehydrotachysterol treatment only resulted in and calcification of the mid-zone of articular cartilage. Chondrocytes examined by TEM showed marked degenerative changes. Matrix vesicles were abundant around necrotic chondrocytes but were not seen around the apatite-like material. The calcified deposits consisted of clumps of needle-shaped crystals in circular and elliptical arrays. None of the biologically deposited calcified material was associated with acute or subacute inflammation. Rather, synovial lining cell and fibroblast proliferation and giant cell formation was prominent in the heavily calcified zones. No crystals were found in the synovial fluid. The HAC deposited in the synovial membrane and articular cartilage were not associated with osteoarthritic change.

6 THE INFLAMMATORY POTENTIAL OF CRYSTALS

There have been relatively few studies relating the physical characteristics of crystals to their phlogistic potential. The effect of crystal size in particular has been poorly researched, although it is the general consensus of opinion that smaller crystals are capable of inducing a more intense inflammatory response than larger crystals.

6.1 Crystal Size

Dunn et al (1978) found that large crystals of HAC, CPPD, urate or silica provoked a lesser degree of acute inflammation than smaller crystals. The parameters measured were extent of PMN infiltration and fluid exudate volume after intrapleural injection in rats. Particles of MSU and CPPD (2-20μ) were were found by Schumacher et al (1975) to be the optimum size for PMN phagocytosis. Kozin and McCarty (1977) showed

a similar degree of protein binding to sonicated MSU crystals *in vitro* as to intact crystals. Sonication caused fracture along the long axis of the crystals so that the increase in surface area was small.

6.2 Crystal Shape

Rupture of internal cell membranes by sharp, pointed crystals such as MSU may confer greater phlogistic potential than that of rounded crystals, according to Dieppe and Doherty (1982).

6.3 The Crystal Surface.

The nature of the crystal surface is an important factor in determining the degree and type of inflammation provoked. Mandel and Mandel (1982) stated:

> "It is important to realize that if a crystal-mediated disease is to be totally defined, the molecular basis of the crystal interactions must be defined at the atomic level."

Crystal growth faces contain the atomic arrays involved in crystal-membrane, crystal-protein and crystal-crystal interactions. Calcium salts, as opposed to MSU, have many non-symmetric intermolecular contacts and have a very complicated molecular packing and bonding scheme. Again, little information is available regarding the inter-relationship between crystal configuration and inflammatory propensity. Positive surface charges would allow binding of complementary arrays of negatively charged proteins, such as albumin, immunoglobins, complement or phospholipids (Reginato et al, 1982a). Coating of the crystal surface by immunoglobin for example, may encourage binding to PMN cell membrane receptors, activate complement and favour phagocytosis. Alternatively, uncoated crystals may form hydrogen bonds

with polar phospholipids of cell membranes and lysosomes, resulting in the release of intracellular enzymes which may act as mediators of inflammation.

The inflammatory potential of crystals may be altered by heating. Crystals used in investigative studies are frequently rendered pyrogen- and endotoxin-free by heating at 200°C for 3 hours. Kozin and McCarty (1977) found that IgG binding to MSU crystals was unaffected by heating although no information regarding the temperature to which the crystals were heated was given. HAC, MSU and CPPD heated to 200°C for 5 hours caused reduced inflammatory swelling when injected into rat foot pads (Denko and Petricevic, 1979). This effect was attributed to heat-induced change of the charge on the crystal surface, causing decreased ability to release prostaglandins from cell membranes.

Cheng and Pritzker (1981) briefly reported changes in Xray diffraction patterns of HAC (heated at 25°C, and 200°C for 3 and 5 hours) MSU and CPPD (both heated at 25°C and 200°C for 3 hours). MSU and CPPD diffraction patterns were altered by heating, whereas heating the HAC reinforced the crystal lattice. Xray diffraction could not detect changes in crystal surface composition and comparative assays of the inflammatory potential of the crystals in vivo was not carried out.

7 HYDROXYAPATITE AS AN IRRITANT

The inorganic component of bone comprises 30% amorphous calcium phosphates, the remainder being crystalline calcium hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ (Cameron, 1972). The crystals are generally thought to be rod-shaped, ranging from 150-1500Å in length and 15-75Å in width.

The organic matrix and HAC form an integrated unit, important to the structure and function of bone. It is of some interest, then, that HAC have been found to be inflammagenic in a variety of experimental models.

7.1 The Irritant Nature of HAC in Humans

Dieppe et al (1976) in their initial report of apatite deposition disease, injected human volunteers intradermally with 0.2ml of sterile saline containing 10mg HAC (crystals 0.5-10μ diameter) and the resulting erythema and induration was measured over 72h. However, no control injections were used The response was similar to the injection of 5mg of uric acid ie. maximum erythema after 24h (mean diameter 26mm).

The ability of healthy persons and those with rheumatic diseases to mount an inflammatory response to a variety of crystals was tested by Dieppe et al (1982). Amounts of 5-10mg of HAC, MSU and CPPD were injected intradermally into forearm skin in a similar way as for Mantoux testing. Inflammation was measured by skin temperature and thickness changes and by the area of erythema that developed for periods of up to 60h after injections.

Saline injections, used as controls, caused an initial erythema that persisted for 2 hours. Crystal injection in 12 healthy volunteers caused an additional secondary response which peaked at 24h for MSU crystals and at 36h for HAC. Persons with OA, RA, chondrocalcinosis and pseudogout responded in an identical manner. Different crystal batches caused different responses but reproducibility between subjects with the same batch was good. The hypothesis that expression of crystal-induced arthropathies depended on varying inflammatory responses to

crystals was not supported by these experiments.

7.2 The Irritant Nature of HAC in Rats

Denko and Whitehouse (1976) investigated the local and systemic inflammatory response to microcrystalline calcium salts using the rat foot pad oedema test and the concurrent evaluation of liver function. An amount of 0.2ml of a 20 mg/ml suspension of crystals was injected into the hind foot pads and the inflammation was quantified over a 24h period. HAC were approximately half as irritant as CPPD as judged by increase in paw thickness at 24h. Similar changes in liver function were measured after 3 days. The authors concluded:

> "Crystal-induced inflammation that is outwardly localized may induce biochemical changes that are similar to changes found in systemic inflammation."

Further confirmation of the irritancy of HAC in an acute inflammatory model came from Glatt et al (1979). MSU, CPPD and HAC (1% solution) were injected intrapleurally into rats and the exudates collected for 72h. Drugs tested in this model included colchicine, indomethacin and dexamethasone. HAC was the most irritant crystal on a weight-for-weight basis. PMN made up 90% of cells during the first 12h of the reaction, after which mononuclear cells became more numerous. Phagocytosis of all 3 crystals occurred. Lysosomal enzyme concentrations peaked before the maximum PMN count, suggesting that resident intrapleural cells were the major source of inflammatory enzymes. The model indicated that HAC could elicit an acute reaction of rapid onset.

Denko and Petricevic (1979) showed that rats depleted of essential fatty acids reacted less floridly to HAC injected into their foot pads.

When HAC were heated to 200°C for 5h, a similar reduction in inflammatory swelling occurred and when physiologic amounts of prostaglandin E_1 was added to the heated crystals, their irritancy returned to normal levels. Control rats injected with saline or prostaglandin E_1 had minor swelling for a short period. Denko and Petricevic hypothesised that HAC induced inflammation was mediated by prostaglandin. Crystal shape was not thought to be an important factor since the 3 crystals tested (MSU, CPPD and HAC) were of varying shapes and all were equally affected by heating and their subsequent exposure to prostaglandin. The electrostatic charge on a crystal may be able to induce inflammation by stimulating cell membrane prostaglandin synthesis. Membrane rupture or crystal phagocytosis are not essential in this theory of crystal-induced inflammation.

7.3 The Irritant Nature of HAC in Dogs

The effect of injecting 15 mg of HAC intra-articularly into dog knees was tested by Schumacher et al (1977). Continuous pressure recordings were made and synovial fluid samples taken hourly for 4h for PMN counts, smears and TEM. Saline injection was used as a control. Joint pressure was increased fourfold in the HAC injected knees and PMN counts were three times higher. Focal PMN infiltration was seen in the superficial synovium for both test and control knees although the infiltration was more intense as a result of HAC injection. Clumps of HAC were found in synovial fluids. Again, the results were comparable with those obtained using urate and CPPD crystals in the same model.

These investigations have tested the inflammagenic properties of HAC using conventional models of acute inflammation. Since HAC have been shown to be present in joint fluids for long periods (Halverson et

al, 1981) without necessarily being associated with acute episodes of OA, it is likely that they exert a chronic inflammatory stimulus that goes undetected by models of acute inflammation.

8 CRYSTAL-SYNOVIUM INTERACTIONS *in vivo*

An experimental model of crystal synovitis in the dog knee joint was introduced by McCarty et al (1966) who injected sterile, pyrogen-free suspensions of sodium urate and CPPD crystals intraarticularly. Changes in intraarticular pressure (IAP), synovial fluid pH and total PMN and crystal concentrations were quantified. Four hours after injection of varying doses of crystals, a dramatic rise in IAP was measured, whereas there were negligible effects after saline injection. The synovial fluid pH decreased as the reaction progressed and the change correlated with the fluid PMN counts. When 15mg of MSU or CPPD was injected, an acute inflammation of similar intensity resulted.

An identical experimental method was used to investigate HAC by Schumacher et al (1977) who injected 15mg into the right knees of four dogs. Their results appear in section 7 of this chapter.

The dynamics of CPPD crystal clearance from normal rabbit and arthritic human knee joints was studied by McCarty et al (1979a) using Ytterbium-169 labeled crystals. Crystals were sized by mechanical sieving and the rate of loss of microgram quantities of crystals was followed by serially counting with scintillation probes over the injected joint for several months. The half life of the isotope was 31 days. Clearance of CPPD crystals from synovium and joint fluid was shown in both species. Rabbit joints cleared crystals 3 to 10 times

more rapidly than human joints despite the smaller mass of synovium relative to dose of crystals injected.

The crystal clearance rate was related to crystal size. In rabbit knee joints, 10-20μ crystals were cleared in 7-12 days, 20-50μ crystals in 16 days and crystals more than 50μ in size took 84 days for half their mass to be cleared.

In the human volunteers, the rate of clearance of 200mg of labeled CPPD crystals was measured. The clearance of free ^{169}Yt was also determined for the same knee once residual radioactivity was less than 1%. Free ^{169}Yt could be detected in urine or plasma 72 hours after intra-articular administration. Half of CPPD crystals were cleared 32-99 days after injection.

This study was followed up by McCarty et al (1979b) using ^{45}Ca and ^{85}Sr labeled CPPD in rabbit knee joints. Although the quantity of crystals injected (40-60mg) was 200-300 times greater than in the previous experiment, the clearance capacity remained very high. Virtually all the CPPD injected into the joint space were endocytosed by fixed synovial macrophages. Migratory phagocytes in the synovium may have also participated in crystal clearance. The mechanism of crystal removal from within the synovial cells was unknown. McCarty et al concluded that crystal endocytosis by synovial cells:

> "is an important, effective mechanism controlling synovial fluid concentration of crystals in patients with CPPD crystal deposition disease."

The concept of homeostasis of intraarticular HAC crystal concentration subsequently developed by Halverson et al (1981) was partly based

on the ability of synovium to efficiently clear crystalline material from the joint space.

Schumacher (1973) investigated the synovial distribution and fate of carbon particles (300Å) injected intravenously in rabbits. Some particles reached the synovial cells and joint space via synovial venules as soon as 30 minutes following injection. The material was also detected in vessel pericytes after 7 days. This finding may be of relevance to rheumatoid arthritis where antigen/antibody complexes or other minute but biologically reactive material may be seeded into joints from the circulation.

9 PROTEIN BINDING TO HYDROXYAPATITE AND OTHER CRYSTALS

9.1 Studies Based on MSU and CPPD Crystals

In an attempt to elucidate the inflammagenic mechanisms of MSU, CPPD and silica crystals, Kozin and McCarty (1976,77) examined their adsorptive properties in serum or protein solutions. Proteins investigated included human IgG, ovalbumin and bovine serum albumin; all proteins were iodonated with ^{125}I so that the amount of protein bound could be determined by liquid scintillation counting. Protein concentrations were also determined chemically. All crystals studied were found to adsorb protein from solution with MSU binding IgG one order of magnitude more avidly than silica and two orders of magnitude more avidly than CPPD. Protein adsorption was greater at 4°C than at 37°C and most protein had been bound after 20 minutes of incubation with the crystals, both findings indicating that protein adsorption

rather than non-specific protein aggregation was occurring.

The amount of protein bound was inversely proportional to the crystal concentration. Smaller crystals such as sonicated MSU bound the same amount as intact crystals. No effect on protein binding was found when crystals had been heated to 190^{o}C for 2h. Finally, decreased pH generally resulted in increased protein binding.

Although the authors compared the binding affinity of the crystals for a range of proteins, the results were presented in terms of the amount of protein bound per milligram of crystalline material. This method may not have reflected the binding affinity accurately since it did not take the molecular weights of the proteins into consideration.

Immunoelectrophoretic studies of MSU crystal incubation with human serum showed that IgG was adsorbed preferentially to other serum proteins and McCarty (1977) suggested that:

> "uncoated crystals do not exist in physiological fluids and that the protein-crystal complex exhibits neither the properties of native protein in solution nor those of the uncoated crystal."

Hasselbacher and Schumacher (1978) examined intact tophi of MSU using immunohistologic techniques and found that the inter-crystalline matrix contained significant amounts of immunoglobin but not albumin or fibrin. Synthetic MSU crystals strongly and specifically bound all major classes of immunoglobins. Fibrin was found in addition to immunoglobin on the surface of MSU aspirated from long-standing disorganized tophi.

Hasselbacher (1979a) showed that the adsorption of IgG to MSU was

a non-random phenomenon related to the charge density of the immunoglobin molecule. Other negatively charged crystals such as HAC, CPPD also adsorbed IgG. The Fab fragment of the IgG molecule may bind preferentially because of its greater positive charge compared with the Fc fragment. MSU adsorbed two to three times more IgG than HAC or CPPD but complement activation was not related to the amount of IgG adsorbed. Heated MSU crystals adsorbed greater amounts of proteins than unheated crystals. HAC caused significant activation of complement (C3), although the level of activation was approximately one fifth of that for MSU (Hasselbacher, 1979b).

A short report by Bardin et al (1982) using TEM, described positive binding of IgG, IgM and Fc fragments to synthetic MSU. The proteins were not bound preferentially to any particular area of the crystals.

9.2 Hydroxyapatite and Chromatography

A significant amount of information regarding the affinity of hydroxyapatite surfaces for proteins has come from its use in preparative biochemistry. Hydroxyapatite chromatography is used for the fractionation and purification of proteins, enzymes, nucleic acids and viruses. It can often resolve complex substances when other separation methods have failed (Bio-Rad Catalogue I, 1983). The relative binding affinities of different proteins are evidently a function of their surface charge density and charge-to-mass ratio.

Tiselius et al (1956) laid the groundwork for the use of hydroxyapatite in chromatography using colored proteins (phycoerythrin and phycocyanin), bovine serum albumin, ovalbumin and human gamma-

globulin amongst others. Specificity was shown for high molecular weight proteins, whereas low molecular weight proteins showed little or no adsorption. The adsorption of IgG also depended strongly on the presence of salts other than phosphate buffer.

The binding sites for negatively charged groups on proteins were probably the calcium ions on the surface of the HAC, according to Bernardi (1971) but to that time the binding sites for positive groups were not known. Bernardi et al (1972) showed that proteins bound to hydroxyapatite in a complicated fashion, involving both calcium and phosphate sites. The phosphate sites probably bound the basic groups on proteins.

The nature of the adsorbance of bovine serum albumin and IgG to HAC was thoroughly investigated by Glueckauf and Patterson (1974). IgG showed a marked change in maximum adsorption capacity as the buffer concentration was increased. At very low buffer concentrations, adsorption was three times greater than at higher concentrations. The authors calculated that an IgG molecule could theoretically cover 940 phosphate sites on HAC and a bovine serum albumin molecule could cover 400 sites. A mathematical model was used to describe the adsorption of proteins to HAC in the presence of phosphate. When a certain phosphate saturation has been achieved on HAC surfaces, proteins are no longer able to make contact with positively charged, strongly adsorptive sites. Weak adsorption of proteins occurs on the negatively charged phosphate ions on the crystal surface. Very small numbers of phosphate ions can stop the adsorption of a protein - a single ion for serum albumin and three ions for IgG on a patch of 13 and 14 adsorption sites respectively.

Two types of protein adsorption occur to HAC:

a) weak adsorption to the natural phosphate surface, occurring independently of phosphate buffer concentration,

b) strong adsorption associated with calcium, resulting in full saturation of the surface independent of the protein concentration of the solution. It is greatly dependent on the phosphate buffer concentration which governs the fraction of the surface available for protein adsorption and which itself is not displaced by protein. A phosphated calcium surface adds weak adsorption to that of the natural HAC phosphate surface. Bovine serum albumin will only be adsorbed to HAC if the contact area is completely free of adsorbed phosphate ions. IgG can still be adsorbed to HAC in the presence of up to two phosphate ions.

It would be tempting to extrapolate these in vitro findings and calculations to the in vivo situation where the concentrations of phosphate salts may fluctuate and are consequently able to influence the amount and type of protein adsorbed to the crystals.

9.3 Protein Binding to Tooth Enamel

The adsorption of protein to tooth enamel (which is 99% hydroxyapatite) is of interest with respect to the formation of dental pellicle and also dental plaque, the causative agent of dental caries and periodontal disease. Numerous studies in this area have assisted in the understanding of the nature of protein-HAC interaction.

Hay (1967) studied synthetic HAC and ground tooth enamel for their capacity to adsorb proteins from human saliva. Disc electrophoresis was used to identify the presence of proteins in the saliva before and

after contact with HAC and enamel. Some salivary proteins were selectively adsorbed to both materials tested and proteins recovered from the surfaces of freshly extracted teeth were found to have the same electrophoretic mobilities. Adsorption took place rapidly, within minutes of exposure to protein. It was suggested that further adsorption of protein may take place non-specifically by protein-protein binding.

McGaughey and Stowell (1971) found that adsorption of salivary proteins (unidentified) to hydroxyapatite was not temperature dependent but that the removal or complexing of calcium ions substantially lowered protein adsorption. More protein was adsorbed at lower pH. Another study by the same authors in 1974 determined the effect of varying physiological concentrations of phosphate ions on protein adsorption to HAC. Salivary phosphate exerted significant inhibition of salivary protein adsorption.

Rölla and Melsen (1975) studied the interaction of synthetic HAC (chromatography grade) with bovine serum albumin and saliva. Fluoride desorbed bound protein, probably by its adsorption to calcium ions on the HAC. Monofluorophosphate also caused protein desorption, presumably by its action as a phosphate. Bacteria adsorbed to HAC could be desorbed in the same way as the proteins, suggesting a similar mechanism of adherence. Rölla et al (1977) found that brief pretreatment of HAC with 1mM sodium fluoride affected the uptake of charged macromolecules. Fluoride treatment resulted in a more negatively charged HAC surface and decreased albumin uptake, whereas treatment with calcium and aluminium caused enhanced albumin adsorption.

The mechanism of fluoride-mediated albumin desorption from HAC was clarified by Eggen and Rölla (1982) using hydroxyapatite column chromatography. Fluoride competed with albumin for receptor sites on the hydroxyapatite surface in the same way as phosphate. Fluoride had higher affinity for surface receptor sites of the HAC (thought to be surface adsorbed calcium) than phosphate. Both ions eluted negatively charged macromolecules by competitive adsorption. Salivary fluoride and phosphate levels could influence the amount of pellicle (salivary glyoprotein) adsorbed to teeth and consequently influence bacterial colonization of the teeth and could be another way in which fluoride acted as a cariostatic agent.

The affinity of hydroxyapatite for proteins has been well documented by workers in divergent fields. However, the relevance of protein binding to HAC crystals *in vivo* to the pathogenesis of apatite deposition disease has not been well established in the literature.

10 CRYSTAL INTERACTIONS *in vitro*

10.1 Crystal Interactions with PMN

There have been few studies on the *in vitro* behaviour of synthetic HAC, whereas urate crystal interactions have been extensively investigated.

Human PMN were exposed to commercially obtained HAC (Sigma Chemical Co.) by Maurer and Schumacher (1979). The clumps of crystals (0.4-15μ) had been heated to remove pyrogen and were incubated with PMN for up to 120 minutes. Endocytosis of HAC and degranulation of PMN occurred 3 minutes after mixture of cells with crystals. The numbers

of necrotic cells increased with time. After 120 minutes, 35% were necrotic compared with death of 19% in control suspensions. Monocytes and eosinophils which were also present in the PMN suspension had endocytosed HAC but did not become necrotic.

Dieppe et al (1983) found that HAC were very toxic to PMN and caused the release of more β-glucuronidase than a similar quantity of MSU. The mechanism whereby PMN became necrotic after crystal endocytosis was not elucidated; most necrotic cells had endocytosed crystal clumps of several microns in length. There was no evidence that the crystals had a protein coating. The investigators had not used other particulate materials as controls for the rate of endocytosis and PMN killing.

10.2 Crystal Interactions with Synovial Cells

A detailed biochemical study of the interactions of human rheumatoid and normal canine synovial cells with HAC and CPPD was carried out by Cheung et al (1981a). The HAC used were obtained from the Sigma Chemical Company and were less than 20μ in diameter. Collagenase, neutral protease, lactic dehydrogenase and prostaglandin E_2 and F_2 activities of the culture media were measured for 7 days after addition of crystals. Both HAC and CPPD were endocytosed by rheumatoid human and normal canine synovial cells and both types of crystals increased collagenase and neutral protease release by the cells. Even small doses of HAC markedly raised the prostaglandin levels, whereas CPPD induced almost negligible prostaglandin release. Dose-response studies for each particle had not been carried out. HAC stimulated release of prostaglandins from synovial cells may play an important role in the pathogenesis of HAC associated joint disease.

Hasselbacher et al (1981) were unable to detect increased prostaglandin E_2 or latent collagenase production when rabbit synovial fibroblasts were cultured in the presence of HAC. Even small amounts of MSU crystals, silica and asbestos particles induced the synthesis of prostaglandin E_2 and latent collagenase by normal rabbit synovial fibroblasts.

Cheung and McCarty (1982) briefly reported that both HAC and CPPD were mitogenic for canine synovial cells over a 3 day period. Maximum incorporation of ^3H thymidine occurred 17 hours after the addition of crystals. The findings were interpreted as a possible explanation for the synovial cell hyperplasia "sometimes found in association with superficial deposits of HAC or CPPD crystals."

10.3 Crystal Interactions with Macrophages and Monocytes

Chambers (1980) studied the interaction of unstimulated mouse peritoneal macrophages and radiolabeled HAC, bone powder and deproteinated bone powder in the presence of diphosphonates. Even high diphosphonate levels in culture medium had no effect on macrophage morphology but in the presence of bone mineral, the toxicity to macrophages was greatly increased. Labeled synthetic HAC were avidly phagocytosed by the macrophages, producing scintillation counts approximately four times higher than bone mineral and 14 times higher than whole bone after a 60 minute incubation. Diphosphonates exerted minimal influence on macrophage uptake of the particulate materials. Chambers suggested that the HAC adsorbed the diphosphonates which resulted in their concentration on the bone surface where they could prove toxic to normal osteoclast function. Bone resorbtion is thereby

inhibited.

Chambers (1981) cultured mouse peritoneal macrophages with radiolabeled bone powder for one hour and found that 20% of bone bound to the cell surfaces was released by contact-dependant resorption. Endotoxin but not prostaglandin E_2 or parathyroid extract caused a significant increase in ^{45}Ca release. These agents are known to increase osteoclast activity *in vivo*.

A similar system for studying bone resorption *in vitro* had been used by Kahn et al (1978), Teitelbaum et al (1979) and McArthur et al (1980). Kahn and coworkers used human circulating monocytes, radiolabeled bone powder (10-105μ particle diameter) and intact labeled calvaria of fetal rats to study the resorption process. Macrophages adhered more readily to the bone matrix than to the collagen matrix in which the particles had been embedded. Resorption of bone particles and calvaria occurred as a consequence of cellular contact and acid phosphatase activity rather than by phagocytosis. This mode of action is similar to that of osteoclasts.

Stimulated mouse peritoneal macrophages were cultured with radiolabeled rat bone powder (particle size <25μ) by Teitelbaum et al (1979). Isotope release began rapidly and continued in a linear fashion for at least 48 hours. Mouse macrophages were able to mobilise isotope more efficiently than rat macrophages. Ultrafilters placed between bone particles and cells caused a marked decrease in the rate of dissolution of bone particles, implicating the importance of cell - bone contact during bone resorption.

McArthur et al (1980) showed that only the adherent cells derived

from a mixed mononuclear cell population could trigger mineral and matrix dissolution of devitalised bone particles. Non-viable adherent cells (frozen and thawed) or metabolically inactive cells did not participate in bone dissolution. Stimulated mouse peritoneal macrophages caused dissolution of both organic and inorganic bone components, confirming the work of Teitelbaum et al (1979). It was hypothesised that sub-populations of macrophages exist that specifically engage in bone dissolution in association with resident osteoclasts.

Kahn and Teitelbaum (1981) found that endotoxin from several species of bacteria suppressed the osteolytic ability of stimulated mouse peritoneal macrophages but had no effect on resident mouse peritoneal macrophages. The authors concluded that osteolysis in chronic inflammatory sites where endotoxins have been found was related to macrophage prostaglandin synthesis and macrophages as putative osteoclast precursors.

Although these studies did not specifically address the consequences of cell-pure HAC interactions in vitro , the inorganic component of the devitalised bone particles is largely HAC. However, in apatite deposition disease, it is likely that the very small HAC that are found in synovial fluid are phagocytosed rather than bound to (macrophage) cell surfaces and dissolved as the devitalised bone particles appear to be.

10.4 Crystal Interactions with Erythrocytes

Suspensions of erythrocytes prepared from fresh human blood were incubated with a variety of crystals by Hasselbacher (1982) to determine their membranolytic potentials. Incubation proceeded at 37°C for

4h and the percent haemolysis was determined by comparing the OD 550λ against an aliquot of cells lysed in distilled water. Quantities of 12.5 mg/ml of crystals were used. In this system, HAC caused 43.6% haemolysis, MSU 11.4% and CPPD only 4.8%. Saline control resulted in 0.8% haemolysis. However, the same preparation of HAC caused only weak prostaglandin E_2 stimulation and no collagenase stimulation (Hasselbacher et al 1981). The results of this experiment indicated that non-specific membrane damage by crystals was probably not an important factor in determining their inflammatory propensities.

10.5 Crystal Interactions with Chondrocytes

A brief report on HAC interaction with rabbit chondrocytes was made by Cheung et al (1981b), Chondrocytes in primary monolayer culture and articular cartilage slices in organ culture were exposed to 1μg of HAC or CPPD. Both types of crystals were endocytosed by the chondrocytes and caused the release of increased amounts of collagenase and β-glucuronidase into the culture medium. HAC caused approximately twice as much enzyme release from cell and organ cultures compared with CPPD crystals.

Rabbit articular chondrocytes were shown by Cheung et al (1983) to be capable of phagocytosing HAC and CPPD *in vitro* . The chondrocytes were from primary cell cultures and the crystals were added at a final concentration if 100 μg/ml of culture medium. The cells were examined by SEM, TEM and phase contrast microscopy. The culture media were tested for collagenase and neutral protease and for prostaglandin levels.

HAC induced a 15 fold increase in collagenase activity and a three

fold increase in the level of neutral protease. CPPD raised collagenase activity by five times that of control cultures. Increased collagenase production was a function of crystal concentration and was dependent on the presence of serum. Nearly all the prostaglandin release occurred in the first 24h after crystal addition, CPPD being less effective per milligram than HAC in raising prostaglandin levels. Cell lysis did not account for the raised enzyme activities. Both types of crystals were endocytosed by the chondrocytes. It was suggested that crystal-induced enzyme release by these cells could account for the destruction of cartilage matrix observed in crystal deposition diseases. Further experiments were being undertaken to assess the response of articular cartilage slices in organ culture to the same crystals.

10.6 Crystal Interactions with Serum

Doherty et al (1981,1983) found that HAC split complement C3 via the alternative pathway, although to a lesser extent than MSU on a equal weight comparison. Amorphous calcium phosphate powders caused an insignificant amount of complement activation. HAC that had been stored for nine months had 22% less complement activating potential than freshly prepared crystals. The authors found that some crystals and particles such as diamond dust and carbonyl iron did not activate C3 and surmised that:

> "Complement activation by active agents depends on some special characteristic(s) of their presenting surface and not merely on their crystalline or particular state."

The fact that MSU, CPPD and HAC could activate complement by the alternative pathway suggested that specific antibody was not required for triggering an inflammatory response. Classical pathway activation

is an adaptive immune response mediated via IgG or IgM combined with specific antigen. It was hypothesised that the initial event in crystal induced synovitis was activation of the alternative pathway following direct interaction between C3 and special crystal surface features, independent of immunoglobins. The products resulting from complement splitting could be chemotactic for phagocytic cells.

11 HYDROXYAPATITE CRYSTAL IDENTIFICATION

Sophisticated methods of identification of HAC in synovial fluid have been introduced because of the submicroscopic size of these crystals. At present, the methodology is directed towards research, but it could soon be applied diagnostically in clinical practice.

11.1 Light Microscopy

Wet smears of synovial fluid using a dust free glass slide and coverslip (dust particles are birefringent) and compensated polarized light microscopy may reveal clumps of HAC (Schumacher, 1977). Shiny globules, purple staining cytoplasmic inclusions with Wright's stain or "occasionally atypical positively or negatively birefringent crystals" were found. However, the individual crystals are submicroscopic in size. HAC are weakly birefringent so that positive HAC identification requires more sophisticated methods.

Staining of synovial fluid samples with alizarin red S was introduced by Paul et al (1983) as a simple method of apatite identification. The fluids were examined microscopically within 3 minutes of staining. Crystal clumps were stained orange-red. Although the stain

was very sensitive, being able to detect synthetic HAC concentrations as low as 0.005 µg/ml, it was not specific for HAC, rather calcium and phosphate salts. There was a strong correlation between intensely positive alizarin tests and the probability of finding apatite-like crystals by TEM. Synthetic CPPD crystals usually stained less intensely than HAC but CPPD in synovial fluid were occasionally found to stain similarly to fluids containing HAC.

Apatite crystals were found in synovial fluid samples from patients with diverse chronic joint diseases. A positive correlation was found between the intensity of positive alizarin stained clumps and the presence of OA. It was suggested that:

> "apatite crystals are commonly associated with articular cartilage degeneration, regardless of the clinical diagnosis."

11.2 Scanning Electron Microscopy

Halverson et al (1981) prepared synovial fluid samples from patients with the "Milwaukee Shoulder" syndrome for SEM and used SEM micrographs for measuring particle size. Microspheroids of HAC were found to be 1.9 to 15.6µ in diameter. Energy dispersive analysis was carried out in conjunction with SEM and confirmed that the microspheroids contained hydroxyapatite. Dieppe et al (1979) centrifuged synovial fluid on to carbon coated electron microscopy grids which were examined by polarized light and SEM. Individual HAC were 50nm long and clusters of overall diameter of 0.3µ were common.

Bjelle et al (1980) suggested the use of millpore filters to collect and prepare particulate and cellular material in synovial

fluids for SEM. SEM with back scattered electron imaging offered another possibility for crystal identification. The effect of sample preparation (ie. dehydration) for SEM on the physical characteristics of synovial fluid HAC is not known.

11.3 Transmission Electron Microscopy

TEM can provide morphological, energy dispersive analysis and Xray diffraction information of samples for more positive identification of HAC in samples. Crocker et al (1976) described a method for the preparation of sections suitable for TEM from pellets obtained by the centrifugation of synovial fluids. Thicker sections than usual were required since particulate material tore or fell out of ultrathin sections. A similar methodology was used by Dieppe et al (1979) for examination of synovial fluid samples. Halverson (1981) found single crystals, crystal clumps and fibrils of native collagen using TEM of synovial fluids. Tearing of thin sections containing crystalline material occurred.

11.4 Energy Dispersive Analysis

Standard preparations of HAC have a calcium/phosphorous ratio of 1.66 (Halverson et al, 1981) as determined by energy dispersive analysis of Xrays. This technology enables the analysis of individual crystals in a mixed population of crystals. One of the main disadvantages of this procedure is that at least 15 different readings should be made of the chosen target. Dieppe et al (1979) called it "a difficult, time consuming technique" but found it to be reliable and reproducible as long as standardized conditions were adhered to. The electron beam could damage the crystals or even hasten the conversion

of octacalcium phosphate to hydroxyapatite. Comparative studies were not possible because of variations in the accelerating voltage used from study to study. Bone fragments could display the same elemental ratio as HAC on energy dispersive analysis.

Schumacher et al (1977) found that the calcium to phosphorous molar ratio of synovial fluid crystals was "very slightly less" than the calculated ratio of 1.66 for hydroxyapatite. These workers noted the importance of exposing their HAC standards to the identical fixation and embedding techniques used for their fluid and tissue samples. Energy dispersive analysis can be used with both SEM and TEM.

11.5 Xray Diffraction

The exact interatomic spacings and relationships of the component atoms of any given crystal may be determined by this method. However, Dieppe et al (1981) thought it difficult to apply to HAC identification because of the diminutive size of the crystals and because it cannot be used to identify individual crystals in a biological matrix. Halverson et al (1981) used Xray diffraction for the identification of HAC in synovial fluid pellets. The results reinforced data obtained by other methods.

11.6 EHDP Binding Technique

A semiquantitative technique for the detection of extracellular HAC in synovial fluids was developed by Halverson and McCarty (1979). The technique used the avid and specific binding propensity of EHDP for HAC. Synovial fluids obtained from 51 arthritic patients were tested and material that bound to EHDP was detected in 29% of non-inflammatory

fluids and none in inflammatory effusions. When joint fluids from three patients were tested on two separate occasions, similar amounts of EHDP binding were measured, suggesting that either HAC remained in the joint fluids for prolonged periods or that they were constantly removed and replenished. The same technique was applied to HAC identification in "Milwaukee Shoulder" patients (Halverson et al, 1981). No further reports of the use of EHDP binding to HAC identification in synovial fluids have appeared to date.

11.7 Infra-red Spectrophotometry

Infra-red spectrophotometry was applied to microanalysis of particulate material by Dieppe et al (1977). It was a quicker and more flexible means of analysis of pure material than Xray diffraction. However, no data relating specifically to HAC identification was presented.

12 EXPERIMENTAL GRANULOMA FORMATION BY IMPLANTATION

12.1 Cotton Pellet Implants

The subcutaneous implantation of sterile cotton pellets in rats was introduced by Meier et al (1950) as a method for assessing the proliferative component of inflammation and the activity of antiinflammatory drugs. The cotton acted as a low grade irritant and elicited a triphasic response, initially involving permeation of the pellet with a noninflammatory transudate followed by protein exudation from the surrounding tissues for 2-3 days and finally a proliferative phase characterized by the ingrowth of granulation tissue (Swingle and

Shideman, 1967). Proteoglycans were produced before collagen and fibroblasts rapidly increased in number between the third and fifth days after pellet implantation.

When more than one pellet was implanted at different locations in the same rat, no gross differences in the development of granulation tissue was observed although this matter had not been studied systemmatically, according to Swingle (1974). Measurement of the anti-inflammatory activity of drugs was largely based on their suppressive effects on the dry weight increase of the granulomae. Swingle and Shideman (1967) found that 50% of the measured dry weight increase could be attributed to the transudative and exudative fluids permeating the pellets shortly after implantation. In general, the cotton pellet granuloma does not seem to be satisfactory for testing the antiinflammatory activity of nonsteroidal agents (Swingle, 1974). Cotton pellets act as irritants in provoking the inflammatory response.

12.2 Viscose Cellulose Sponge Implants

Viscose cellulose sponge implants weighing 170-190 mg were used by Viljanto and Kulonen (1962) to study the tensile strength of granulation tissue. The sponges were found to be homogenous, suitable for mechanical and chemical study, sterilizable and easily impregnable with various solutions. However, pretreatment of the sponges was necessary to remove sulphur and to soften them. After implantation the increase in tensile strength paralleled the amount of collagen being formed within the sponges. Sponges soaked in soluble collagen resulted in granulation tissue of higher tensile strength.

Kaltiala and Heikkinen (1971) also implanted viscose cellulose

sponges (weighing 73 mg) in rats for up to 16 days to study the histamine content of granulation tissue. Four sponges were implanted in each rat. The histamine content of early granulation tissue was high but the level fell by over 50% as the tissue matured. Phenylbutazone and salicylic acid administration caused inhibition of granulation tissue ingrowth but resulted in increased levels of histamine.

12.3 Polyvinyl Sponge Implants

Polyvinyl sponges were investigated by Grindlay and Waugh (1951) as inert, non-resorbable materials for use in surgical reconstructions. Sponges were carefully washed to remove glycerine added by the manufacturer and sterilized prior to implantation in 37 areas of 28 dogs for 1 - 18 months. The sponges were well tolerated at all implantation sites and were not associated with chronic inflammatory reactions. Histologically, the sponges "had the appearance of a strange but somehow normal-appearing organ." Clinical use of polyvinyl sponges in the surgical treatment of large abdominal aneurysms in four patients was briefly described.

Moore and Brown (1952) implanted 1x0.5x0.5 cm polyvinyl sponges subcutaneously in mice for up to 6 months. Hair loss and crusting of the overlying skin occurred and was thought to be due to ischaemia created by the increasing pressure of the sponge as it became more fibrous. Smaller sponges did not cause these problems. The sponges were surrounded by a fibrous capsule and were penetrated by granulation tissue containing few inflammatory cells. No tumours were observed in the animals used for implantation. Polyvinyl sponge was found to be inert, non-toxic and non-carcinogenic and as such, suitable as

subcutaneous prostheses.

Sterile polyvinyl sponges were implanted into rats, rabbits and humans by Boucek and Noble (1955). The sponges for human implantation weighed 200-300mg and were implanted subcutaneously in the submammary area along the anterior chest wall. Rabbits had 10 sponges implanted via five separate incisions, adult rats had 2 sponges implanted. During the first week, sponges were encapsulated and fibroblastic ingrowth into the interstices of the sponge matrix commenced. Giant cells were found in 30 day specimens. The wet tissue weight showed no correlation with the duration of implantation. The lipid content was 30% of the dry weight of the older granulomae (80 days plus), a surprising finding since few tissues other than brain have values of this order. The polyvinyl did not interfere with biochemical assays. No histological or biochemical data from the human sponge implantations were presented.

Polyvinyl sponges were implanted subcutaneously in rats by Edwards et al (1975) to study the histology and biochemistry of healing tissues. The 2x2x0.8 cm sponges were infiltrated by granulation tissue whose collagen fibre composition decreased 30 days after implantation, being partially replaced by fatty connective tissue. Biochemically, hexosamine content of sponges was approximately twice that of open wounds at the same stage of "healing", whereas the amount of sponge collagen was half that of healing wounds. The sponge model offered a unique opportunity to study dry weight gain of a <u>defined</u> area. Soaking the sponges in saline rather than in distilled water prior to their implantation resulted in delayed and varied ingrowth of granulation tissue. One of the advantages of using sponges was that wound exudate could be sampled earlier and more readily than from open

wounds.

Polyvinyl sponges, both plain and impregnated with autogenous bone chips or particles were implanted in the periosteal bed of young adult dogs by Struthers et al (1955). The sponges were recovered at one, three and five month intervals and examined histologically and radiographically. Plain sponges were well tolerated by osseous tissue and showed no degenerative changes. They were initially infiltrated by granulation tissue and slowly, by bone which grew from the adjoining cancellous bone surfaces but never completely filled the sponges. Sponges containing scattered chips of autogenous cancellous bone demonstrated bone formation throughout after 4 weeks. The bone arose from periosteum, cancellous bone and from the bone chips. Sponges impregnated with autogenous bone powder also demonstrated extensive formation and ingrowth of bone.

Sponges implanted away from skeletal tissue did not exhibit bone formation even when impregnated with autogenous bone particles or powder. This method of inducing extra-skeletal bone formation was seen as having potential use in the repair of bony defects.

Another report of bone formation occurring within polyvinyl sponges came from Amler et al (1958). A trough measuring 5x2x0.5mm was made in the femur of adult rats into which a T-shaped piece of sponge was inserted and held in place with sutures. The implants were studied for up to 24 weeks. Parts of the sponges that had been in contact with the marrow were infiltrated by bone. The sponge material was gradually replaced by well organised bone. Small areas of unresorbed sponge (constituting 15% of the original sponge) remained within the matrix of the bone.

Polyvinyl sponges were used by Bole and Robinson (1962) to stimulate and study granulation tissue formation in guinea pigs. Histological and biochemical changes with time, rate of tissue ingrowth, influence of the implantation site and sex of the host were some of the factors studied. The sponges ranged from 16-160mg in weight and up to 10 pairs were implanted subcutaneously in each animal for up to 144 days. Dry weight increases varied greatly with the site of implantation; sponges implanted in the dorsolumbar area gained less weight than the interscapular sponges for all time periods of implantation and for all sponge sizes studied. The ingrowth of granulation tissue was measured on histologic serial sections but the method used was not described.

Wound fluid initially permeated the sponges. Proliferative tips of granulation tissue started to advance into the sponges 7 days after implantation. Macrophages and giant cells became prominent between 10 and 14 days and were most frequently observed apposed to the sponge surface. Mast cells were rarely observed before 10 days and the few that were noted subsequently were confined to the sponge periphery. No evidence of fatty infiltration was found even after 144 days. Older implants (4-7 months) showed degenerative changes characterized by collagen "fragmentation" and increasing numbers of macrophages loaded with intracellular particulate material. Evidence of cytonecrosis was seen in many areas of the tissue. The sponge material itself became more labile to the fixative used. The authors concluded that the polyvinyl sponge model enabled the study of the constantly changing events of the inflammatory process.

Dasler et al (1960) examined the variability in lipid content of

polyvinyl sponges implanted at different subcutaneous sites in rats. Sponges implanted in the peritoneum became embedded in extraperitoneal inguinal fat after 7 weeks. Sponges implanted closest to the subscapular fat deposit had higher lipid contents than other sponges and it appeared that fat within sponges was formed from pre-existing adipose tissue rather than from cells present within the sponges. The authors concluded:

> "Care must be taken in selecting comparable sites for subcutaneous sponge implantation. Our observations indicate that the number of sponges that can be implanted in comparable sites in a single adult rat is strictly limited to a relatively small area of the mid-back."

Bole and Heath (1967) found that 6-mercaptopurine interfered with normal capillary formation, caused dysplasia in collagen fibre deposition and suppression of connective tissue organization of granulation tissue growing into polyvinyl sponges. An ocular micrometer was used to measure the depth of penetration of the granulation tissue. Other parameters measured included number of giant cells, total protein and hydroxyproline content of the sponges.

Polyvinyl sponges and cotton pellet induced granulomae were used by Boyle and Mangan (1980) in a comparative histological and biochemical study. After 15 days, the capsules of the cotton pellets were more organized than the infiltrated sponges. There was a sparsity of giant cells associated with the sponges suggesting that the sponge model was "an irritant rather than a foreign-body granuloma." The ease with which sponges could be dissected from the surrounding tissues was claimed to account for the greatly reduced variation in individual results and it was "a more sensitive model for the detection of subtle

change in collagen metabolism."

12.4 Polyether Sponge Implants

Parnham (1980) injected lymph node cells (derived from adjuvant-treated rats) into polyether sponges implanted in normal syngeneic rats. Granuloma formation was enhanced as measured by increase in dry weight and histological parameters. This effect was related to lymphocyte-mediated activation of granulation tissue. The sponges offered a unique matrix within which the cellular interactions could be studied. Parnham saw such granuloma models as:

> "a long lasting attempt by the organism to phagocytose and/or isolate a foreign body."

A constituent of polyether sponges that was extracted by boiling the sponges in ethanol for 30 minutes was discovered by Parnham et al (1977) to have antioxidant and antiinflammatory properties. Boiled sponges soaked in carrageenan induced more proliferation of granulation tissue and more fluid exudate after 8 days than unboiled, soaked sponges. The extract was thought to contain a phenolic material, commonly used as an antioxidant in polymer manufacture.

12.5 Polyurethane Sponge Implants

Unlike most polymers such as polyethylene, polystyrene and polyvinyl chloride, which are polymers of monomeric units (ethylene, styrene, vinyl chloride respectively), polyurethanes do not have repeating units of urethane in the regular manner and do not have an empirical formula that is representative of all. They are polymers containing the urethane linkage (-NH-CO-O-).

Paulini et al (1974) carried out a quantitative histological, biochemical and autoradiographical study on polyurethane sponge implants contained within PVC tubes. Six implants were placed in each rat for periods of up to 16 weeks. The tissue response occurred in three phases, the first being an exudative phase in the initial seven days after implantation. Cell turnover was slow. The second phase occurred between one and four weeks after implantation and was characterized by the proliferation of granulation tissue. The third phase, seen after four weeks, was a chronic proliferative inflammation. Cell turnover was high. The authors concluded that the reaction to the implants represented three models of inflammation in one and it was therefore suitable for the study of the effects of antiinflammatory agents on the different phases.

Polyurethane sponge implants, impregnated with dead tubercle bacilli (TBC) were implanted subcutaneously in rats which were treated with a variety of antiinflammatory agents by Clarke et al (1975). The sponges weighed 17mg, were impregnated with 0.33mg of TBC and were implanted for up to 14 days. Sponge dry weight gain and extent of cellular invasion were the parameters used to assess the efficacy of the antiinflammatory agents tested. The sponges themselves were claimed to be "biologically relatively inert." They enabled the fluid and cellular phases of inflammation to be studied separately. The dry weight gain of sponges implanted for 5 days was attributed to the protein content of the fluid exudate. The extent of infiltration of the sponges by inflammatory cells was measured on sections cut from the central part of the sponges with a calibrated eyepiece at 10 positions around each section. This method gave an indication of the number of cells within the sponges. The fluid and cellular phases of acute in-

flammation were reduced by treatment with phenylbutazone, aspirin and prednisolone. However, sodium aurothiomalate and D-penicillamine only suppressed the cellular phase (mainly PMN) of inflammation.

Woodland et al (1977) implanted polyurethane sponges impregnated with TBC in normal rats. Sponges soaked in tuberculin were implanted in rats sensitised 10 days earlier with Freund's complete adjuvant. An image analyzing computer was used to measure the density of cellular infiltration into the sponges in sections of sponge stained for nuclear DNA. An eyepiece graticule was used to measure the depth of cellular infiltration. Flurbiprofen was found to be of comparable potency to prednisolone in suppressing both fluid and cellular phases of inflammation. Sodium aurothiomalate effectively suppressed fluid exudates into tuberculin impregnated sponges.

* * *

These studies of the implantation of artificial materials have been included in this review since the present series of investigations have involved the implantation of polyurethane sponges as a means of studying the inflammatory properties of HAC.

Chapter Three

Materials, Methods and Results

CHAPTER THREE

MATERIALS METHODS AND RESULTS

The wide range of experiments undertaken in this study were based on a semi-quantitative model of inflammation to test the inflammagenic properties of HAC *in vivo* . Both synthetic and biologically deposited HAC were studied. Corroborative morphological investigations of HAC-macrophage and synovial cell interactions were carried out *in vitro* . The materials, methods and results are presented consecutively for each experiment.

* * *

Since the majority of experiments involved the implantation of polyurethane sponges, the methodology described below was used for the preparation, implantation, removal, processing and staining of the sponges. In addition, techniques for the measurement of sponge dry weight gain and the extent of granulation tissue ingrowth were developed and applied to each sponge implantation experiment.

A Preparation of Polyurethane Sponges for Implantation

Sheets of polyurethane sponge measuring 2x2m and 6mm in thickness were purchased from Olympic General Products, Adelaide, South Australia. The material was a high density polyether urethane, type 31.1.80 and was light green in colour. Squares of sponges for implantation measuring 1.5 x 1.5cm were cut using a scalpel blade guided by a steel template. The blades were quickly dulled by the sponge material. Individual sponges were weighed and segregated into weights within 1mg of each other. Sponges in the weight ranges 39-40, 40-41, 41-42 and

42-43mg were used for implantation. Sponges of the same weight range (and consequently similar volume) were used in individual experiments.

Removal of surface contaminants was achieved by soaking the sponges in 70% ethanol for 30 minutes, followed by several changes of deionised water at room temperature and finally, deionised water at 80°C for 2 hours. Sponges were dried at 37°C on stainless steel trays and packaged. The cut sponges were stored in the dark since ultraviolet light made the sponges more brittle and their colour changed to light yellow.

This regime of sponge preparation resulted in a high degree of biocompatability with tissues surrounding and developing within the implants. No cases of infection attributable to sponge contamination were evident.

B Sponge Implantation Technique

The following procedure was used in all implantation experiments. A sterile technique was used throughout. Under general anaesthesia induced by a mixture of Halothane, nitrous oxide and oxygen administered through a plastic mask, an incision, 1cm long was made in the midline of the dorsum of each animal. Subcutaneous pouches in the flanks were developed by blunt dissection on each side of the midline. Care was taken to prepare the pouches in similar areas in all animals since the site of implantation appears to affect the quality and quantity of tissue growing into the sponges (Bole and Robinson, 1962). Sponges were compressed between the beaks of a pair of fine tweezers and were inserted into the subcutaneous pouches. In most experiments, one left- and one right-sided sponge was implanted in each rat. Following

implantation, the midline incision was closed with a 12mm Aesculap Michel clip. The procedure took no more than two minutes to perform per animal.

C Sponge Removal

Anaesthetised rats were sacrificed by intracardiac injection of sodium pentothal. The dorsal fur was soaked in alcohol and a midline incision 4cm long made at the site of placement of the Michel clip. Lateral incisions were made on each side to expose the sponges which were carefully separated from the surrounding tissues by blunt dissection. In general, sponges implanted for up to 5 days had not developed attached capsules and were removed without adherent tissue. Sponges implanted for more than 5 days were carefully dissected from the surrounding capsular tissues.

D Processing of Sponges for Histological Examination

Pieces of sponge approximately 5mm in thickness were cut from the sponge portions set aside for histology. The pieces were orientated in plastic processing cassettes (Lab Tek, Miles Labs. Naperville Il. USA) so that the section through the approximate centre of each sponge faced the perforated plastic part of the cassette.

Automatic histological processing of the sponges was carried out under vacuum in an Autotechnicon Ultra II tissue processor set on a 12 hour cycle. Sponges were embedded in wax and orientated so that the microtome cut through the approximate centre of each sponge.

Sections 6μ in thickness were cut on an American Optical Co.

microtome using disposable blades. The sections were floated on to albumin coated glass slides and dried.

E Staining Techniques

1. Haematoxylin and Eosin (HE)

Sections were dewaxed and hydrated in two changes of xylene, 100% alcohol, 95% and 70% alcohol (5 minutes in each). After washing in running tap water and rinsing in distilled water, the sections were stained in modified Lillie-Mayer haematoxylin for 7 minutes. The excess stain was washed in running tap water, differentiated in acid alcohol and left in tap water for 10 minutes to "blue". Following a rinse in 70% alcohol, the sections were stained in an eosin-phloxine mixture for 3 minutes and rinsed in 95% alcohol. The sections were dehydrated, cleaned in xylene and finally mounted in PIX5.

Recipe for modified Lillie-Mayer Haematoxylin

20g hematoxylin	2800ml distilled water
200g ammonium alum	4g sodium iodate
1200ml glycerine	80ml glacial acetic acid

Dissolve haematoxylin in small amount of absolute alcohol.
Dissolve alum in 1 litre distilled water with heat.
Allow to cool and add haematoxylin solution.
Add remaining ingredients and filter.
The eosin-phloxine solution was made according to Luna (1968).

2. Von Kossa (VK) for phosphate

Sections were taken to distilled water, missing the wash in running tap water. Staining for 30-60 minutes occurred in 0.5% silver nitrate in sunlight or 100 watt lamp directed at the container. (The slides were held in all-plastic holders.) Following a rinse in distilled water, the sections were counterstained with HE.

3. Stain for Mast Cells (neutral-red/haematoxylin)

See Allen (1960) for method.

4. Martius Scarlet Blue (MSB) for fibrin

The method of Culling (1974) was used with the following modifications:

a. Sections of formalin fixed tissues were treated with picro-mercuric-alcohol (saturated alcoholic solution of picric acid containing 3% mercuric chloride) for 24h after dewaxing.

b. Nuclei were stained with Weigert's iron haematoxylin for 10 minutes.

4. Alizarin red S for calcium

Sections were taken to distilled water as for HE and stained for 5 minutes in 2% aqueous Alizarin red S (adjusted to pH 4.2 with 10% ammonium hydroxide). After rinsing in 4-5 changes of distilled water the sections were dehydrated, cleared and mounted as usual.

5. Mallory's Aniline Blue stain for collagen

See Raphael (1976) for method.

F Measurement of Dry Weight Gain of Sponges

To minimise the numbers of animals required for the experiments described below, one portion of each sponge was used for the estimation of dry weight gain and the other portion for histological study. It was assumed that the granulation tissue that had penetrated the sponges was homogenous and that an equal rate of ingrowth had occurred in both

portions of the sponges. Standardization of the plane of bisection of sponges was achieved by marking the sponge surfaces with a felt-tipped pen along the dorso-ventral plane before their removal.

The sponges were bisected into portions (designated A and B) with a scalpel blade, and both portions weighed. One portion (A) was weighed wet, dried to a constant weight at 60°C, and reweighed. Portion B was placed in 10% formal-saline and processed for histological examination. The total dry weight of the sponge was calculated from:

$$\text{Total dry weight} = \frac{\text{dry weight portion A}}{\text{wet weight portion A}} \times \text{total wet weight (A+B)}$$

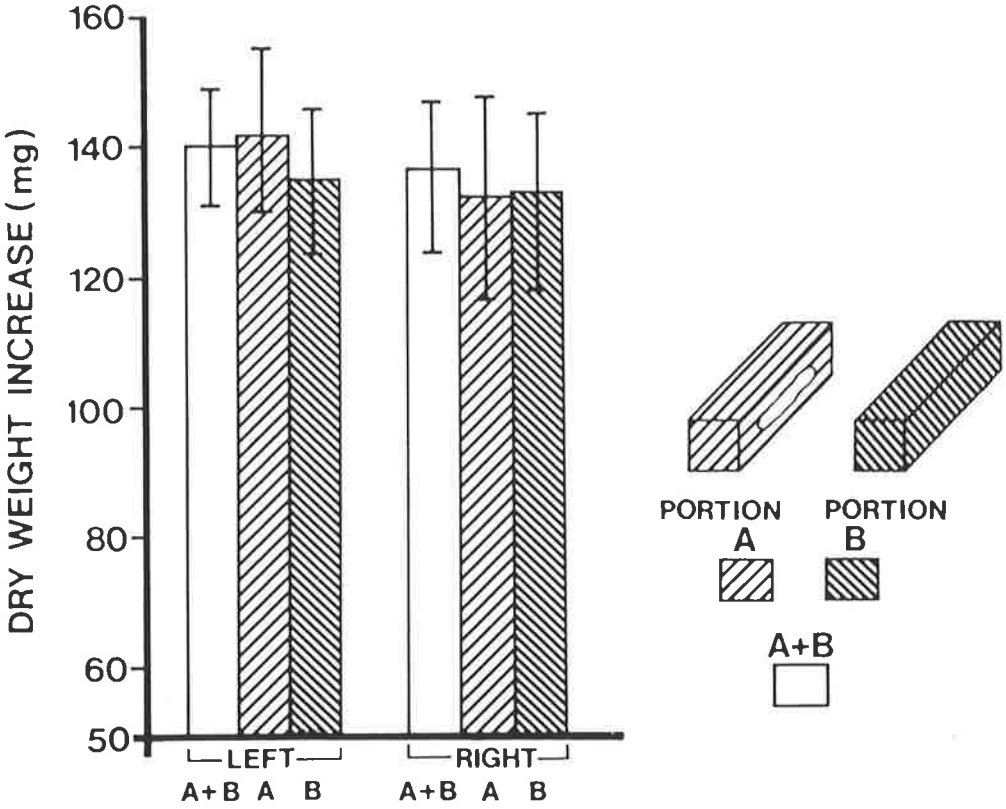
The dry weight increase of each sponge was then determined by subtracting the dry weight of the sponge prior to implantation from the total dry weight as calculated above.

The validity of the assumption that both portions of the sponges had an essentially homogenous granulation tissue content was tested by the implantation of one left- and one right-sided plain sponge in 4 male Porton rats for 14 days. On removal and bisection of the sponges, both portions were weighed wet and dry and the total dry weight increase compared with that calculated from each portion, and that by adding the dry weights of portion A and portion B.

Results (Table F and Histogram F)

Calculation of the dry weight increase of sponges implanted for 14 days by weighing the whole sponge (dried) or by estimation from portions A or B resulted in a similar finding. There was also no statis-

tically significant difference in the dry weight gain of left- and right-sided sponges, no matter which method had been used to estimate this parameter.



Histogram F
Dry weight gain of left and right sided plain sponges implanted for 14 days in Porton rats as estimated by weighing the whole sponge and by calculation from the wet and dry weights of sponge portions A and B.

G Measurement of Extent of Infiltration of Sponges by Granulation Tissue

The extent of granulation tissue ingrowth into the sponges was assessed in the following manner. A HE/VK stained histological section from the centre of each sponge was enlarged seven times using a transparency projector. The outline of the whole section and the limit of granulation ingrowth was traced on to a sheet of blank paper. The total area of the section (A) and the area unoccupied by granulation tissue (B) were traced by a Cursor System on a Hewlett Packard 9874A XY

digitiser interfaced with a 98/30 programmable calculator. Numerical integration of coordinate impulses from the digitiser was performed using the trapezoidal rule for area. This gave an area measure in arbitrary units. The percentage area of the section occupied by granulation tissue was calculated from the formula:

$$\frac{A - B}{A} \times 100$$

The error involved in this estimation was determined by studying eight randomly chosen sections from the pool of all the slides used in Experiment 1. The section outlines were traced five times using the method described above and each tracing was digitised four times (20 measurements per section). The data were subjected to single factor analysis of variance testing, after Snedecor and Cochran (1980).

Source	SS	DF	MS	F	P
Slides	169977669.000	7	24282524.200	16215.767	< 0.001
Error	227614.500	152	1497.464		

The total variance due to error was calculated thus:

MS (within measurements) $= \delta_w^2 = 1497$

MS (between slides) $= \delta_w^2 + 20.\delta_B^2$

$$\delta_B^2 = \frac{\text{MS (between)} - \delta^2 \text{ (within)}}{20} = \frac{24282524 - 1497}{20}$$

$$= 1214051$$

$$\delta^2_{Total} = \delta^2_{Within} + \delta^2_{Between}$$

$$= 1214051 + 1497$$

$$= 1215548$$

$$\%\text{Total variance due to error} = \frac{\delta^2_{Within}}{\delta^2_{Total}} = \frac{1497 \times 100}{1215548}$$

$$= 0.12\%$$

H Statistical Methods

Data for Experiments 1-9 were subjected to testing for normal distribution and were subsequently subjected to parametric statistical analysis. Analysis of variance was used to test for treatment differences in each experiment. The level for significance was set at $p<0.05$ using one-tail or two-tail tests where appropriate. Data processing was carried out on both a Hewlett Packard model 9830A and an Apple II computer using commercial statistical software. The number of rats assigned to the separate experimental treatment groups was limited to four because of the cage size (4 per cage) and a restricted number of rats available from the supplier.

Data are presented in tabular form as an Appendix and in graphical form in the text. The error bars shown on the histograms and figures represent the standard error of the means.

I Ethical Implications

All of the techniques used in this project have received approval from the Animal Ethics Committee of the Institute of Medical and Veterinary Science. Those techniques which have been utilised in the Medical School, University of Adelaide, have received approval from the University's Animal Ethics Committee.

EXPERIMENT 1

Aim

To assess the response of 3 rat strains to the subcutaneous implantation of polyurethane sponges.

Method

One left- and one right-sided plain sponge was implanted in male Porton, Lewis and Dark Agouti rats weighing 200-250g. Four animals of each strain were randomly assigned to each experimental group. The effect of implantation of sponges for 5, 7, 10 and 14 days was assessed by measuring the dry weight gain of the sponges, the area of sponges occupied by granulation tissue and the histological appearance of sections stained for mast cells, collagen and with HE. Samples of sponges implanted for 14 days were prepared for TEM.

Tissue samples were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer at pH 7.6 for 2 hours at room temperature. They were transferred to a 0.1M cacodylate buffer containing 6% sucrose which was changed twice before post-fixing in 2% osmium tetroxide in cacodylate buffer. Another wash in cacodylate sucrose buffer followed. The fixed tissues were dehydrated through a graded series of alcohols from 35 to 100%.

The dehydrated tissues were exposed to two changes of propylene oxide followed by 1:1 and 1:3 mixtures of propylene oxide and TAAB

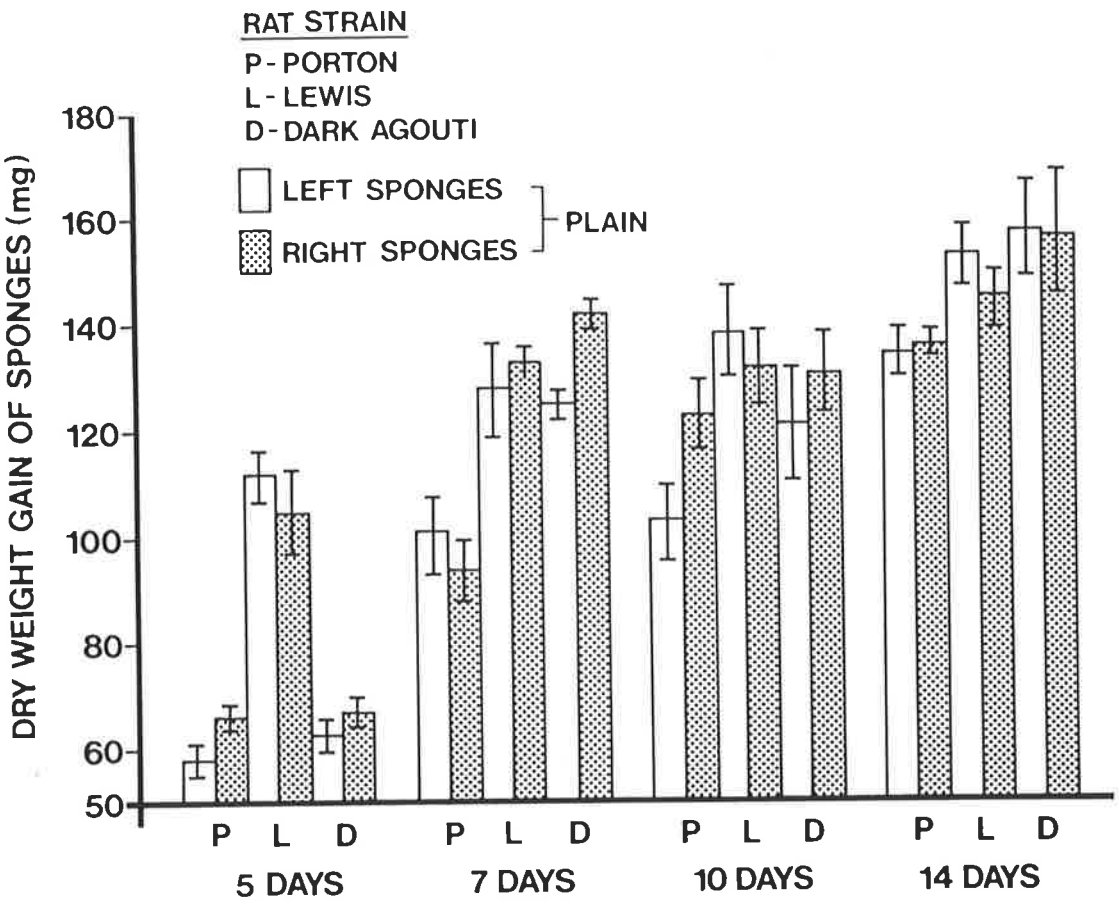
embedding resin, then two changes of resin alone. Polymerization occurred at 60°C overnight.

Sections 1μ in thickness were cut with a glass knife and were stained with Toluidin blue. Appropriate sections for TEM were chosen by examination with a light microscope. A diamond knife mounted in an ultramicrotome was used to cut ultrathin sections (approximately 60nm thick) which were mounted on 3mm copper grids, stained with uranyl acetate and lead citrate and finally carbon coated. The grids were placed in a JEOL 100CX TEMSCAN electron microscope, the accelerating voltage set at 80kV and the images examined visually and recorded photographically.

Results

1a. Sponge Dry Weight Gain (Table 1a and Histogram 1a)

Left- and right-sided sponges showed similar dry weight gain in all rat strains and at all time periods. However, significant differences in sponge dry weight gain were measured across the four time periods for each rat strain ($p<.001$). In all cases, sponge weight increased with increasing time of implantation, this effect being most pronounced in Porton and Dark Agouti rats with sponge weight gain doubling between 5 and 14 days. In Lewis rats, sponge weight gain was nearly twice as much as that for the other rat strains five days after implantation. There was no interaction between the implantation site and the duration of implantation on sponge dry weight gain in any of the rat strains tested.

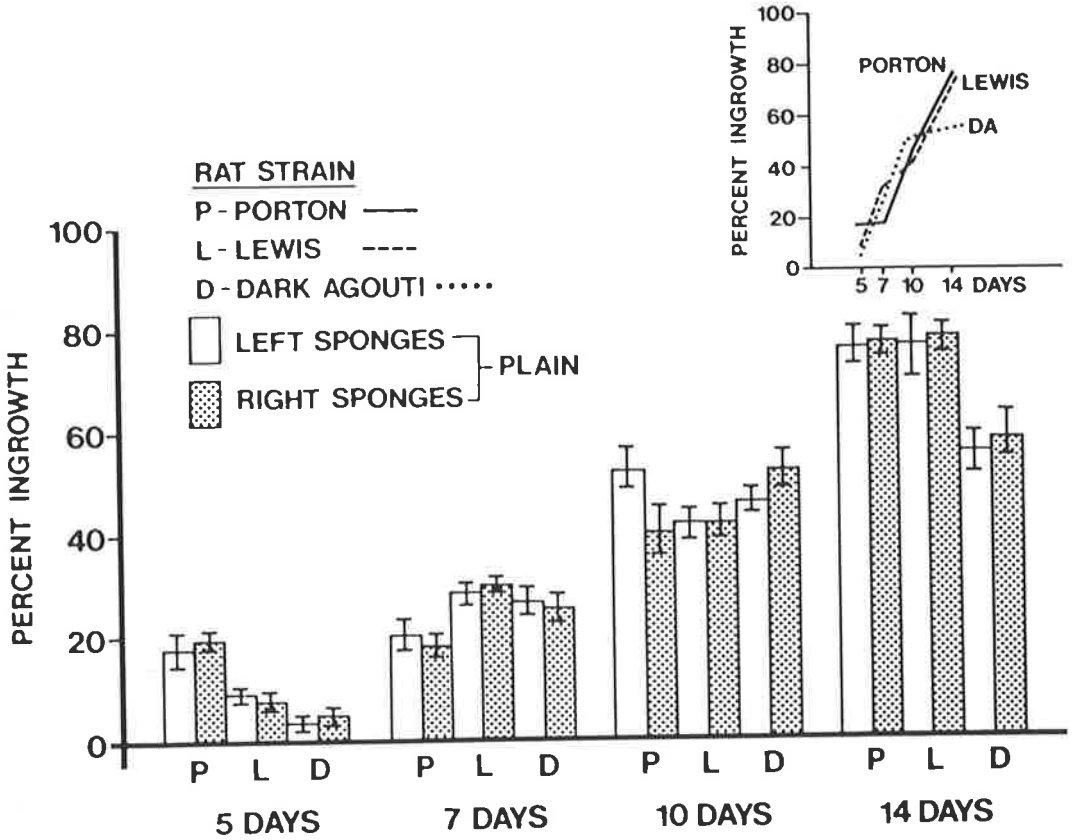


Histogram 1a
Dry weight gain of plain sponges implanted in Porton, Lewis and Dark Agouti rats for 5, 7, 10 and 14 days.

1b. Ingrowth of Granulation Tissue (Table 1b and Histogram 1b)

Left- and right-sided sponges had been permeated by a similar amount of granulation tissue in all rat strains and at all time periods. A statistically significant increase in the amount of granulation tissue occupying the sponges occurred across the four time periods for each rat strain ($p<.001$). Five days after implantation, ingrowth ranged from 4.4% in Dark Agouti rats to 19% in Porton rats. By 14 days, sponges implanted in Porton and Lewis rats had 77% of their sectional area occupied by granulation tissue, whereas the figure in Dark Agouti rats was only 57%. There was no interaction between

implantation site and the duration of implantation on granulation tissue ingrowth in any of the rat strains tested.



Histogram 1b
Ingrowth of granulation tissue into left and right sided plain sponges implanted in Porton, Lewis and Dark Agouti rats for 5, 7, 10 and 14 days. Inset shows rate of tissue ingrowth for the 3 rat strains over the same time period.

1c. Histology

The histological appearance of implanted sponges at each time interval was similar in all rat strains. Artefactual shrinkage spaces around the sponge matrix were seen in all sections.

5 days

There was evidence of organization at the periphery of the sponges with stellate fibroblasts (fig.1.1) and capillary budding (fig 1.2). Condensation of cells occurred around the sponge matrix. A few PMN in various

stages of degeneration were apparent within the central zone of the sponges.

7 days

The granulation tissue was well-established with stellate fibroblasts, prominent vascularization and scattered inflammatory cells (fig.1.3). Degenerative PMN were seen in the centre of the sponges (fig.1.4). There was a condensation of flattened cells around the sponge matrix.

10 days

Fibrosis at the sponge periphery and condensation of fibrous tissue and flattened cells around the sponge matrix were prominent (fig.1.5). Residual granulation tissue was present in the sponge interstices. Within the central zone, fibroblasts extended into a loose network of precipitated protein.

14 days

The peripheral junction of the sponge with surrounding tissues was delineated by a layer of mature fibrous connective tissue with fat cells prominent in some areas (fig.1.6). Intact mast cells were present in this layer but were absent from the granulation tissue within the sponge. Further towards the central zone in the older granulation tissue, fibroblasts were comparatively reduced in numbers and the collagen fibre content was increased with some condensation around the sponge matrix (fig.1.7). Moderate aggregations of macrophages and giant cells surrounded the sponge matrix. The young granulation tissue around the "unoccupied" central zone was moderately cellular with a modest collagen fibre content (fig.1.8). Fibroblasts were the most common cell. Occasional lymphocytes and plasma cells were also present.

Fig.1.1 Polyurethane sponge implanted for 5 days showing sponge matrix (S) and organization at the periphery. HE x45

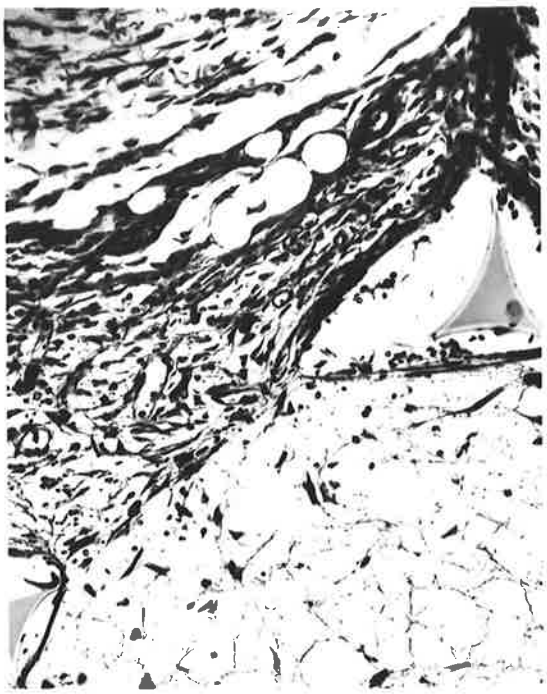


Fig.1.2 Capillary budding and fibroblastic proliferation at the periphery of a sponge implanted for 5 days. HE x145

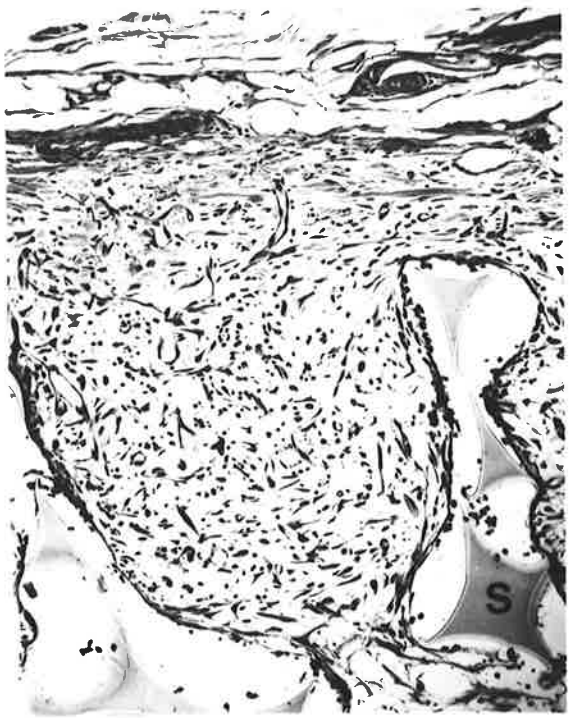


Fig.1.3 Well established granulation tissue in sponge 7 days after implantation. The spaces around the sponge matrix (S) are artefactual. HE x145

Fig.1.4 PMN in various stages of degeneration in the central zone of the sponges. HE x370

1d. Transmission Electron Microscopy

TEM of sponges implanted for 14 days showed the presence of collagen fibres in transverse and longitudinal section. Cells with elongated nuclei and cytoplasm were closely associated with the collagen (fig.1.9). The cells were characteristic of fibrocytes and fibroblasts; macrophages with pseudopods and lysosomes were present (fig 1.10). Capillaries and venules were prominent (fig.1.11). Multinucleated giant cells containing dilated rough endoplasmic reticulum and mitochondria were seen around the sponge matrix (fig.1.12). No cell membrane was evident at the junction of sponge and MNGC.

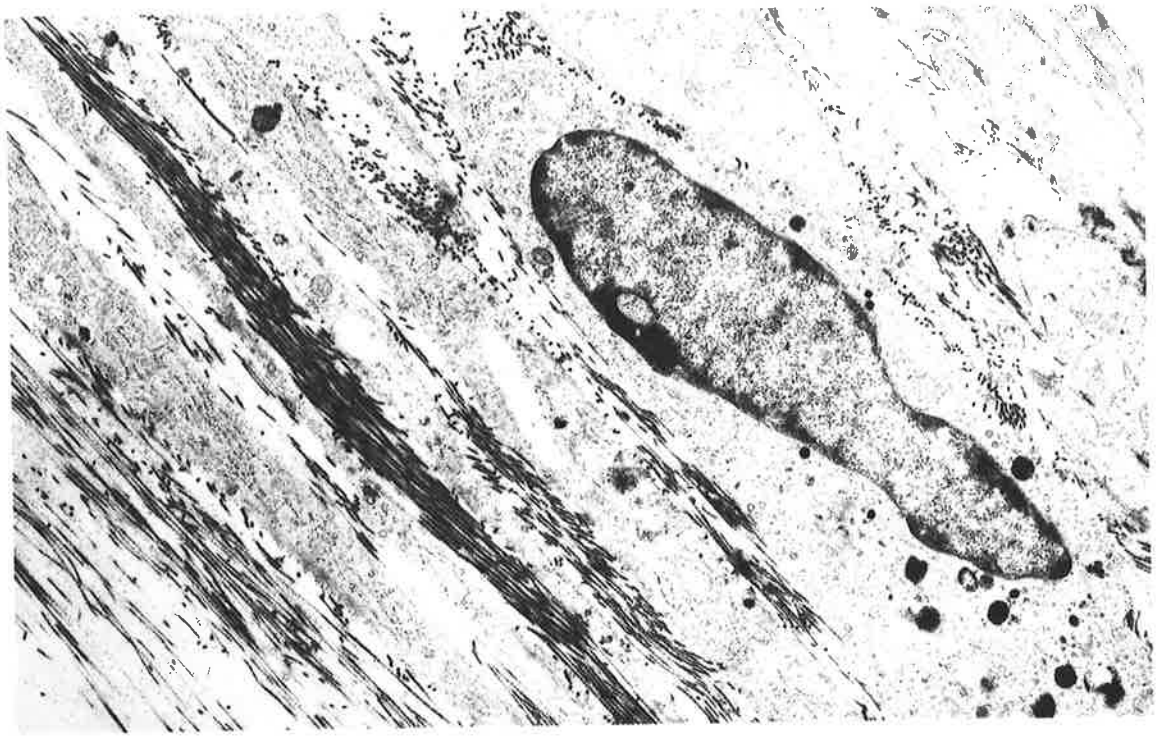


Fig.1.9 Electron micrograph of granulation tissue in sponges implanted for 14 days. Bundles of collagen and part of a fibrocyte are seen. x9,000

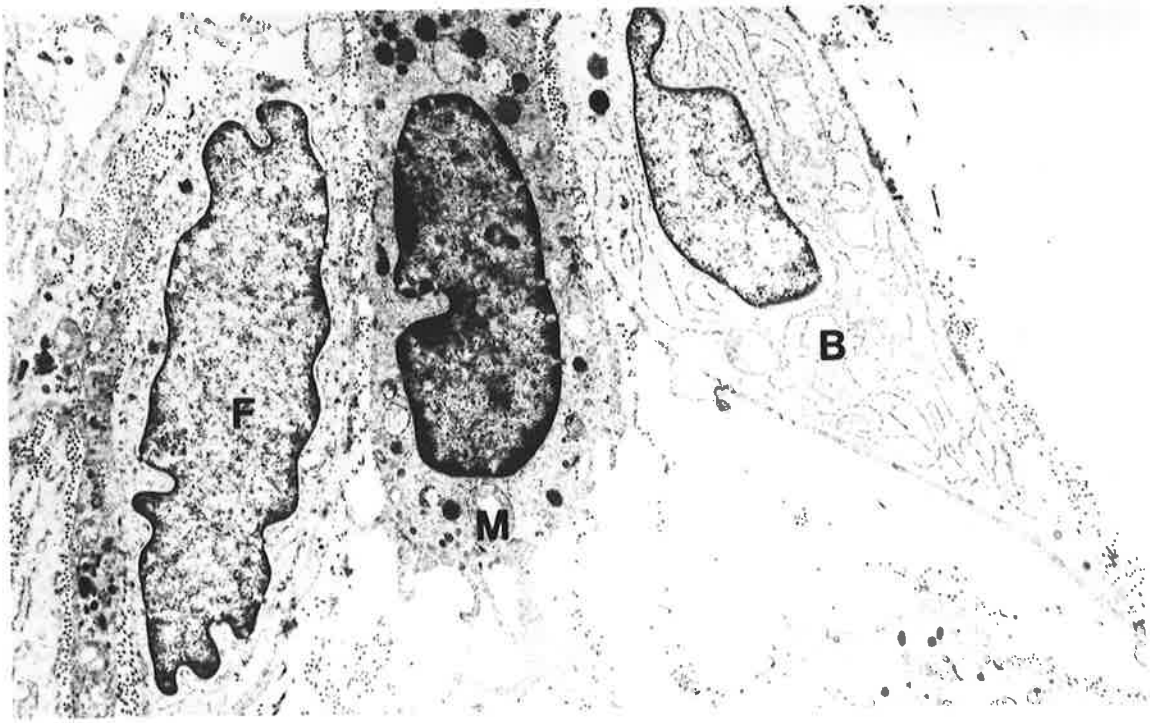


Fig.1.10 Electron micrograph of granulation tissue in sponges implanted for 14 days showing a fibrocyte (F), macrophage (M), fibroblast (B) and transversely cut bundles of collagen. x9,000

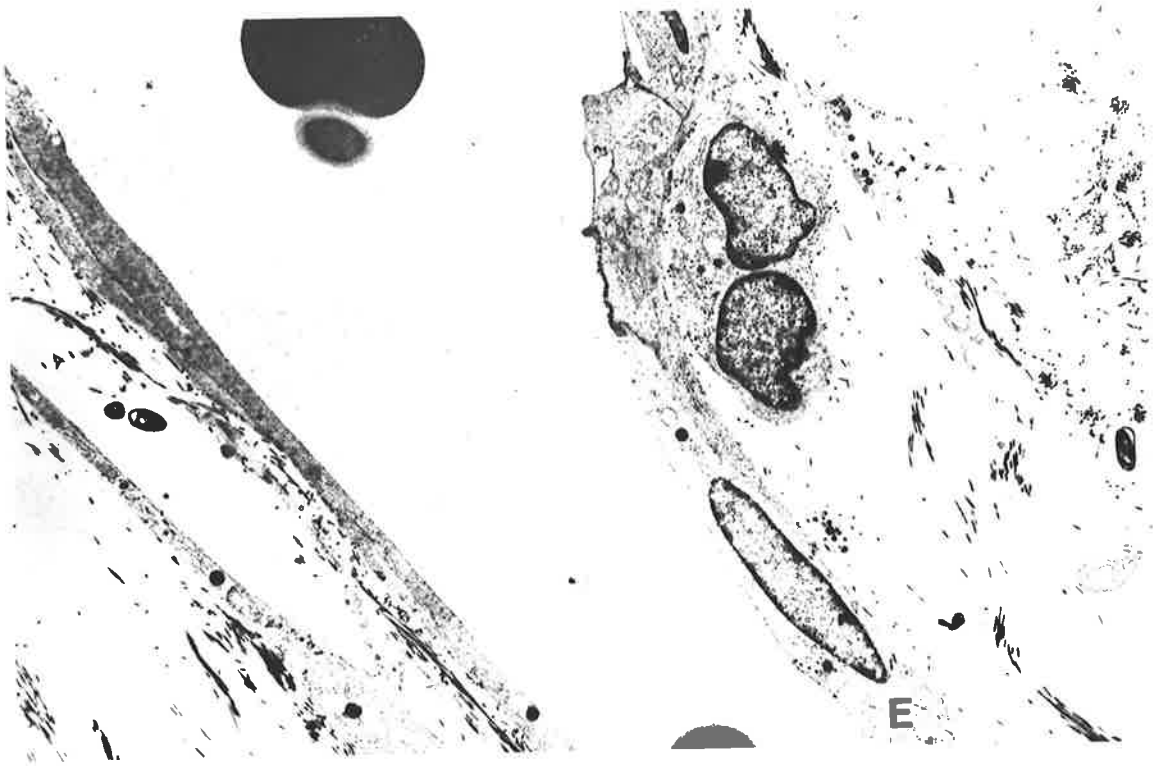


Fig.1.11 Electron micrograph of granulation tissue in sponges implanted for 14 days featuring a post-capillary venule, containing red blood cells, lined by endothelial cells (E). x4,860

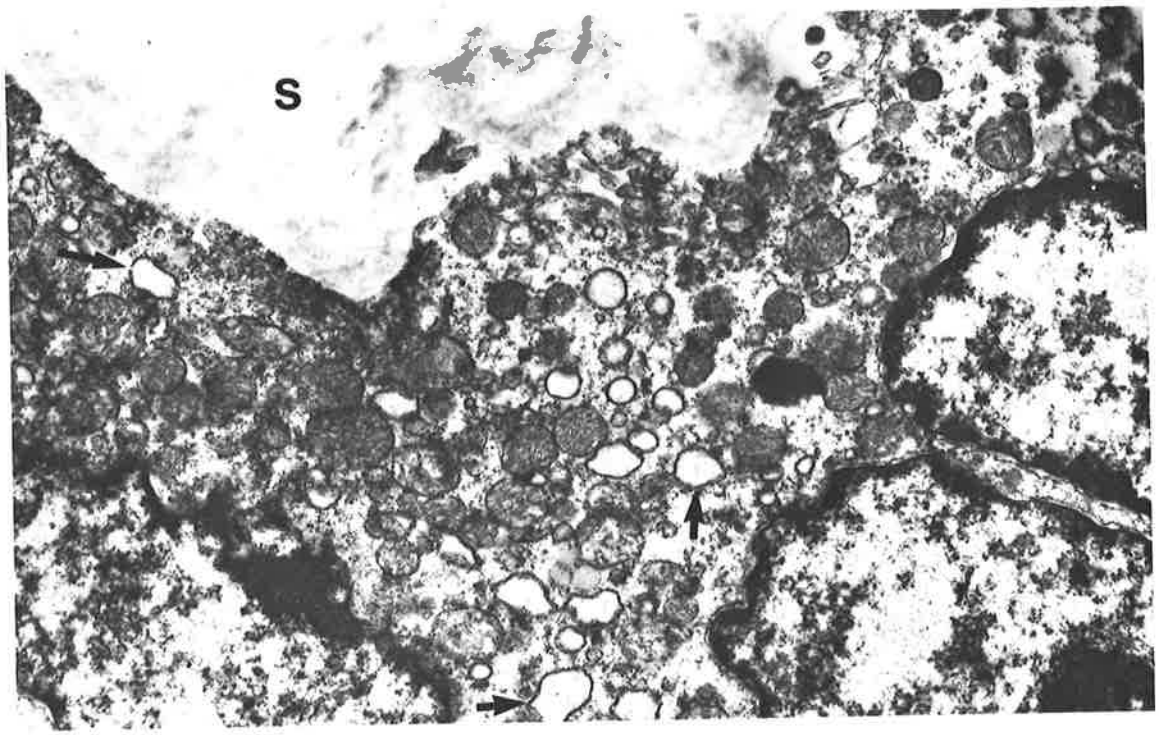


Fig.1.12 Electron micrograph of a MNGC adjacent to sponge matrix (S) in a sponge implanted for 14 days. Numerous mitochondria and dilated rough ER (arrows) are present. No cell membrane is present at the junction of sponge and MNGC. x12,500

EXPERIMENT 2

Aim

To assess the response of Porton rats to the subcutaneous implantation of four polyurethane sponges per rat.

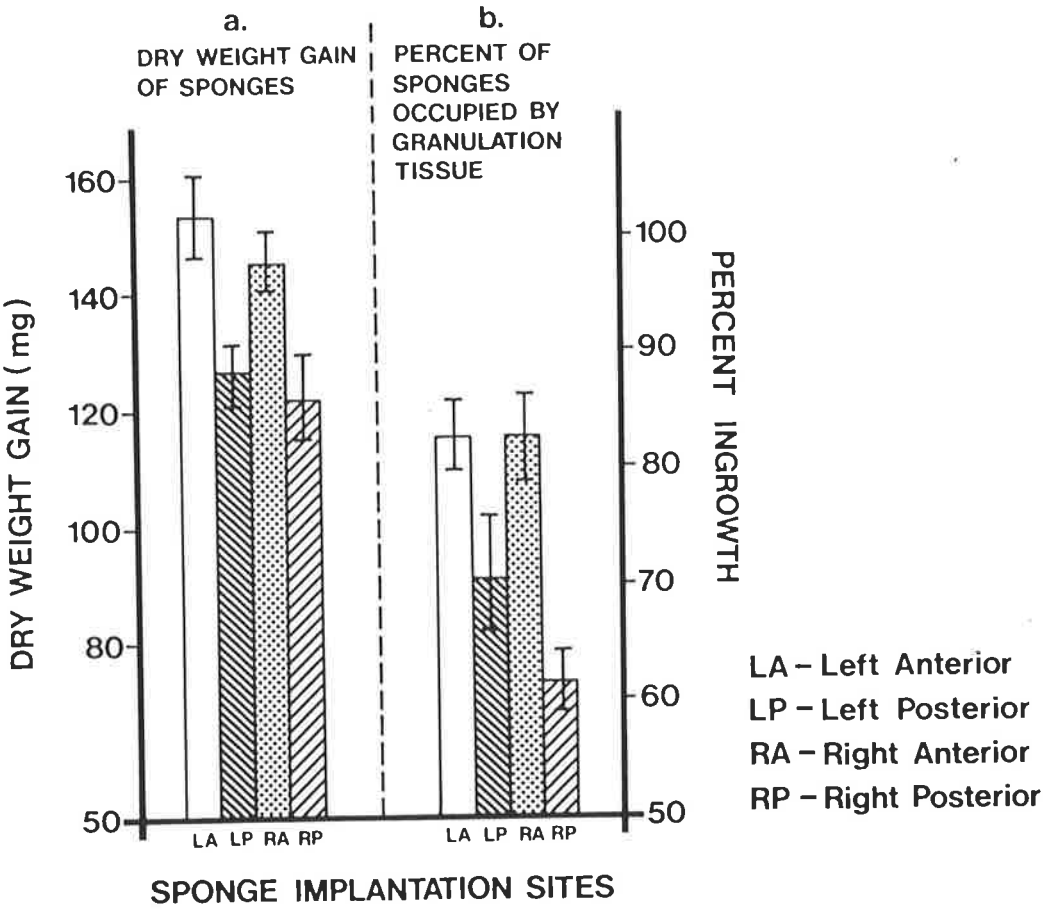
Method

Four male Porton rats weighing 200-250g had two left- and two right-sided plain sponges implanted. The sponges implanted in anterior sites were positioned as previously described. Posterior sponges were implanted through an additional midline incision made towards the base of the animals' backs. Subcutaneous pouches were developed and wound closure was carried out as before. The sponges were removed 14 days after implantation and the parameters measured were sponge dry weight gain, ingrowth of granulation tissue and histological assessment.

Results

2a. Sponge Dry Weight Gain (Table 2a and Histogram 2a)

Sponges implanted at anterior sites gained significantly more weight than sponges placed at posterior sites ($p=.01$). Anterior sponges gained an average of 20mg more weight than posterior sponges. No significant difference was found between left- and right-sided sponges at both anterior and posterior implantation sites.



Histogram 2
a. Dry weight gain of plain sponges implanted for 14 days at left and right, anterior and posterior sites in Porton rats.
b. Ingrowth of granulation tissue into the same sponges.

2b. Ingrowth of Granulation Tissue (Table 2b and Histogram 2b)

Sponges implanted at anterior sites were infiltrated by a significantly increased amount of granulation tissue than sponges implanted at posterior sites ($p=.006$). Anterior sponges had been infiltrated by an average of 17% more granulation tissue than posterior sponges. Left and right anterior sponges had similar amounts of granulation tissue ingrowth whereas right-sided posterior sponges had 8% less ingrowth than the left-sided posterior sponges.

2c. Histology

The granulation tissue in all sponges was similar in appearance to that in 14 day sponges described in Experiment 1. However, sponges implanted at posterior sites had less tissue infiltration than those from anterior sites.

EXPERIMENT 3

Aim

To assess the effect of implanting sponges impregnated with varying amounts of hydroxyapatite on the quality and quantity of tissue growing into the sponges.

A Source of Synthetic Hydroxyapatite

The synthetic HAC used throughout this and subsequent experiments were obtained from the Sigma Chemical Company, St. Louis Mo. USA, (Type 1 No.H0252) in 0.001M phosphate buffer, pH 6.8. The crystals were thoroughly washed in deionised, distilled water, dried at 60°C and stored dry at 4°C in an airtight container.

The crystals were prepared for SEM examination by making a suspension in deionised water. After brief sonication, the larger clumps were allowed to settle. A drop of the suspension was placed on a Formvar/carbon coated copper grid. Crystals were allowed to settle for 30 seconds and excess fluid was drawn off with a filter paper. The grid was coated with carbon (evaporated) then sputter coated with gold. Specimens were examined in a JEOL 100CX TEMSCAN electron microscope at 40kV.

The SEM appearance of these crystals (fig.3.1) showed the heterogeneity of crystal size and shape. These crystals were aggregates of microcrystals. The size of the crystal aggregates ranged from approximately 0.1-30μ. TEM (figs.3.2 and 3.3) showed the microcrystalline nature of the aggregates with individual well formed rod shaped crystals.

Fig.3.1 Scanning electron micrograph of Sigma hydroxyapatite as supplied by the manufacturer. x1000

Fig.3.2 Electron micrograph of a cross section through an aggregate of Sigma hydroxyapatite crystals revealing the internal structure of the particles seen in Fig.3.1. x25,000

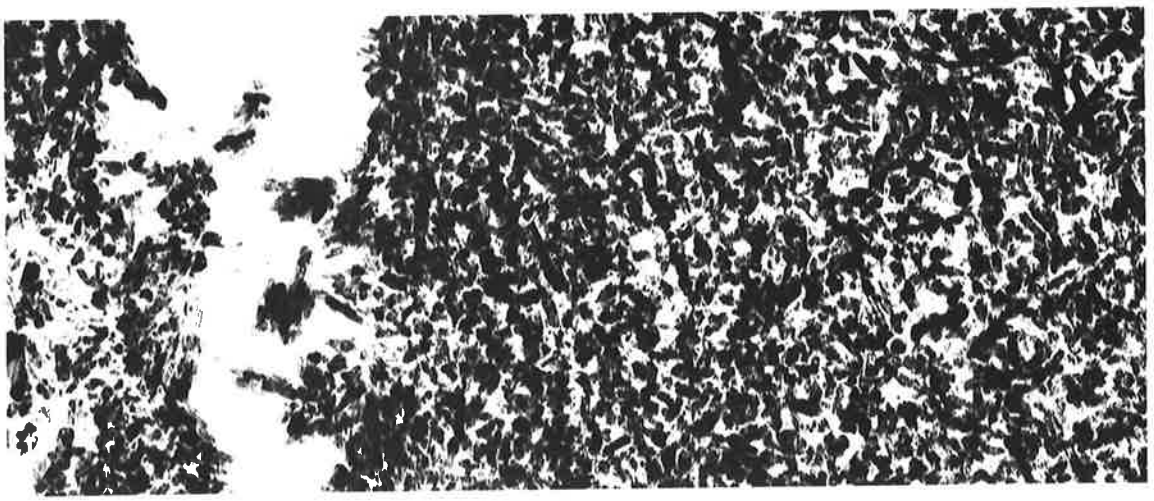


Fig.3.3 Higher magnification of Sigma HAC showing individual well-formed rod-shaped crystals. x70,000

B Sponge Impregnation

Sponges were impregnated with a suspension of Sigma HAC that contained a large range of particle sizes. The procedure used to impregnate the sponges with 20mg of hydroxyapatite was as follows:

An amount of dried Sigma HAC was weighed, ground to a powder with an agate pestle and mortar , added to 90% alcohol and sonicated briefly to disperse the larger clumps of HAC. The quantity of HAC used was determined by multiplying the number of sponges by 1.5ml (the amount of fluid absorbed by each sponge). The sponges were compressed between the beaks of a wide-ended pair of sterile tweezers, immersed in the HAC suspension and released, allowing the sponges to soak up the solution. Care was taken to constantly agitate the mixture to avoid settling of the HAC particles. The sponges were placed on a stainless steel tray in an oven at 60°C and were turned every two minutes until they were dry.

Several plain and HAC impregnated sponges were prepared for SEM examination by brief immersion in liquid nitrogen followed by bisection with a scalpel blade. One half of the sponge was attached to a copper stub (using double sided adhesive) so that the cut surface faced upwards. Silver dag adhesive was also used to glue sponge to stub. The sponges were coated with carbon and gold and examined as before.

SEM of plain sponges (fig.3.4) demonstrated the appearance of the sponge network. The sponge matrix had concave surfaces. In sponges containing 20mg of HAC, the crystal aggregates were evenly distributed throughout the sponges (figs.3.5 and 3.6). The HAC particles lodged

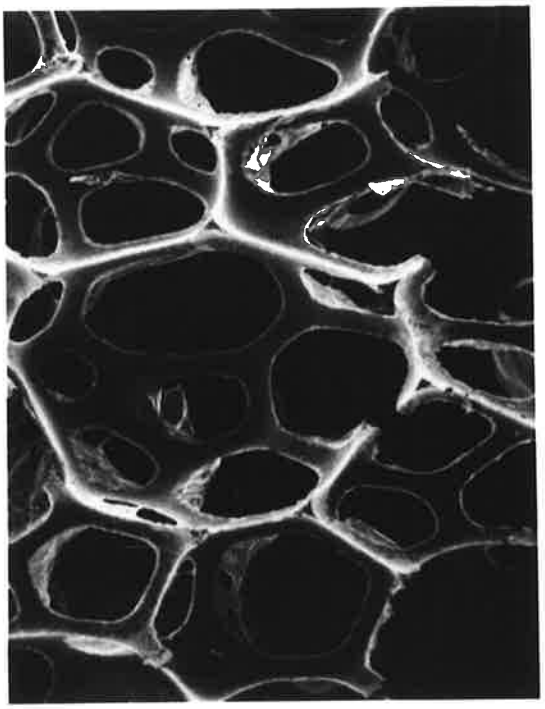


Fig.3.4 Scanning electron micrograph of a plain polyurethane sponge highlighting the concave surfaces of the sponge matrix. x30

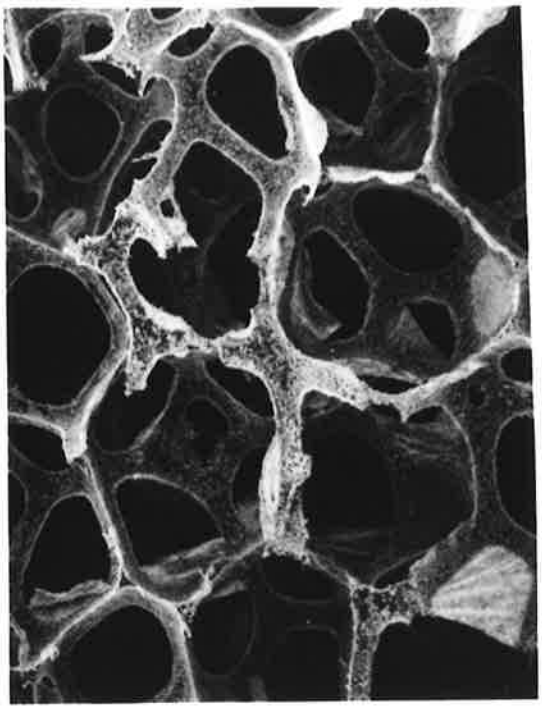


Fig.3.5 Scanning electron micrograph of a sponge impregnated with 20mg of HAC. The material has been evenly deposited on the sponge surface. x30

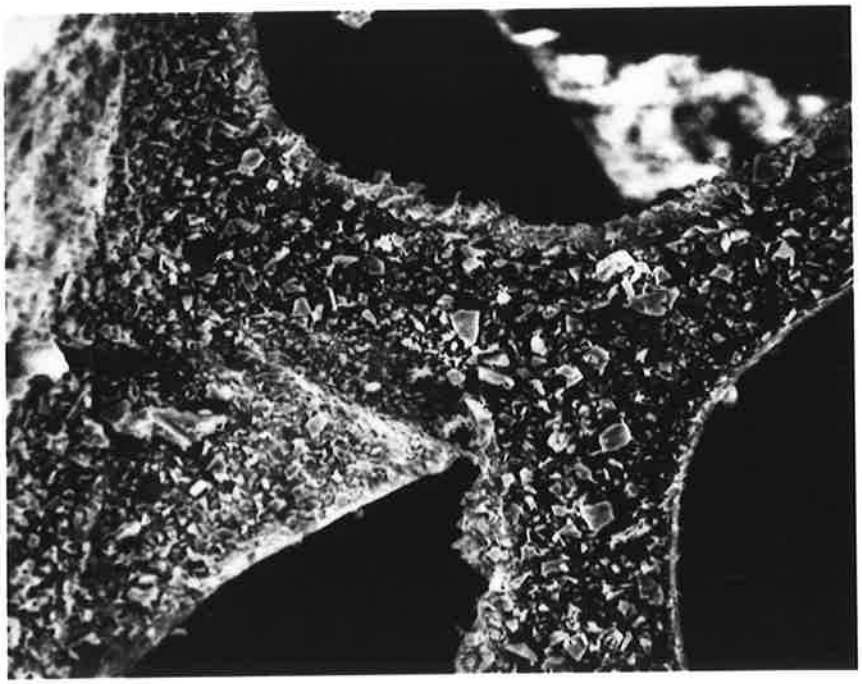


Fig.3.6 Higher magnification of sponge impregnated with 20mg of HAC. The particles are lodged on the concave surfaces of the sponge. x200

upon the concave surfaces of the sponge matrix. It would appear that 20mg of HAC was sufficient to coat all surfaces of the sponge.

Alcohol was selected as a suspension medium because it evaporated quickly during the drying process and did not cause dissolution of the HAC particles. When dry, the sponges were re-weighed to ensure that they held the required amount of hydroxyapatite and were stored in an airtight container.

Method

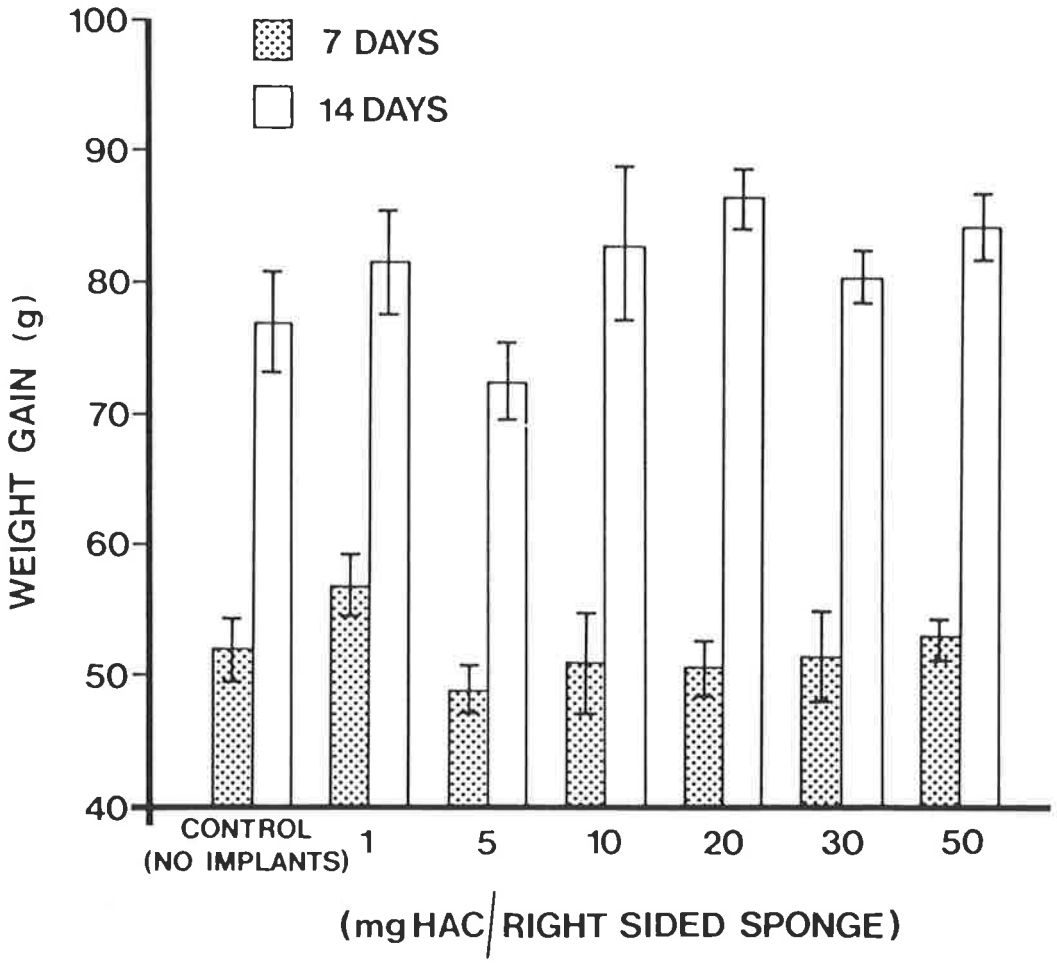
Groups of 4 male Porton rats weighing 170-230gm had sponges implanted for 14 days. Sponges implanted on the left side were plain (control) sponges and right-sided sponges contained varying amounts of HAC as detailed below.

Group	Weight of HAC per sponge(mg)
1	1
2	5
3	10
4	20
5	30
6	50

Upon removal, the sponges were bisected, weighed and one portion processed for histology. Histological sections were stained with HE/VK. Parameters assessed were sponge dry weight gain, area of infiltration of the sponge by granulation tissue and histological response. Increase in animal weight was also measured, using animals implanted with plain sponges only as controls. One extra group of rats had sponges implanted for eight weeks for assessment of histological changes only.

Results

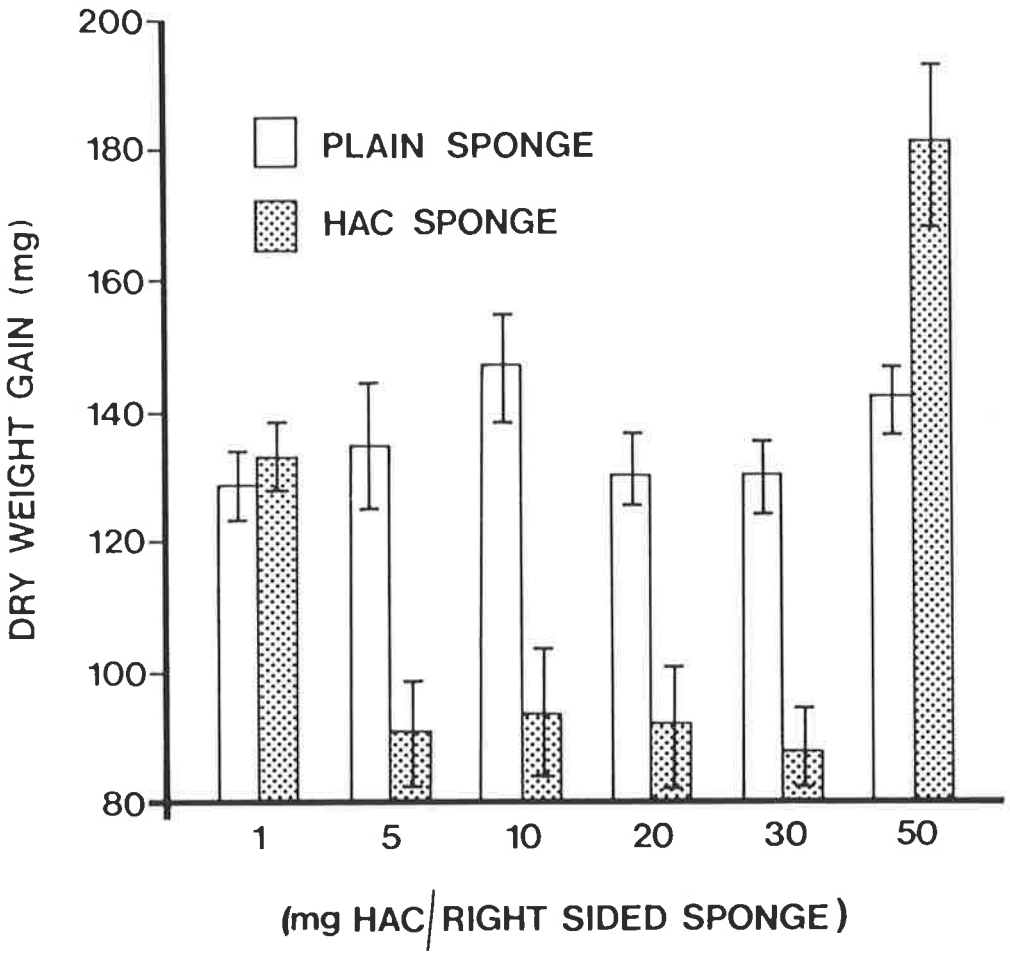
3a. Rat Weight Gain (Table 3a and Histogram 3a)



Histogram 3a
Effect of implantation of sponges impregnated with various amounts of HAC on the weight gain of Porton rats at 7 and 14 days after implantation of sponges impregnated with various amounts of HAC.

Rat weight gain was measured at 7 and 14 days after sponge implantation. While there were no significant differences in rat weight gain beteen control and test animals at the same time period, there was a statistically significant difference in rat weight gain between the two times of measurement ($p<.001$). Control and test rats gained approximately 30g in the last 7 days of the experiment.

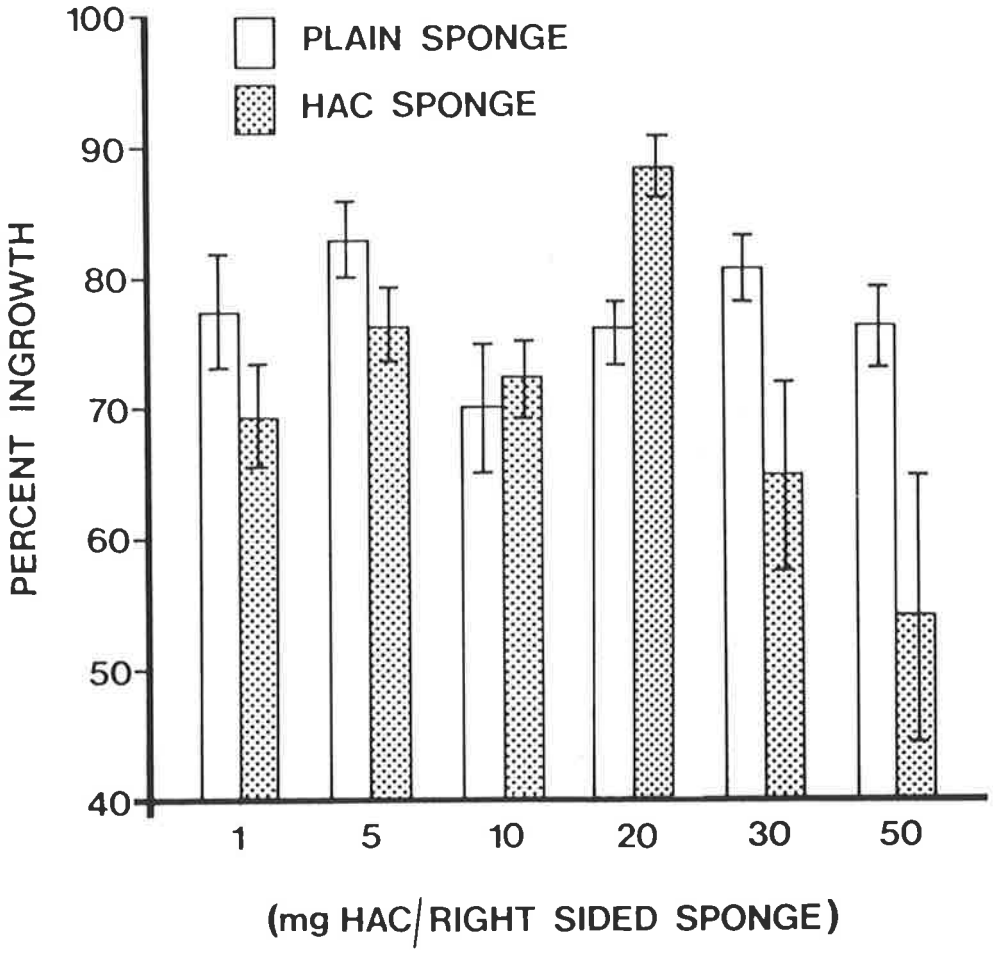
3b. Sponge Dry Weight Gain (Table 3b and Histogram 3b)



Histogram 3b
Dry weight gain of plain and HAC impregnated sponges implanted for 14 days in Porton rats.

Control (left-sided) sponges gained 136mg on average and there was no statistically significant difference between the experimental groups. Sponges containing 1mg of HAC gained a similar amount of weight as control sponges. However, sponges containing 5, 10, 20 and 30 mg of HAC gained significantly less weight (40mg) than control sponges. Sponges containing 50mg of HAC gained 50mg more weight than control sponges.

3c. Ingrowth of Granulation Tissue (Table 3c and Histogram 3c)



Histogram 3c
Ingrowth of granulation tissue into plain and HAC impregnated sponges implanted for 14 days in Porton rats.

Plain sponges were infiltrated to an average extent of 77% by granulation tissue and the response to left-sided sponges of all experimental groups was similar (p=.25). Ingrowth into right-sided sponges was variable; the main trend was a decreased amount of granulation tissue ingrowth into sponges containing 30 and 50mg of HAC (12 and 22 per cent less ingrowth than controls respectively). Incorporation of 20mg HAC per sponge had a slight inductive effect on granulation tissue ingrowth (6% more than controls).

3d. Histology

One milligram of HAC per sponge resulted in one or two clumps of VK positive material (the HAC) in each sponge section (fig.3.7). A few mononuclear cells including macrophages were seen around the clumps.

With 5mg of HAC per sponge, the HAC was sparsely scattered throughout the sponges and it had evoked a macrophage response confined to the immediate vicinity of the HAC (fig.3.8).

With 10mg of HAC per sponge, the clumps were larger and more numerous and were surrounded by macrophages and MNGC (fig.3.9) resulting in a foreign body granulomatous reaction.

With 20mg of HAC per sponge, the clumps of HAC were widely distributed and most were adjacent to the sponge matrix (fig.3.10) in all parts of the sponge. There was little evidence of a recent cellular response to the HAC in the central part of the sponges that was unoccupied by granulation tissue (fig.3.11).

With 30mg of HAC per sponge, a similar histological appearance was observed. In addition there was an intensified lymphocytic infiltrate in the granulation tissue between the HAC deposits (fig.3.12).

With 50mg of HAC per sponge, foreign body granulomae with endocytosed HAC were found throughout the sections (fig.3.13). Some breakdown of the HAC clumps into a fine granular material contained within cells was seen (fig.3.14). Artefactual tears in the sections were prominent because of the large amounts of calcified material present. There was no evidence of an acute inflammatory response.

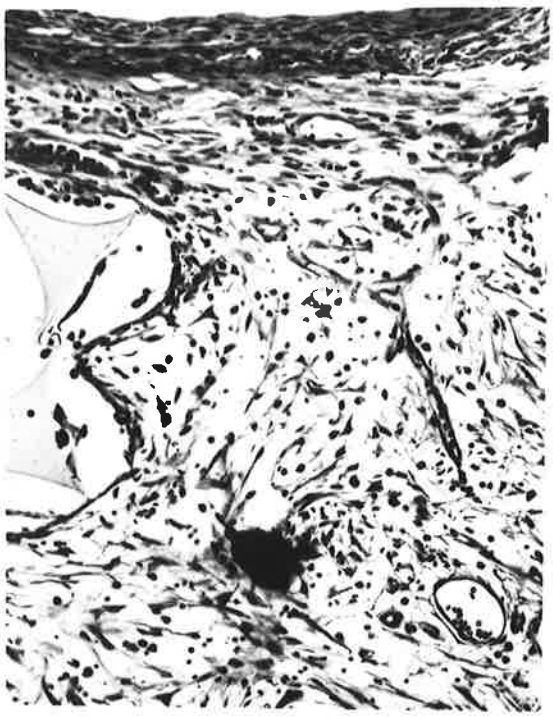


Fig.3.7 Sponge impregnated with 1mg of HAC which has formed an isolated clump in the granulation tissue. HE VK x145

Fig.3.8 Sponge impregnated with 5mg of HAC. The HAC is sparsely scattered and is asociated with a macrophage response in its immediate vicinity. HE VK x90

Fig.3.9 Sponge impregnated with 10mg of HAC. The HAC is surrounded by macrophages and MNGC. HE VK x145

Fig.3.10 Sponge impregnated with 20mg of HAC showing distribution of the HAC near the sponge matrix. HE VK x90

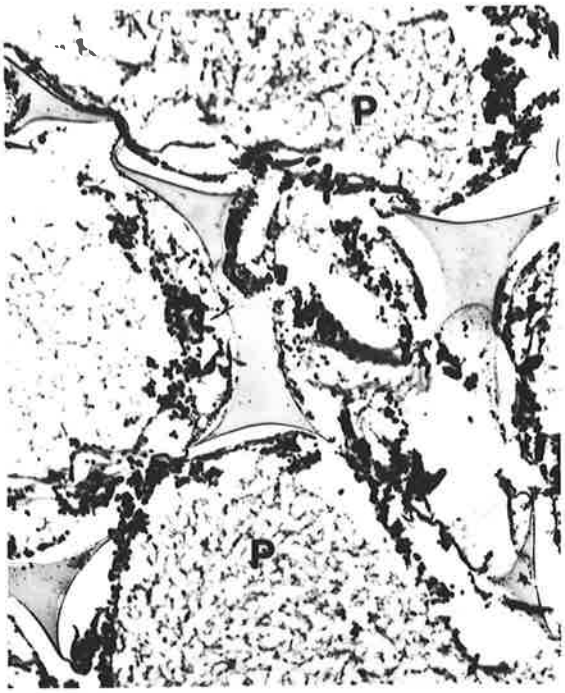


Fig.3.11 Central zone of sponge impregnated with 20mg of HAC. There is no evidence of a recent cellular response. A proteinaceous precipitate (P) is widespread. HE VK x90

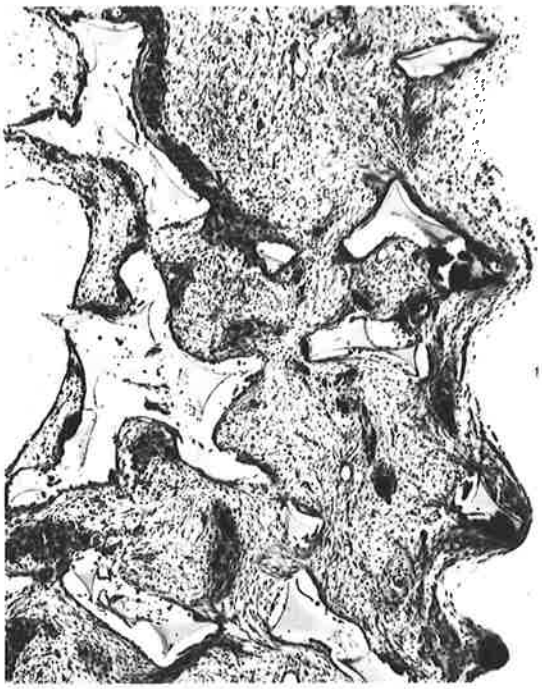


Fig.3.12 Sponge impregnated with 30mg of HAC. There is an infiltration of lymphocytes between the HAC deposits. HE VK x35

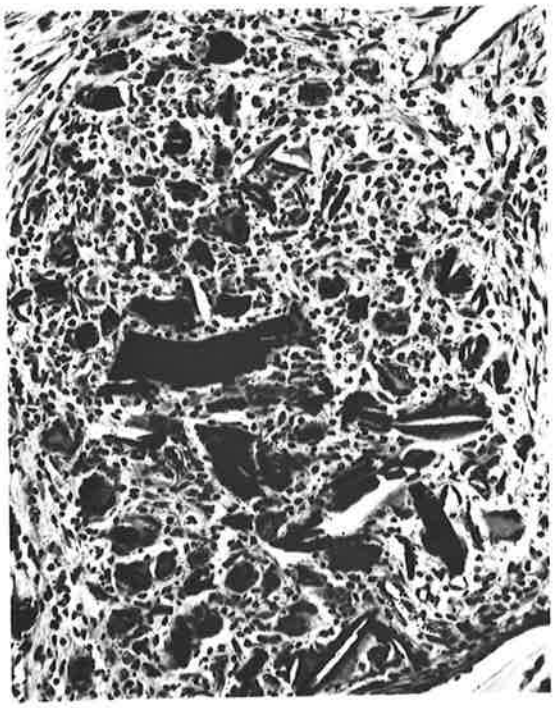


Fig.3.13 Intense macrophage and MNGC response in sponges impregnated with 50mg of HAC. HE VK x290

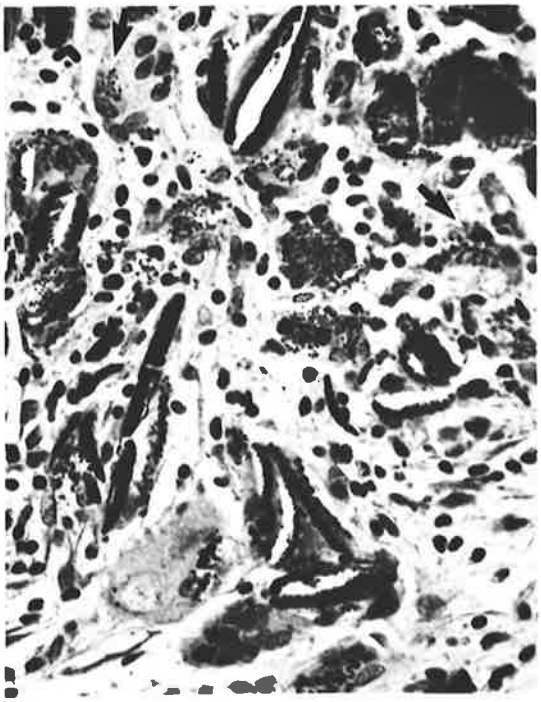


Fig.3.14 Disaggregation of clumps of HAC (arrows) within macrophages and MNGC in sponges impregnated with 50mg of HAC. HE VK x370

EXPERIMENT 4

Aims

4a. Modification of the Shape and Size of HAC Aggregates

4b. To Assess the Tissue Response to Modified HAC Aggregates

EXPERIMENT 4a

Methods

1. Sonication

One gram of dried Sigma HAC was briefly ground in an agate mortar and pestle and added to 50ml of phosphate buffered saline (pH7.2, 0.001M phosphate) in a pyrex beaker. The suspension was sonicated in an MSE ultrasonic cabinet tuned to deliver maximum power. Sonication for at least 45 minutes was required to cause fragmentation of the crystal aggregates.

2. Grinding

One gram of dried Sigma HAC was ground by hand in an agate mortar and pestle for 15 minutes with the application of the largest amount of force possible over this time.

3. Smashing

One gram of Sigma HAC was briefly ground and added to the well of

a stainless steel mortar and pestle which contained liquid nitrogen, the piston placed and a sledgehammer was used to apply a large compressive force to the crystal-liquid nitrogen mixture.

4. Sedimentation

Suspensions of HAC in PBS underwent clumping a few minutes after the addition of the crystal aggregates, resulting in their falling out of suspension. A variety of detergents, both anionic and cationic at different concentrations were added to the PBS crystal suspension but were unsuccessful in preventing clumping of the HAC. Addition of 5% protein in the form of normal rat serum to the PBS was successful in preventing clumping of HAC.

One gram of dried Sigma HAC was briefly ground and added to 200ml of PBS (made with glass distilled water) containing 10ml of serum obtained from normal adult male Dark Agouti rats. The serum had been inactivated by immersion in a water bath at 56°C for 60 minutes. The mixture was sonicated for 3 minutes to ensure dispersal of the crystal aggregates and left undisturbed at 4°C overnight. The larger aggregates settled to the bottom of the flask, leaving the smallest crystal aggregates in suspension. Light microscopic examination revealed the small particles to be in a state of Brownian motion. The supernate containing this material was syphoned into centrifuge tubes and centrifuged at 3,000rpm for 20 minutes. The pellets were washed in several changes of PBS, dried at 60°C and stored in airtight containers.

Results

Physical Characteristics of Modified Crystal Aggregates

1. Sonication

Figure 4.1 shows the effect of sonicating the crystal aggregates for 45 minutes. Sonication caused the sharp edges of the crystals to be sheared away and all the resulting aggregates have a rounded-off appearance. Particle sizes ranged from approximately 0.1 to 15 microns.

2. Grinding

Figure 4.2 shows that prolonged grinding caused fragmentation of most large crystal aggregates into smaller rounded particles, most of which were 2-5μ in diameter.

3. Smashing

Figure 4.3 shows that compression of crystal aggregates by very high forces caused fragmentation of some of the larger particles to form irregularly-shaped fragments. These ranged in size from 1 to 5μ. Particles of approximately 30μ were also seen, although their edges were not as clearly defined as in the control specimen (fig.3.1).

4. Sedimentation

Figure 4.4 shows the effectiveness of particle size separation by sedimentation of HAC in the presence of protein. The majority of the particles were less than 0.4μ in diameter and the largest only 3μ. No evidence of protein coating of the HAC could be seen by SEM examination.

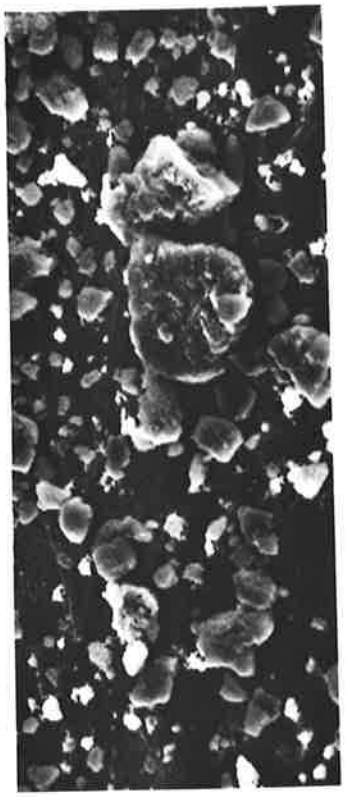


Fig.4.1 Scanning electron micrograph of sonicated HAC aggregates. x1,000

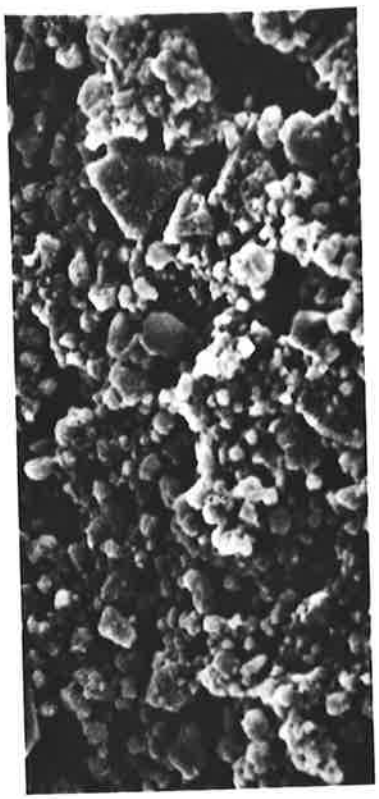


Fig.4.2 Scanning electron micrograph of ground HAC aggregates. x1,000

Fig.4.3 Scanning electron micrograph of smashed HAC aggregates. x1,000

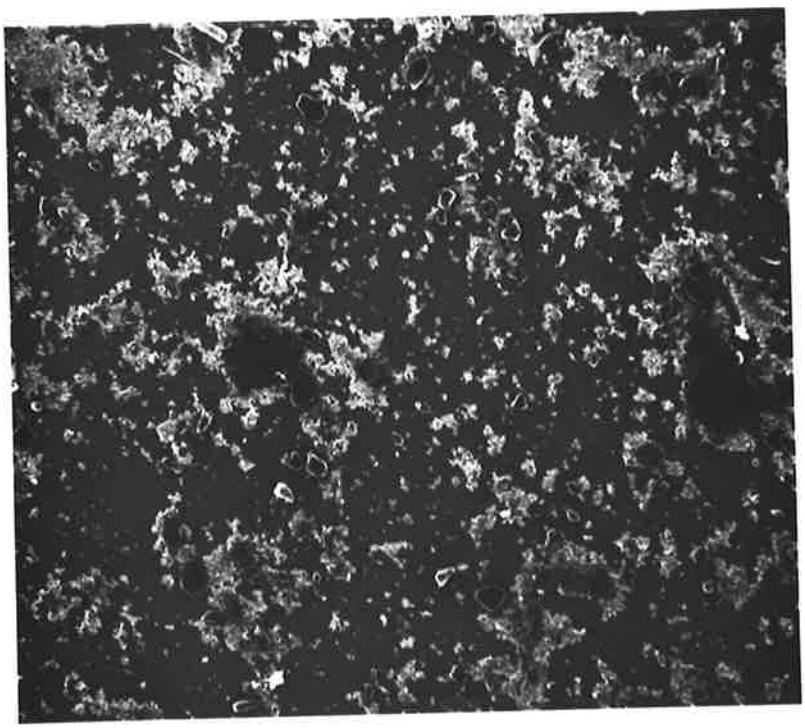


Fig.4.4 Scanning electron micrograph of sedimented HAC aggregates. Particle sizes range from 0.4 to 3 microns. x1,000

EXPERIMENT 4b

Method

Polyurethane sponges were impregnated wth 20mg of the four modified HAC preparations in addition to HAC as supplied by the manufacturer. Groups of 4 male Porton rats weighing 300-350g had sponges implanted for 14 days using the following protocol:

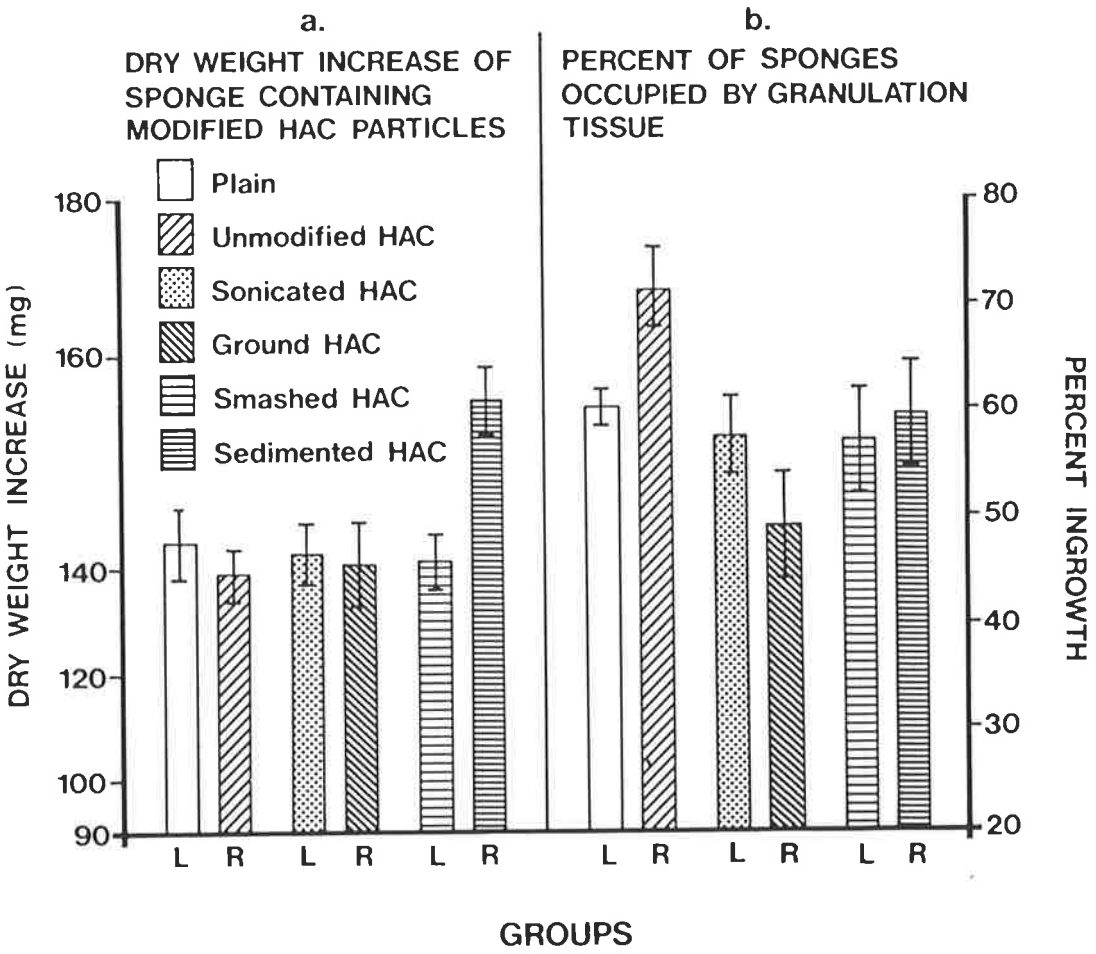
	Left Side	Right Side	
Group 1	Plain Sponges	Unmodified HAC	
Group 2	Sonicated HAC	Ground HAC	
Group 3	Smashed HAC	Sedimented HAC	
Group 4	Plain Sponge	Sedimented HAC	- implanted for 8 weeks (histology only)

Sponge dry weight gain, area of infiltration by granulation tissue and histological reaction was assessed. TEM was carried out in unmodified HAC and ground HAC impregnated sponges only.

Results

4a. Sponge Dry Weight Gain (Table 4a and Histogram 4a)

Sponges impregnated with HAC crystal aggregates prepared by sedimentation gained 30mg more weight than control sponges over the 14 day implantation period. Sponges impregnated with the other HAC preparations gained a similar amount of weight as control sponges.



Histogram 4
a. Dry weight gain of sponges impregnated with various physical modifications of HAC aggregates. Sponges were implanted for 14 days in Porton rats.
b. Ingrowth of granulation tissue into the same sponges.

4b. Ingrowth of Granulation Tissue (Table 4b and Histogram 4b)

Sponges impregnated with sonicated, smashed and sedimented HAC had similar amounts of ingrowth of granulation tissue as control sponges. Sponges containing unmodified HAC had an additional 11% of granulation tissue ingrowth whereas sponges with ground HAC had 11% less tissue ingrowth.

4c Histology

Unmodified HAC elicited a foreign body granulomatous reaction in the granulation tissue (fig.4.5a). Macrophages and MNGC were found in foci around the HAC. In the central zone of the sponges, the HAC was surrounded by non-viable PMN (fig.4.5b). The cellular response was similar to that observed to this material in previous implantations.

Sonicated HAC formed moderate sized clumps which attracted a macrophage and MNGC response in addition to prominent infiltration of lymphocytes (fig.4.6a). In the central sponge zone, cells around the HAC were sparse (fig.4.6b).

Ground HAC formed large clumps which were surrounded by macrophages and MNGC and a few lymphocytes. Fibroblasts around the clumps had become flattened and collagen fibres appeared to be walling off the deposits (fig.4.7). There were few cells around the HAC in the central sponge zone.

Smashed HAC also formed large clumps which were surrounded by macrophages and MNGC (fig.4.8a). An extensive infiltrate of non-viable PMN was found in the central sponge zone (fig.4.8b).

Sedimented HAC formed clumps around which small numbers of macrophages were seen. A MNGC response was prominent (figs.4.9a,b). Smaller clumps of HAC had attracted a more intense cellular response (fig.4.10). A few non-viable PMN were scattered around the HAC in the central sponge zone.

After 8 weeks, plain sponges showed extensive fibrosis with collagen fibres encircling the sponge matrix. The macrophage and MNGC

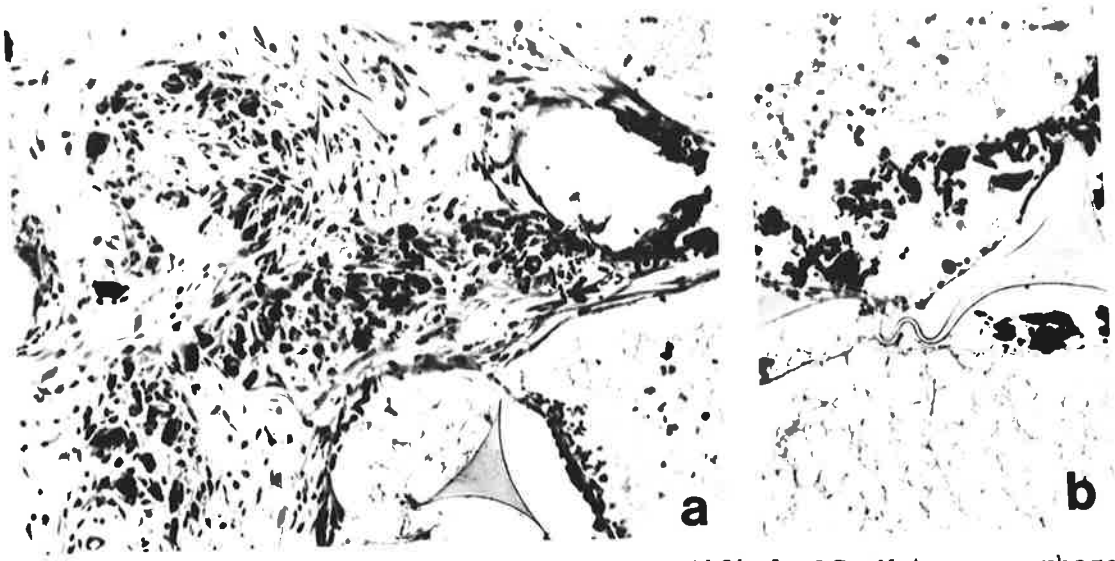


Fig.4.5 a. Sponge impregnated with unmodified HAC. Note macrophage and MNGC response in the granulation tissue. HE VK x145
b. Non-viable PMN surrounding HAC lodged in the central zone of the sponge. HE VK x180

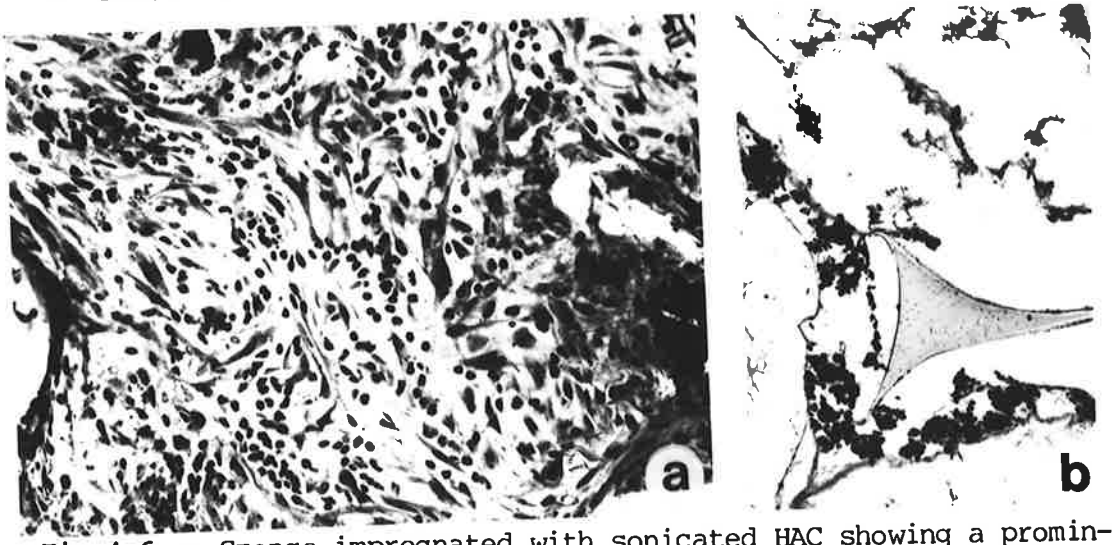


Fig.4.6 a. Sponge impregnated with sonicated HAC showing a prominent lymphocytic infiltration of granulation tissue. HE VK x145
b. Sparse cellular reaction to sonicated HAC in the central zone of the sponge. HE VK x180

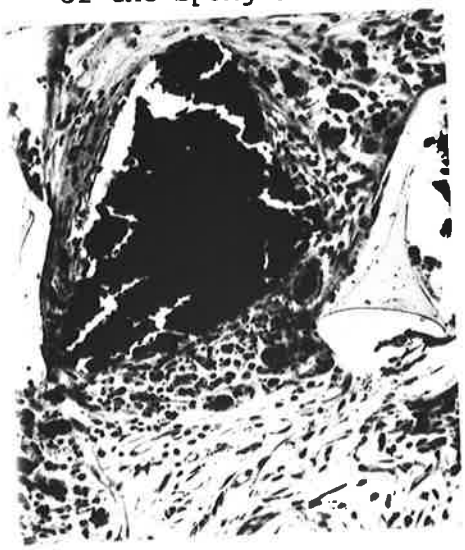


Fig.4.7 Sponge impregnated with ground HAC showing the formation of large clumps surrounded by macrophages, MNGC and collagen fibres. HE VK x145

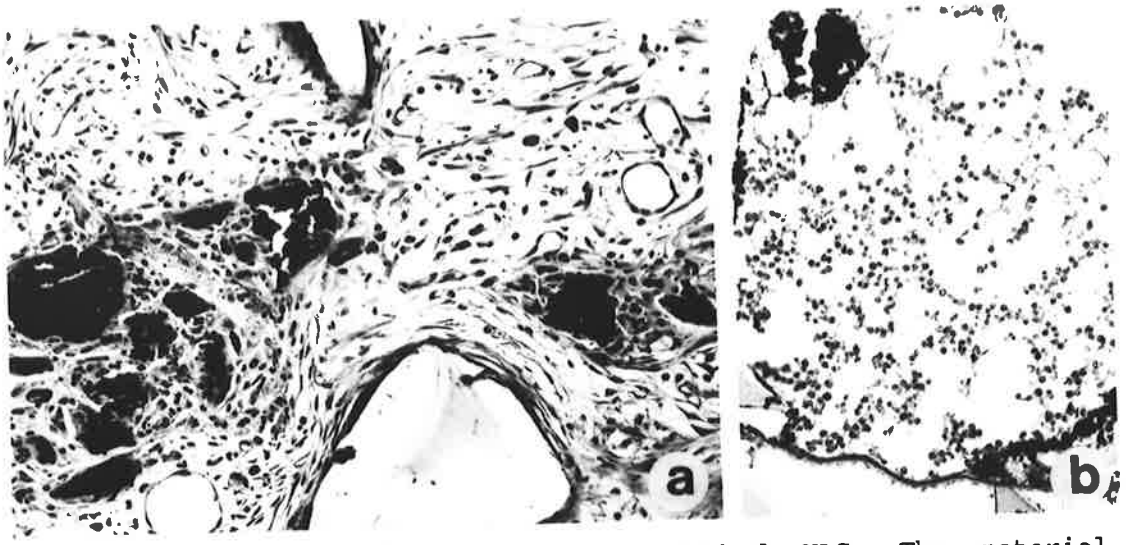


Fig.4.8 a. Sponge impregnated with smashed HAC. The material is surrounded by macrophages and MNGC. The granulation tissue distant from the HAC has a minimal inflammatory infiltrate. HE VK x145
b. Masses of non-viable PMN in the central sponge zone. HE VK x145

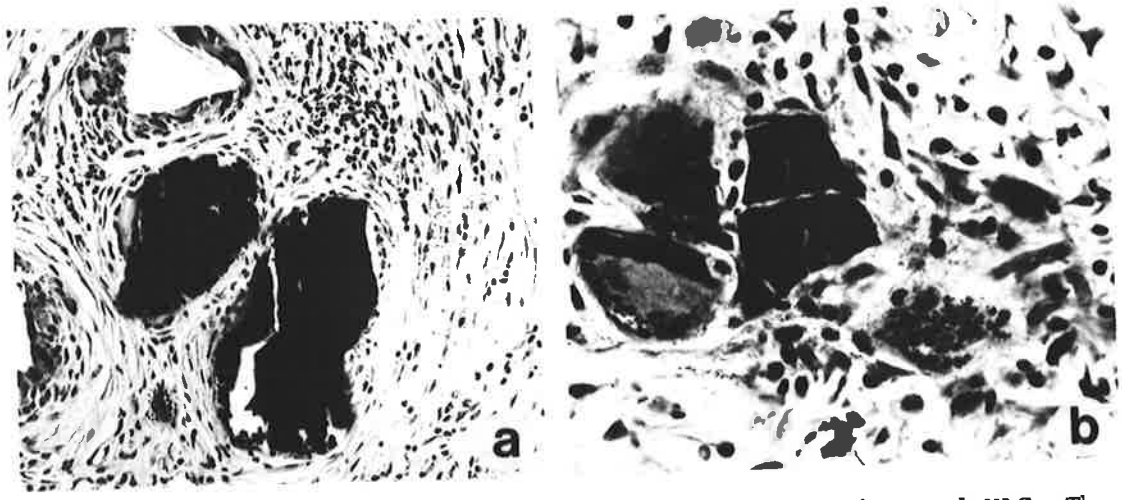


Fig.4.9 a. Sponge impregnated with sedimented HAC. The material is present in large clumps and has been walled-off by collagen fibres. HE VK x145
b. Macrophage and MNGC response to sedimented HAC. HE VK x370

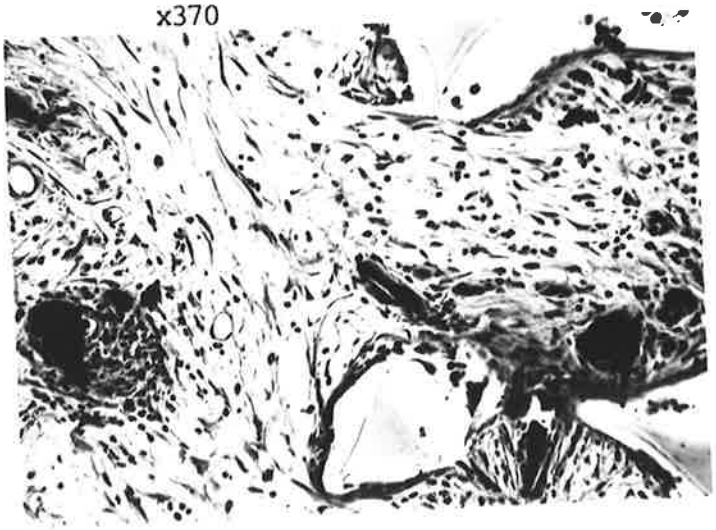


Fig.4.10 The smaller clumps of sedimented HAC were associated with a more intense cellular response. HE VK x145

response to the sponge was more prominent than after 14 days and fragmentation of the sponge was evident (fig.4.11a)

The large clumps of sedimented HAC found after 8 weeks had been encapsulated by fibrous tissue (fig.4.11b). The cellular response consisted of a few macrophages.

4d Transmission Electron Microscopy

Endocytosis of unmodified HAC aggregates by macrophages (figs.4.12 and 4.13) and MNGC (fig.4.14) was confirmed by TEM examination of impregnated sponges 14 days after implantation. Cells that had endocytosed large amounts of HAC showed no degenerative changes. Phagosomal membranes were not apparent. There was no alteration in crystal morphology compared with controls (fig.3.2) Disaggregation of the HAC clumps was observed (fig.4.13) and the smaller particles were contained within membrane bound vesicles. Lysosomes were closely associated with the crystals.

Ground HAC aggregates (figs.4.14 and 4.15) were found within macrophages and were invariably bounded by a phagosomal membrane. The endocytosed material was in close proximity to the cell nucleus. Ground HAC was less electron dense than unmodified HAC. No control TEM analysis of ground HAC was undertaken. There was some evidence of segregation of the crystalline material into smaller membrane bound parcels (fig.4.15).

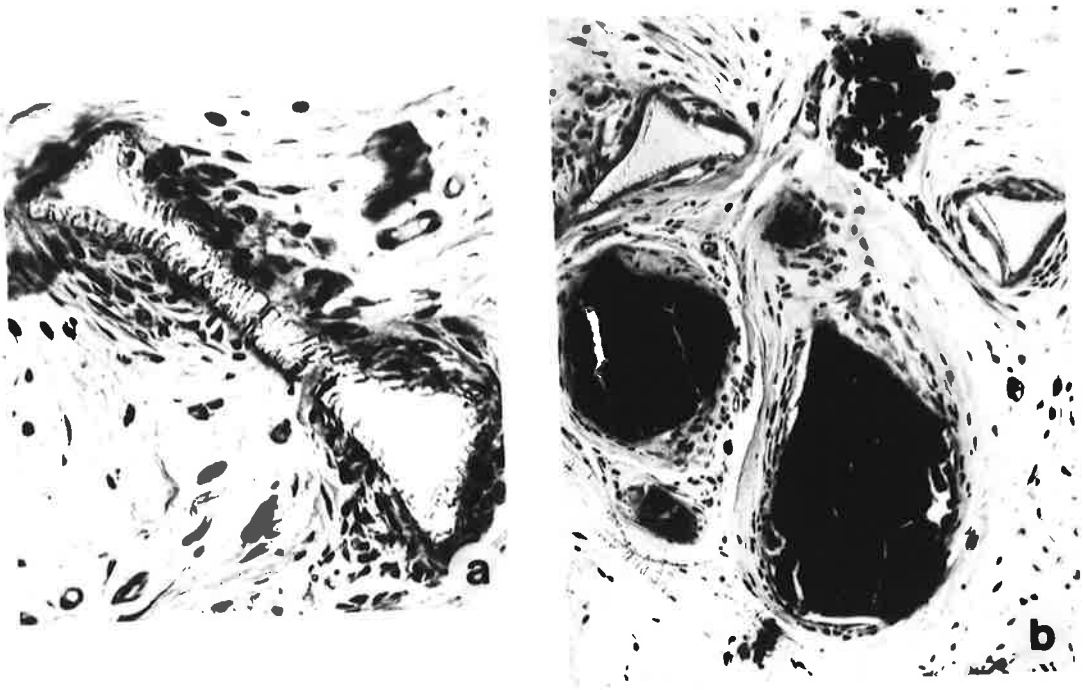


Fig.4.11 a. Sponge impregnated with sedimented HAC 8 weeks after implantation. The sponge matrix is splitting as a result of MNGC activity. HE VK x290
b. Dense fibrous capsule formation around large clumps of sedimented HAC, 8 weeks after implantation. HE VK x145

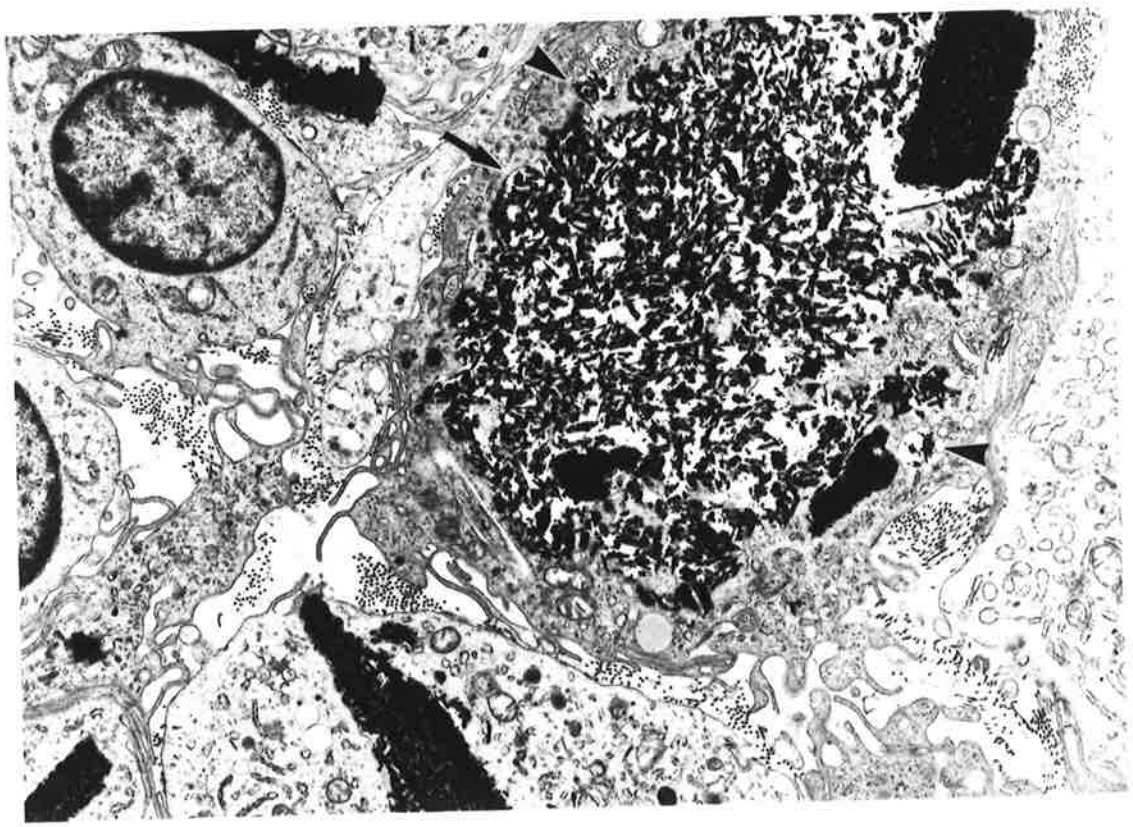


Fig.4.12 Electron micrograph of a sponge impregnated with unmodified HAC implanted for 14 days. Most of the cytoplasm of the macrophage is occupied by disaggregated HAC which is bounded by a unit membrane (arrow). Smaller phagosomes containing non-aggregated crystals are also present (arrowheads). x7,940

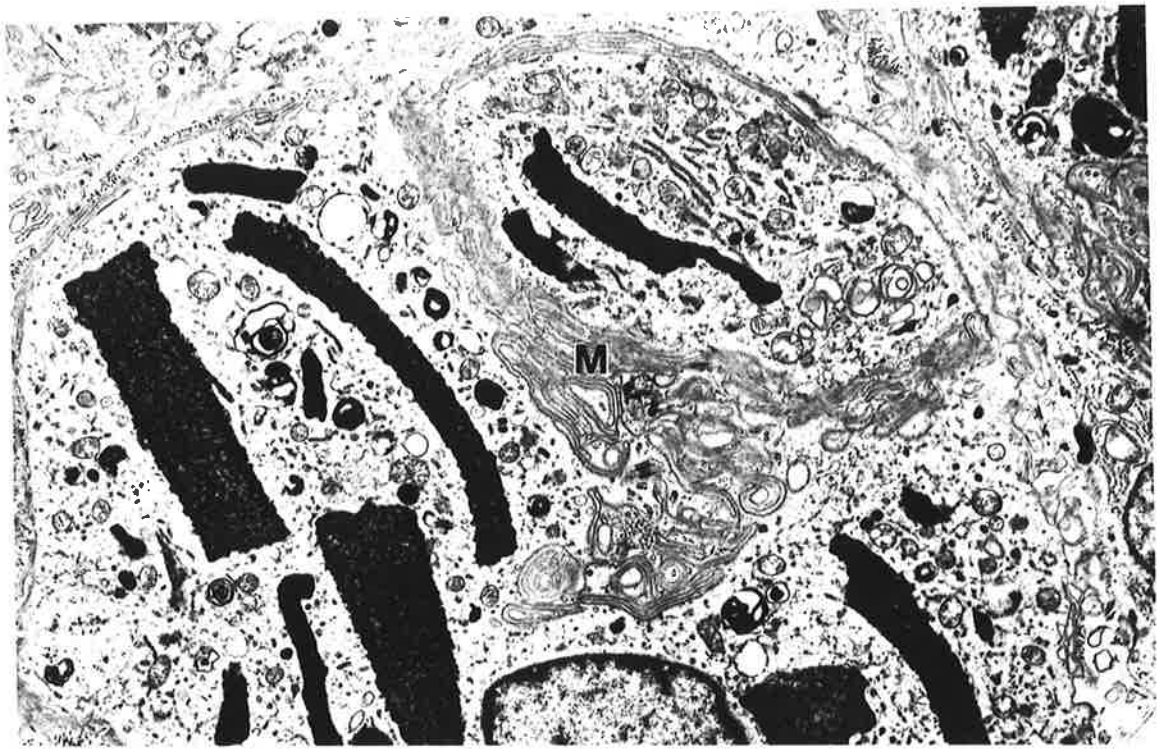


Fig.4.13 Electron micrograph of sponge impregnated with unmodified HAC showing two macrophages containing crystal aggregates. The cell processes showed prominent interdigitation (M). x4,600

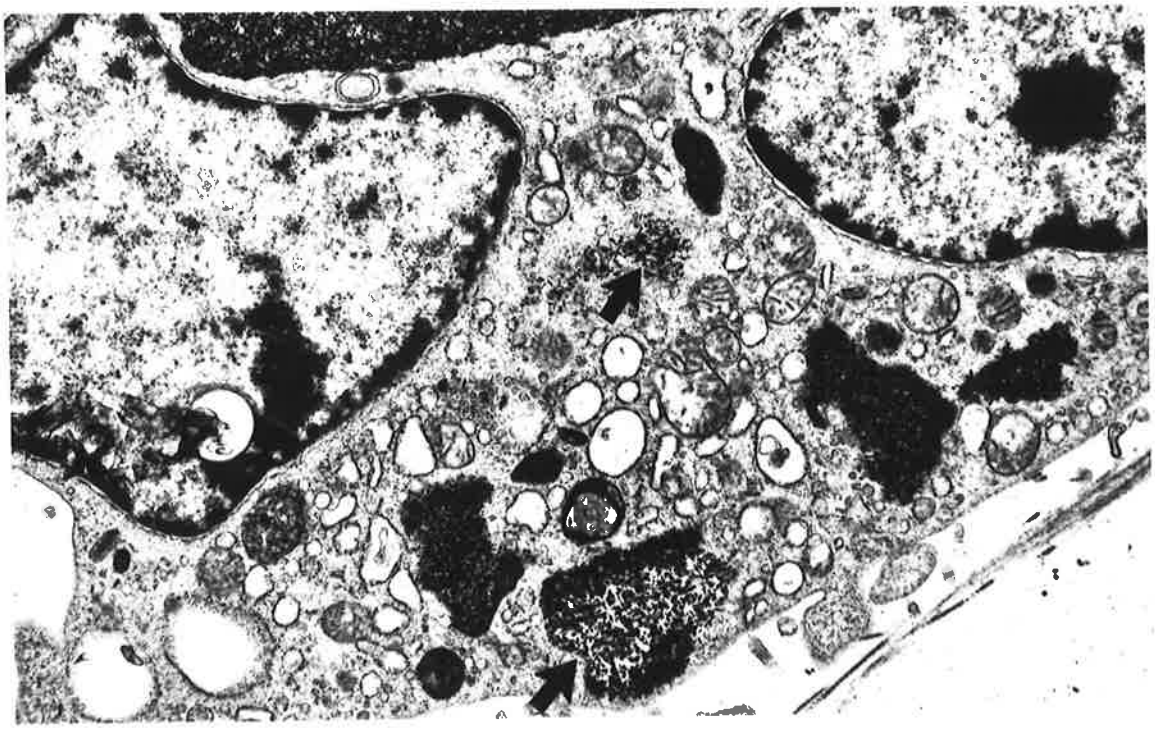


Fig.4.14 Electron micrograph of sponge impregnated with unmodified HAC. A MNGC has endocytosed the crystal aggregates and disaggregation of some crystal clumps has occurred. x13,200

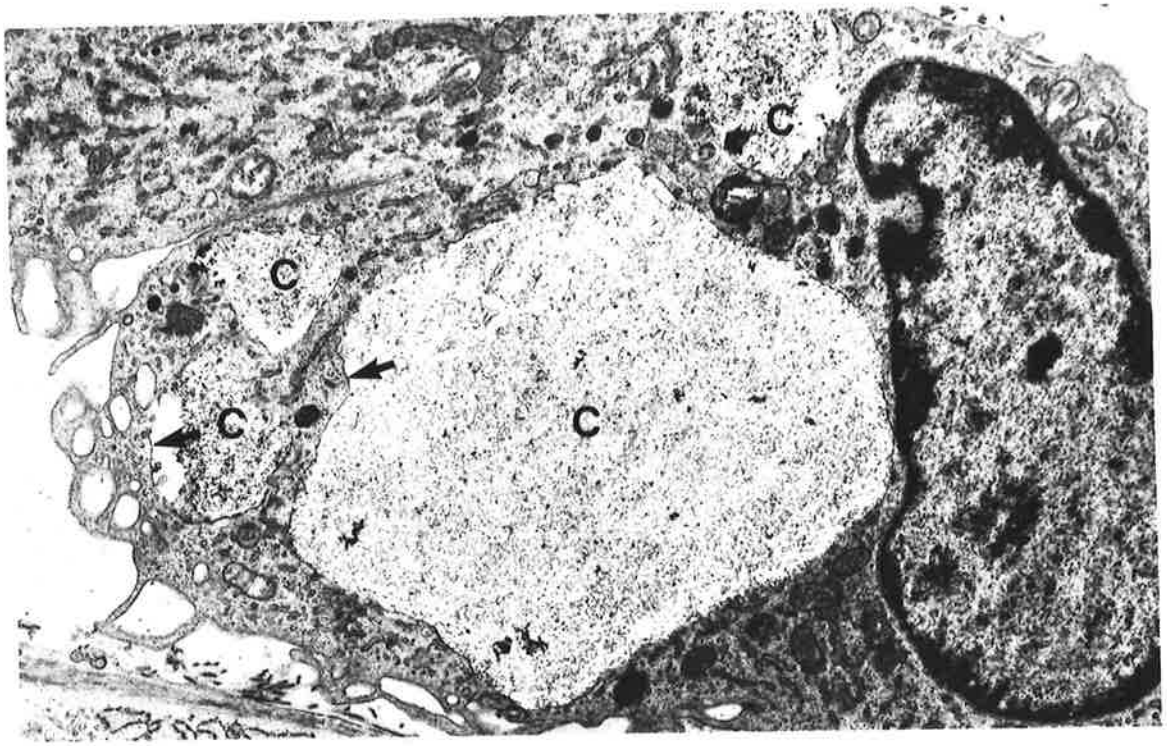


Fig.4.15 Electron micrograph of sponge impregnated with ground HAC aggregates. Most of the cytoplasm of this macrophage is occupied by crystal aggregates (C) bounded by unit membranes (arrows). The aggregates are much less electron dense than unmodified HAC. x10,650

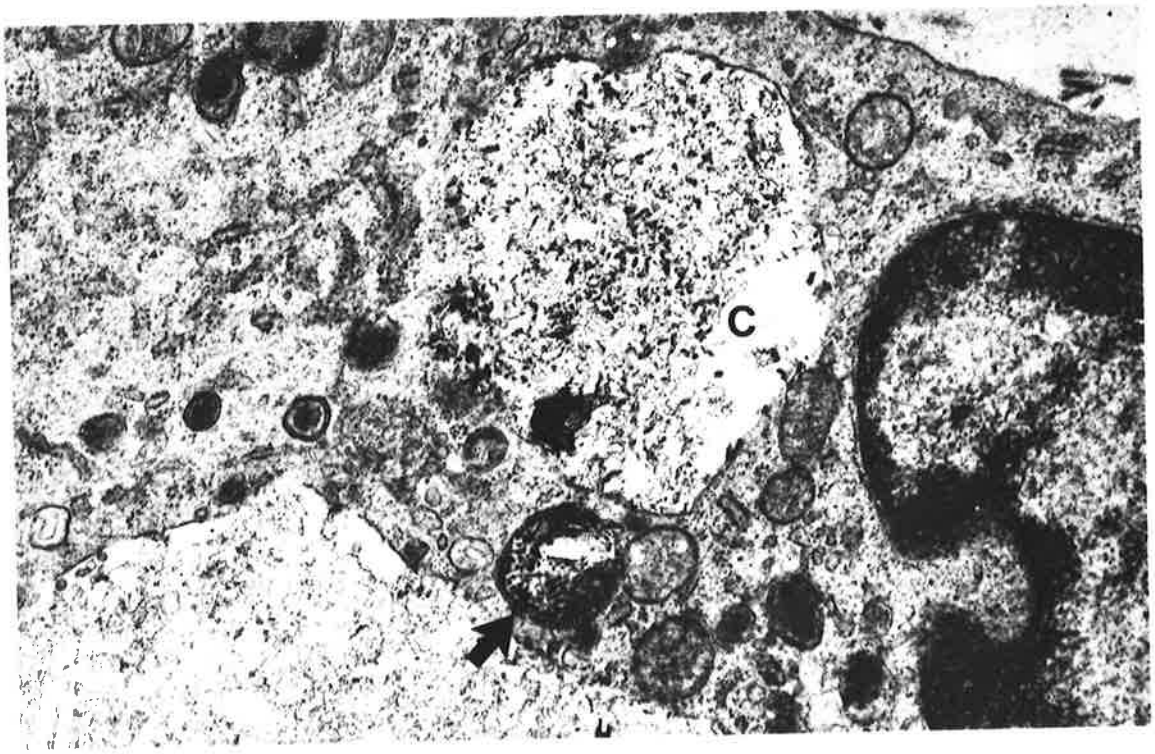


Fig.4.16 Higher magnification of fig.4.15 showing crystals (C) in a phagosome and a phagolysosome (arrow) also containing crystalline material. x25,670

EXPERIMENT 5

Aim

To assess the response of Dark Agouti rats to subcutaneously implanted sponges impregnated with HAC.

Method

Groups of four male Dark Agouti rats weighing 200-250g had plain sponges implanted in their left flanks and sponges implanted with 20mg of HAC (as supplied by the manufacturer) in their right flanks. Sponge dry weight gain, extent of granulation tissue ingrowth and histology were studied 7 and 14 days after implantation.

Results

5a. Sponge Dry Weight Gain (Table 5a and Histogram 5a)

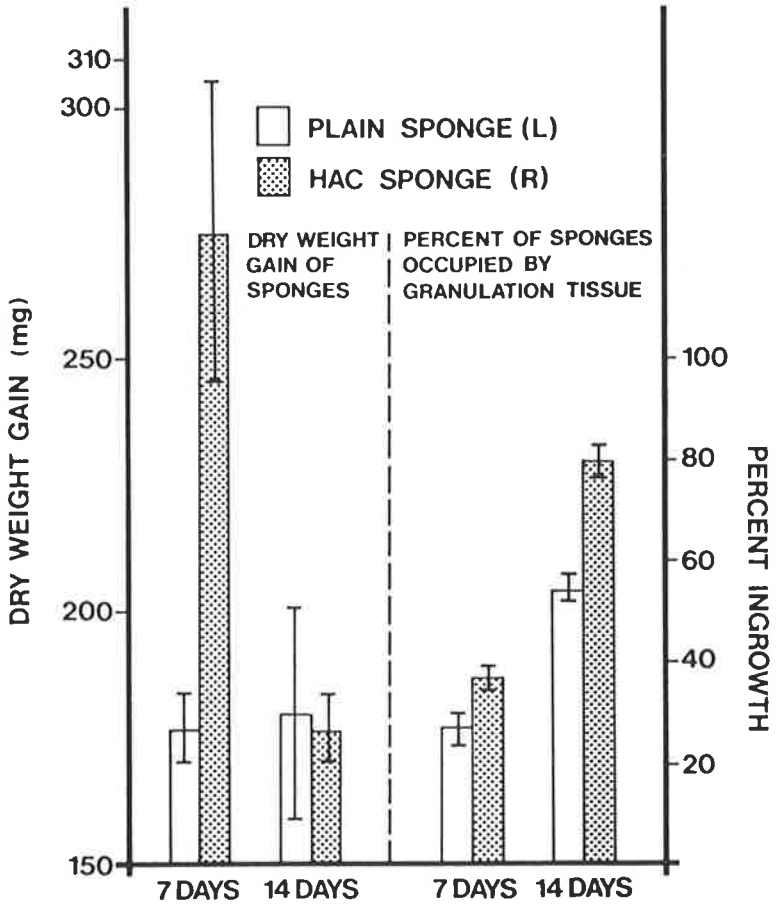
HAC containing sponges implanted for 7 days gained one and a half times more weight than plain sponges. However at 14 days, there was no significant difference in the weight gain of left and right sponges. Plain sponges gained a similar amount of weight at 7 and 14 days.

5b. Ingrowth of Granulation Tissue (Table 5b and Histogram 5b)

Statistically significant differences in the extent of granulation

tissue ingrowth between plain and HAC impregnated sponges were observed at both 7 and 14 days after sponge implantation ($p<.001$). HAC sponges had 10% and 25% additional granulation tissue ingrowth than plain sponges at 7 and 14 days respectively.

Between seven and 14 days after sponge implantation, plain sponges had a 28% increase in granulation tissue whereas HAC sponges had an extra 43% tissue ingrowth in this time period. These differences were statistically significant ($p<.001$).



Histogram 5
a. Dry weight gain of plain and HAC impregnated sponges implanted for 7 and 14 days in Dark Agouti rats.
b. Ingrowth of granulation tissue into the same sponges.

5c. Histology

Plain sponges implanted for 7 days in DA rats showed early proliferation and infiltration of granulation tissue. Some fat cells were prominent at the perimeter of the sponges (fig.5.1a). A delicate lace-like network of precipitated protein was seen throughout the parts of the sponges unoccupied by granulation tissue. Few inflammatory cells were found in this zone.

HAC sponges implanted for 7 days showed advancing granulation tissue ingrowth. There were numerous stellate fibroblasts, prominent capillary formation and a predominantly macrophage response to the HAC (fig.5.1b). In the central part of the sponges, the HAC was surrounded by a moderate PMN infiltrate. Widespread deposition of a precipitated material (presumably protein containing inflammatory exudate) was also found in this sponge zone (fig.5.1c).

Plain sponges implanted for 14 days (fig.5.2a) showed the ingrowth of a vascular, poorly organized granulation tissue. The inner limit of the granulation tissue in these sponges was marked by the formation of a single layer of flattened cells.

HAC sponges implanted for 14 days (fig.5.2b) showed the formation of extensive amounts of well organized granulation tissue with prominent bundles of collagen and a moderate lymphocytic infiltrate. An intense macrophage and MNGC reaction was directed at the HAC aggregates (fig.5.2c).

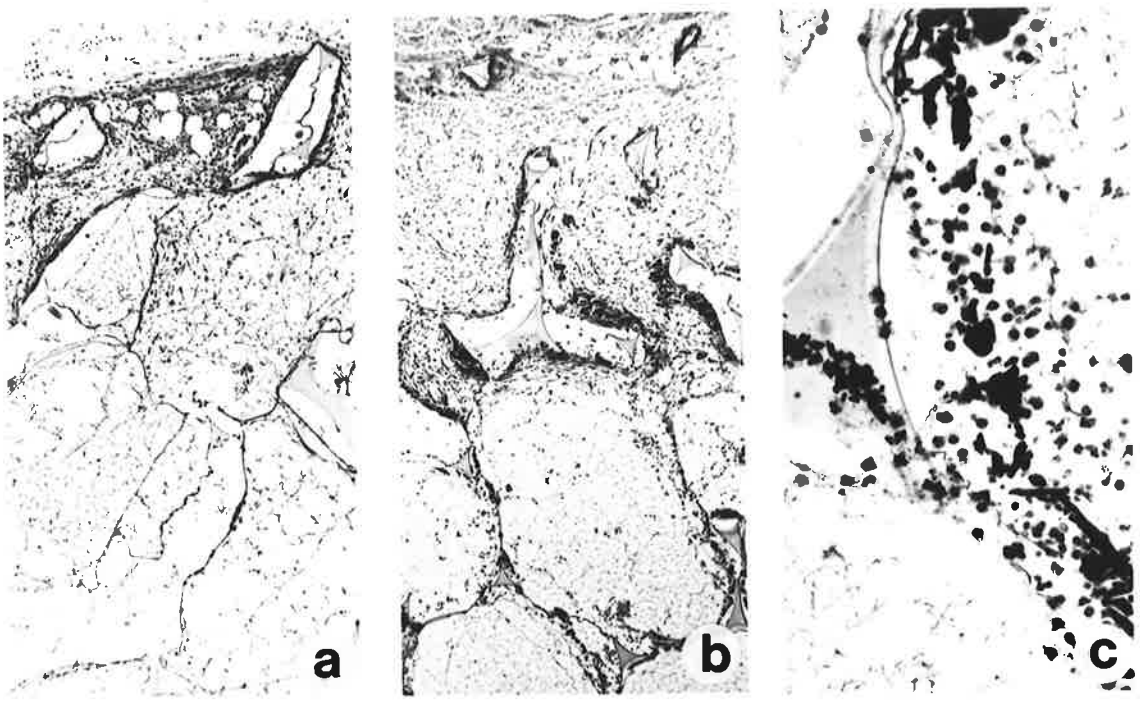


Fig.5.1 Sponges implanted for 7 days in DA rats.
a. Plain sponge in which fat cells are prominent at the periphery and early proliferation of granulation tissue has occurred. HE VK x35
b. HAC impregnated sponge with mature granulation tissue and large amounts of precipitated protein in the central zone. HE VK x35
c. Central zone of HAC impregnated sponge with a moderate PMN infiltrate around the clumps of HAC. HE VK x230

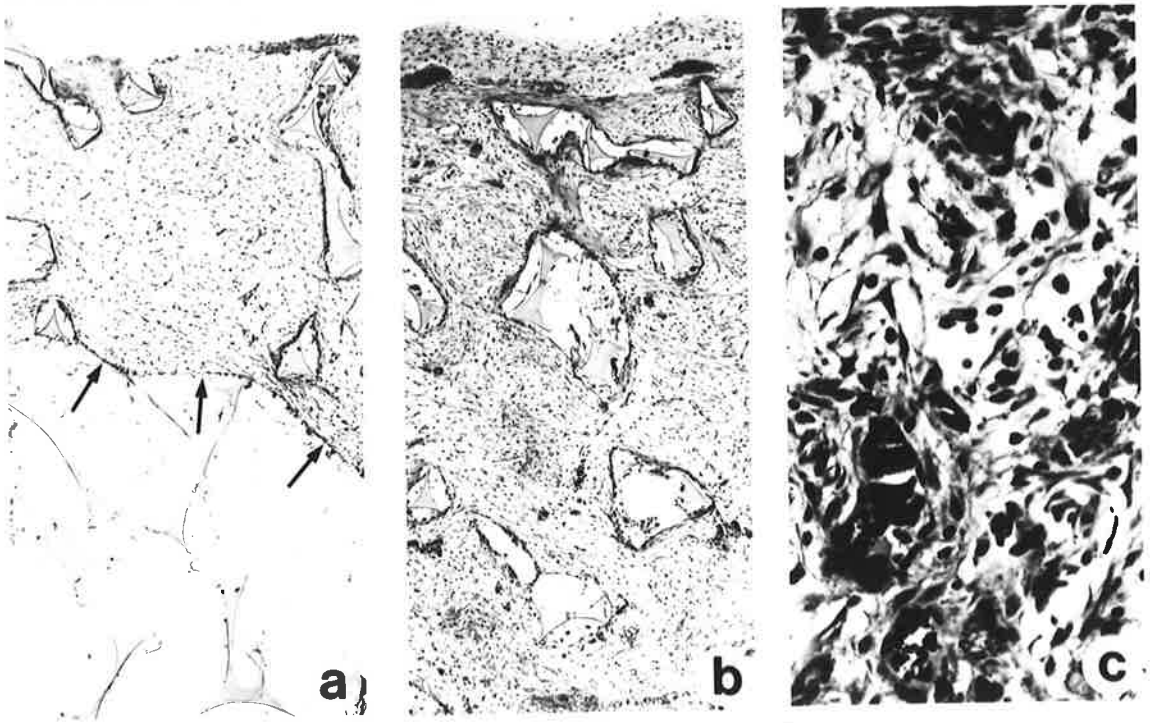


Fig.5.2 Sponges implanted for 14 days in DA rats.
a. Plain sponge containing poorly organized granulation tissue, its inner limit being formed by a single layer of flattened cells (arrows). HE VK x35
b. Extensive infiltration of well organized granulation tissue in HAC impregnated sponge. HE VK x35
c. Intense macrophage and MNGC reaction to the HAC. HE VK x290

EXPERIMENT 6

Aim

To assess the effect of antiinflammatory and antirheumatic drugs on the response to plain and HAC impregnated sponges implanted in Dark Agouti rats.

Method

Male Dark Agouti rats weighing 260-320g had plain sponges implanted in their left flanks and sponges impregnated with 20mg of HAC (as supplied by the manufacturer) implanted in their right flanks. Each rat had one plain and one HAC sponge implanted. The following regime of drugs was administered to the animals (four rats per treatment group):

Group	Drug	Daily Dosage
1	Saline	0.2ml
2	Prednisolone(Glaxo)	10mg/kg
3	D-penicillamine(Sigma)	200mg/kg
4	Copper/D-pencillamine	50mg/kg(D-pen).
5	Sodium aurothiomalate (May and Baker)	12.5mg/kg (predosed 4 days)
6	Adjuvant Oil C30 (squalane) containing heat killed M.tuberculosis	50µl injected once into tail tendon

D-penicillamine (DPA) (200mg/ml) was dissolved in a 0.25M sodium bicarbonate solution. The mixture was prepared in the following way:

34mg of $NaHCO_3$ was dissolved in 8ml of distilled water.

42mg of $CuCl_2$ was added, producing a cloudy suspension.

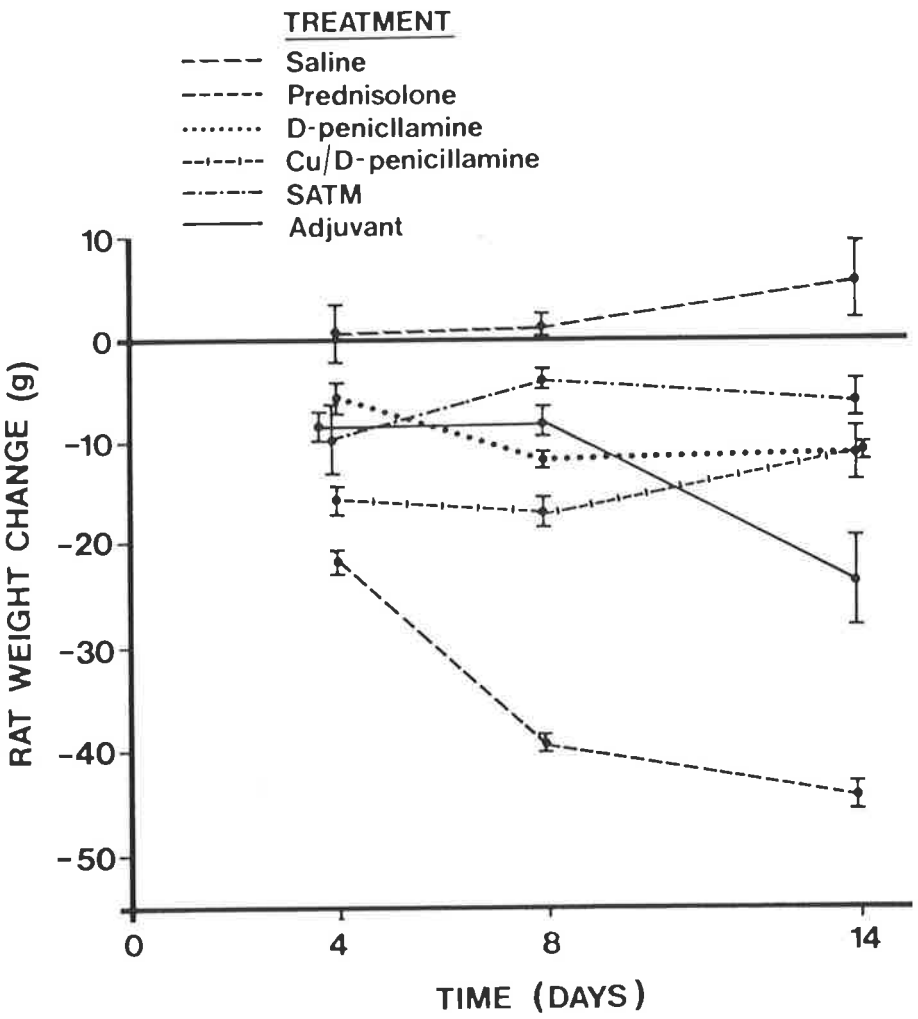
50mg of DPA was dissolved in 2ml of distilled water and added drop wise to the first solution. The pH was adjusted to 7 with 1N NaOH,resulting in a deep purple coloured solution. The mixture was prepared fresh daily.

To achieve effective blood gold levels at the time of sponge implantation, sodium aurothiomalate (SATM) was given daily for four days prior to the start of the experiment.

All drugs were administered by intraperitoneal injection at the same time of day for 14 days. The adjuvant treated group were given 50µl of squalan oil (Art.9766 Merck, Darmstadt, W.Germany) in which 10mg/ml of heat-killed delipidated human M.tuberculosis had been dispersed by subcutaneous injection into the tendon of the dorsal side of the tail near its root. The adjuvant was given at the time of sponge implantation.

The parameters assessed were: animal weight changes, sponge dry weight gain, extent of granulation tissue ingrowth and the comparative cellular responses by histological examination.

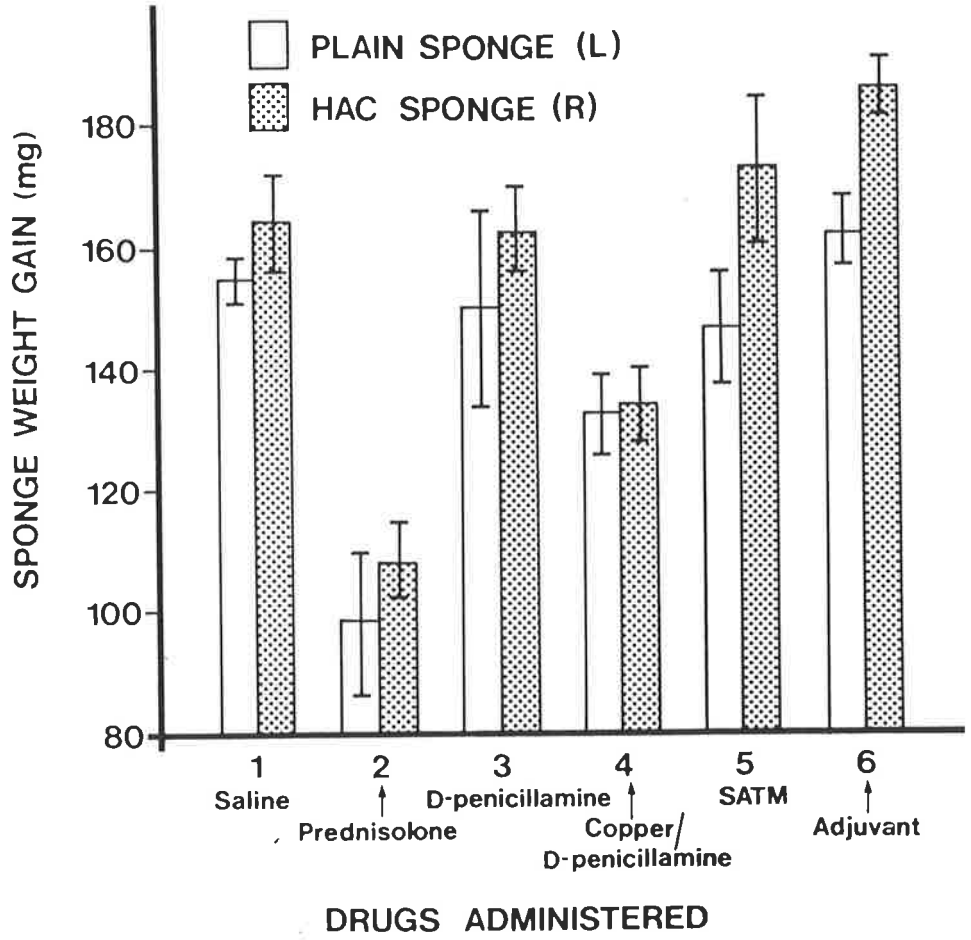
Results 6a. Rat Weight Changes (Table 6a and Figure 6a)



Histogram 6a
Weight changes of Dark Agouti rats treated with various drugs over 14 days.

The weight gain of saline treated animals was 5g during the 14 day experimental period. All other treatments resulted in the animals losing a statistically significant amount of weight compared with controls ($p<.001$). Weight loss ranged from 6g to 44g in the SATM and prednisolone treated groups respectively.

6b. Sponge Dry Weight Gain (Table 6b and Histogram 6b)

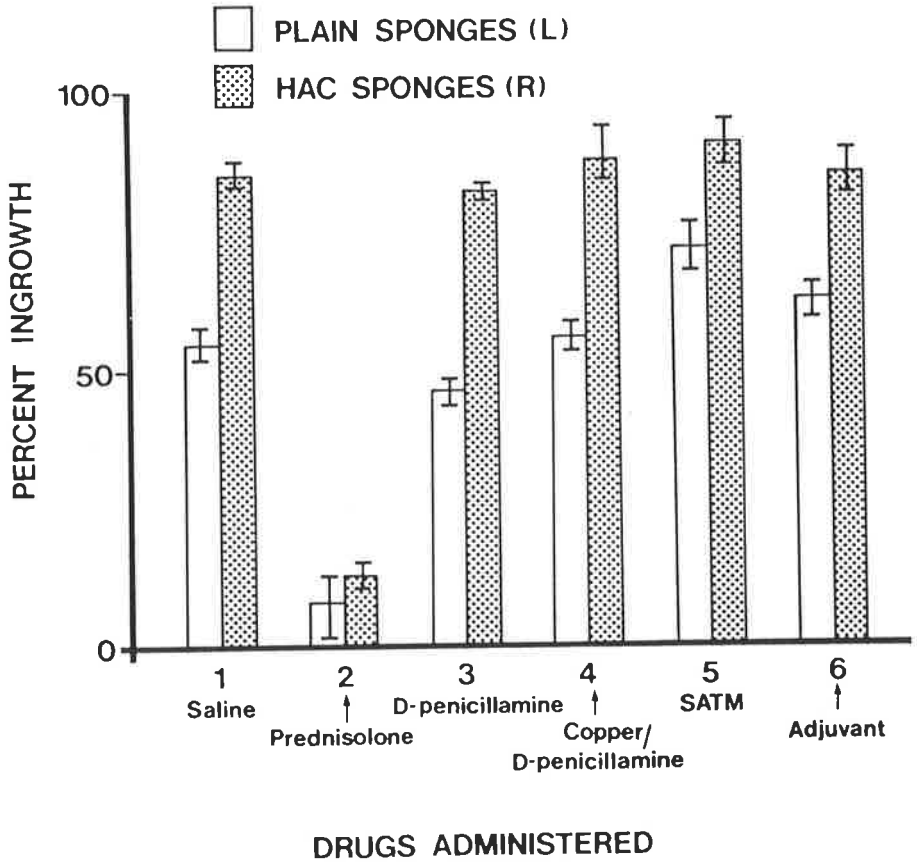


Histogram 6b
Dry weight gain of plain and HAC impregnated sponges implanted in drug treated Dark Agouti rats for 14 days.

Dry weight gain of plain sponges in saline, DPA, SATM and adjuvant treated rats was similar. However, prednisolone treatment resulted in a 37% reduction and copper/DPA a 15% reduction in sponge weight gain. Dry weight gain of HAC impregnated sponges in saline, DPA and SATM

treated rats was similar. However, prednisolone and copper/DPA caused a respective 33% and 18% reduction in sponge weight gain. Adjuvant treatment resulted in a 13% increase in sponge weight gain. There was a statistically significant different in weight gain between plain and HAC sponges across the experimental groups (p=0.008).

6c. Ingrowth of Granulation Tissue (Table 6c and Histogram 6c)



Histogram 6c
Ingrowth of granulation tissue into plain and HAC impregnated sponges implanted in drug-treated Dark Agouti rats for 14 days.

The extent of ingrowth of granulation tissue into plain sponges in saline and copper/DPA treated rats was similar. Prednisolone and DPA reduced the growth of tissue by 46% and 6% respectively. SATM and adjuvant treatment raised the tissue ingrowth into plain sponges by 18% and 9% respectively.

The extent of ingrowth of granulation tissue into HAC containing sponges was similar in saline, DPA, copper/DPA, SATM and adjuvant treated rats. Prednisolone treatment resulted in 72% less tissue ingrowth into the sponges.

There was a statistically significant difference in the ingrowth of granulation tissue into plain and HAC sponges for all treatments other than prednisolone ($p<.001$).

6d. Histology

Sponges implanted in saline treated rats responded in the same way as sponges in untreated rats (Experiment 5, 14 day implantations).

Sponges implanted in prednisolone treated rats

In plain sponges (figs.6.1a,b) the granulation tissue had only infiltrated through the outermost interstices of the sponges. It was poorly developed, hypocellular and poorly vascularized with virtually no cellular condensation around the sponge fibres. There was a minimal inflammatory response in the central part of the sponges. A loose fibrous capsule had formed at the periphery of the sponges.

HAC sponges (fig.6.2a) showed the formation of a dense fibrous capsule around the sponges. There was less granulation tissue ingrowth than in plain sponges. A macrophage and MNGC reaction occurred around HAC particles that were entrapped in the fibrous capsule (fig.6.2b). There was no cellular response to HAC within the sponges (fig.6.2c).

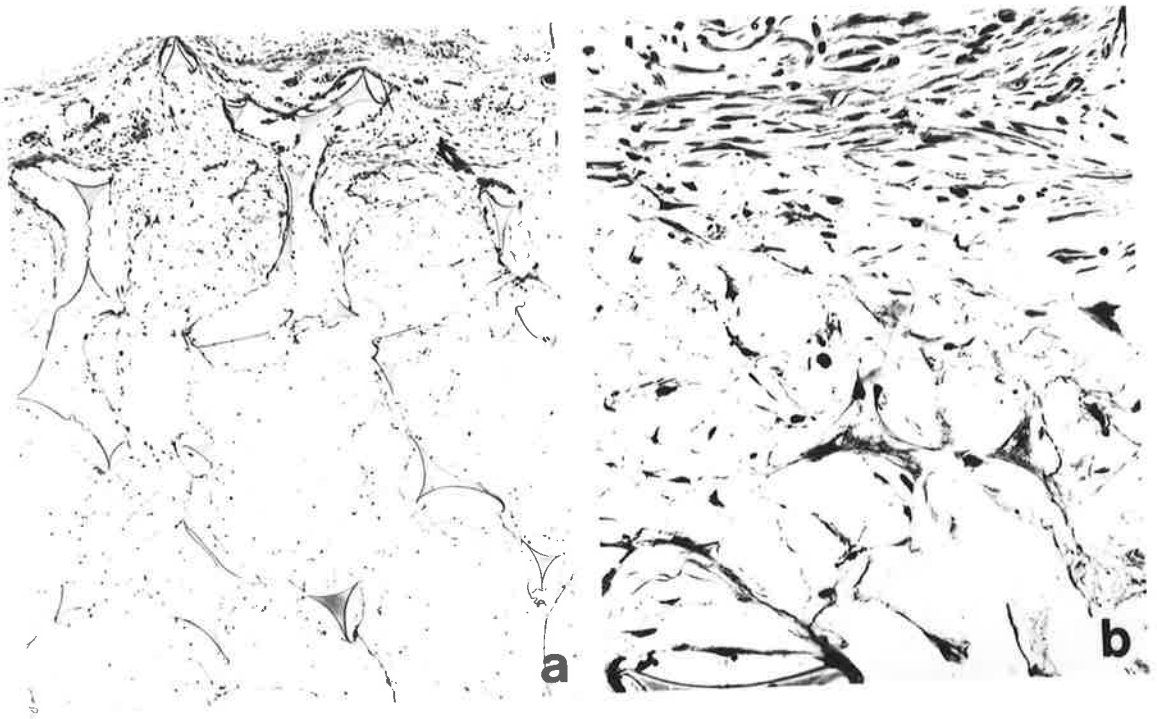


Fig.6.1
Plain sponges implanted for 14 days in prednisolone treated DA rats.
a. Poorly developed, hypocellular granulation tissue extending into the outermost part of the sponge. HE VK x35
b. Loose fibrous capsule (top) and lack of organization of granulation tissue. HE VK x145

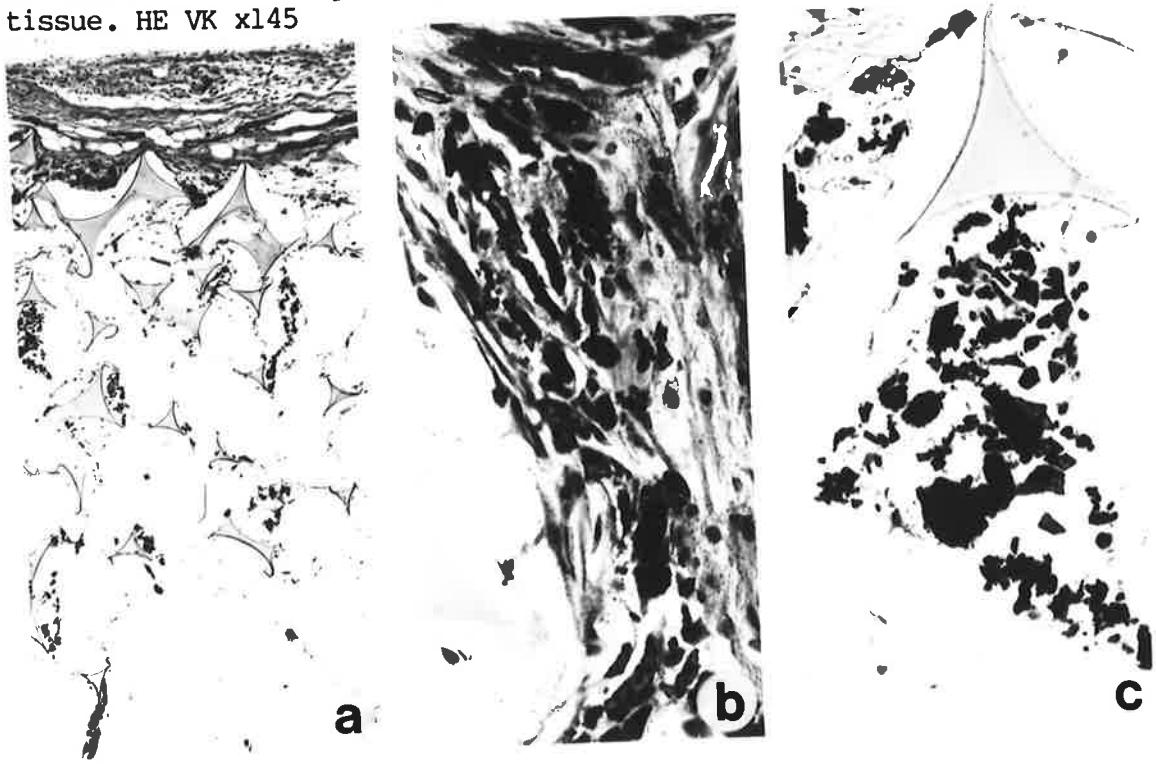


Fig.6.2
Sponges impregnated with HAC implanted for 14 days in prednisolone treated rats.
a. Formation of a dense fibrous capsule around the sponge. Granulation tissue ingrowth has been suppressed. HE VK x35
b. Macrophage and MNGC response to HAC in capsular tissues. HE VK x290
c. Lack of cellular response to aggregates of HAC in the central sponge zone. HE VK x230

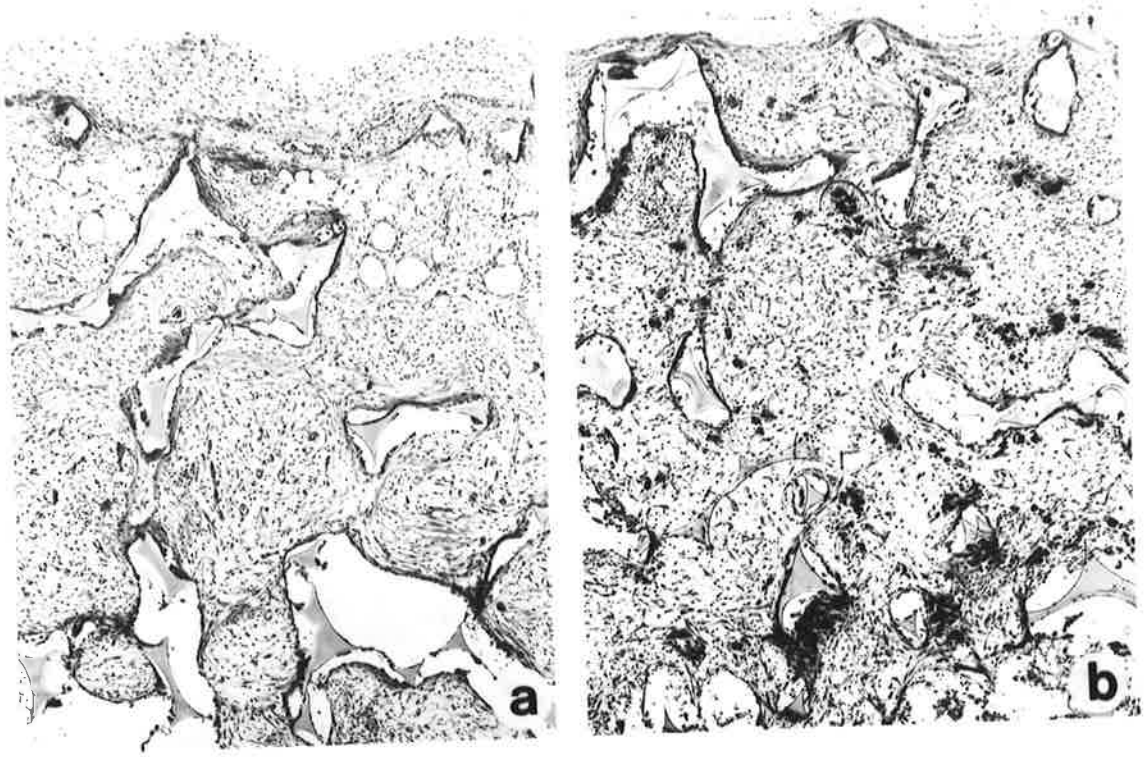


Fig.6.3 Sponges implanted for 14 days in DPA treated DA rats.
a. Plain sponges with well collagenized granulation tissue and prominent fat cells in the peripheral zone. HE VK x35
b. HAC impregnated sponge with localization of cells (mainly macrophages) around the aggregates of HAC. HE VK x35

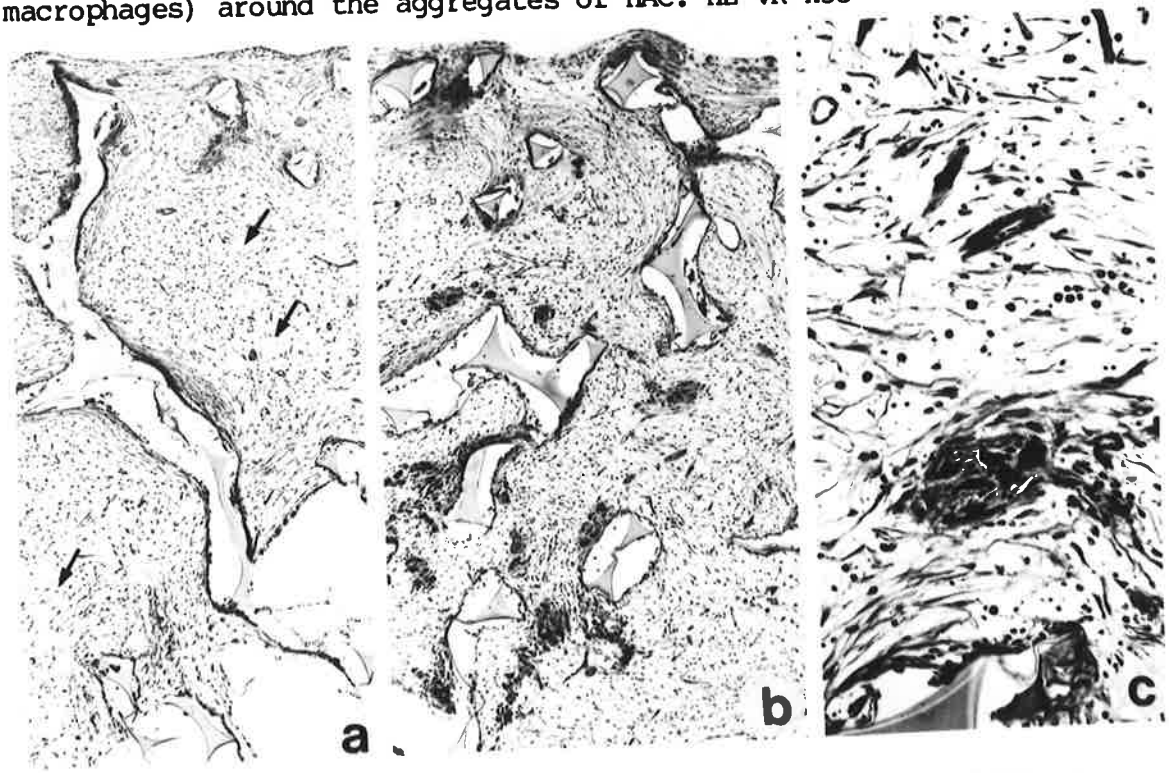


Fig.6.4 Sponges implanted for 14 days in Cu/DPA treated DA rats.
a. Plain sponge containing granulation tissue with focal oedema (arrows) and delayed fibroblastic proliferation. HE VK x35
b. HAC impregnated sponge infiltrated by granulation tissue lacking well-formed, orientated collagen bundles. HE VK x35
c. HAC impregnated sponge showing the loose arrangement of the tissues. Lymphocytes are scattered throughout. HE VK x145

Sponges implanted in DPA treated rats

Plain sponges (fig.6.3a) were similar to those in saline treated rats. Collagen fibre bundles were prominent and fat cells were seen towards the sponge periphery.

HAC sponges (fig.6.3b) attracted a similar response to those in saline treated rats. The HAC was randomly distributed and was aggregated into small clumps. Macrophages and MNGC were prominent around the HAC.

Sponges implanted in copper-DPA treated rats

In plain sponges (fig.6.4a) the granulation tissue showed focal oedema and an apparent delay in fibroblastic proliferation compared to saline treated rats.

HAC sponges (figs 6.4b,c) showed a moderate macrophage and MNGC response around the scattered HAC aggregates. The reaction seemed less intense than in HAC sponges in saline treated rats. The granulation tissue distant from the HAC was not as well-structured as the control tissue, lacking well-formed collagen fibre bundles of definitive orientation. Lymphocytes were scattered through the tissue.

Sponges implanted in SATM treated rats

Plain sponges (fig.6.5a) were infiltrated by well-established granulation tissue with a similar degree of collagenisation as plain sponges in saline treated rats.

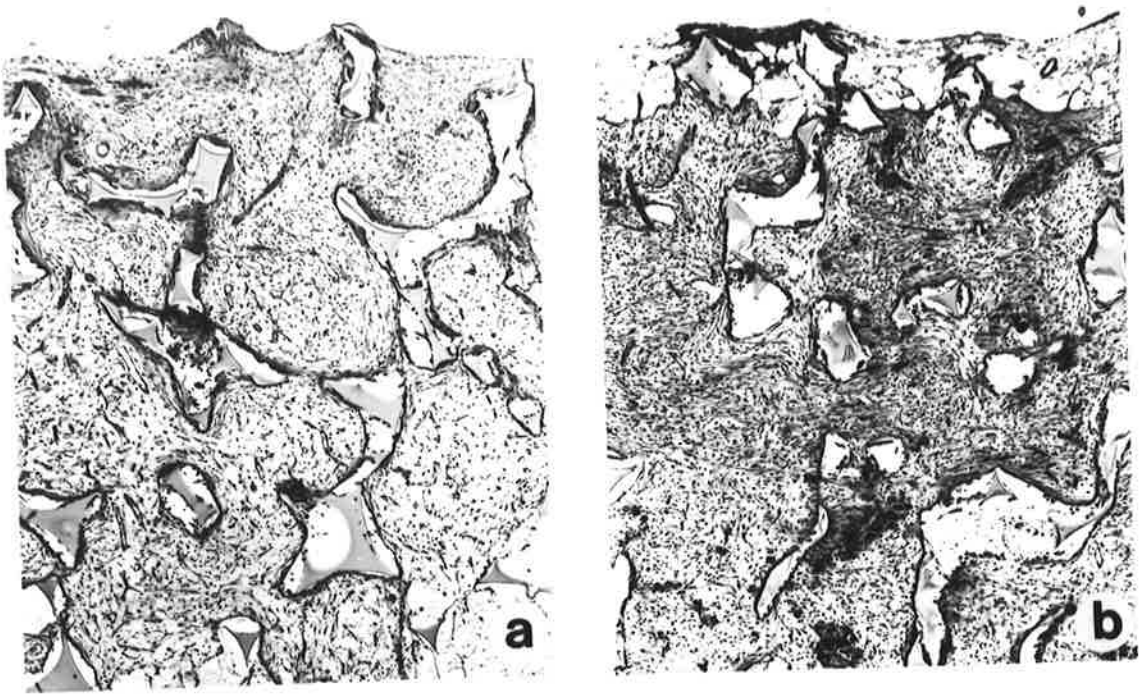
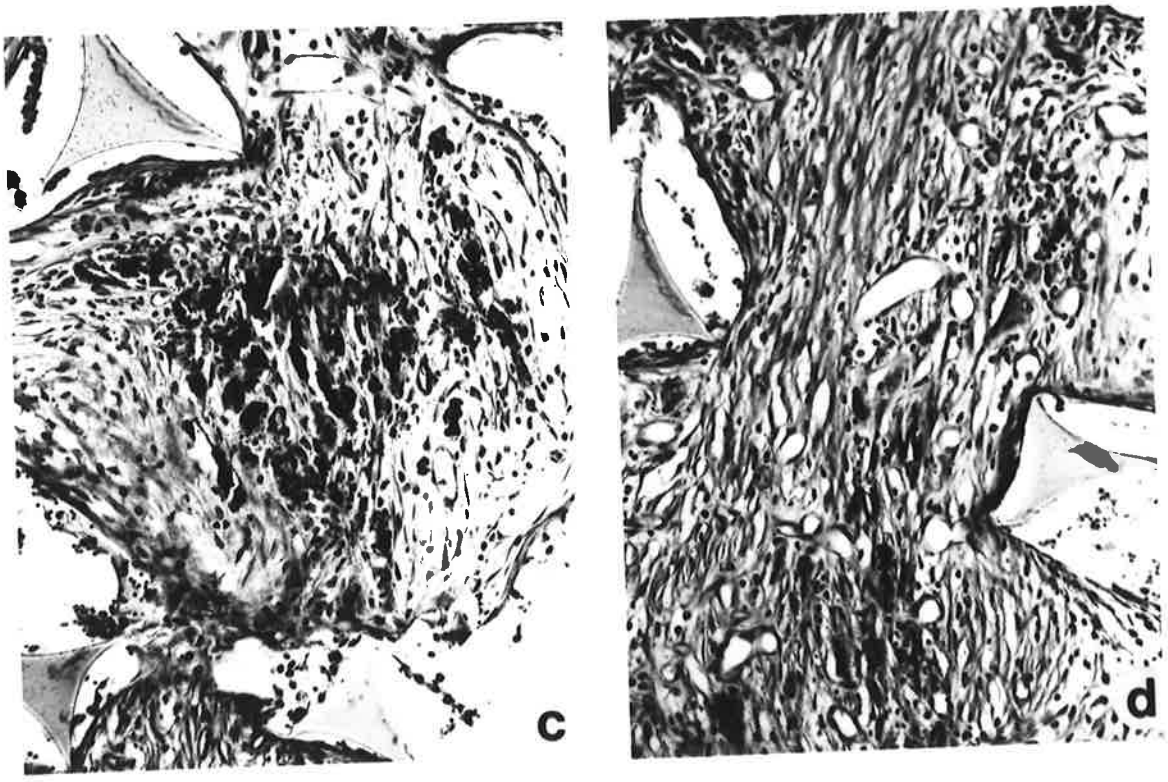


Fig.6.5 Sponges implanted for 14 days in SATM treated DA rats.

a. Plain sponge infiltrated by well-established granulation tissue with a similar degree of collagenization as controls. HE VK x35.

b. HAC impregnated sponge infiltrated by granulation tissue with prominent fibroblastic proliferation and focal sclerosis. HE VK x35



c. HAC aggregates surrounded by macrophages, MNGC and lymphocytes. HE VK x145

d. Condensation of the connective tissue in the vicinity of the HAC aggregates. HE VK x145

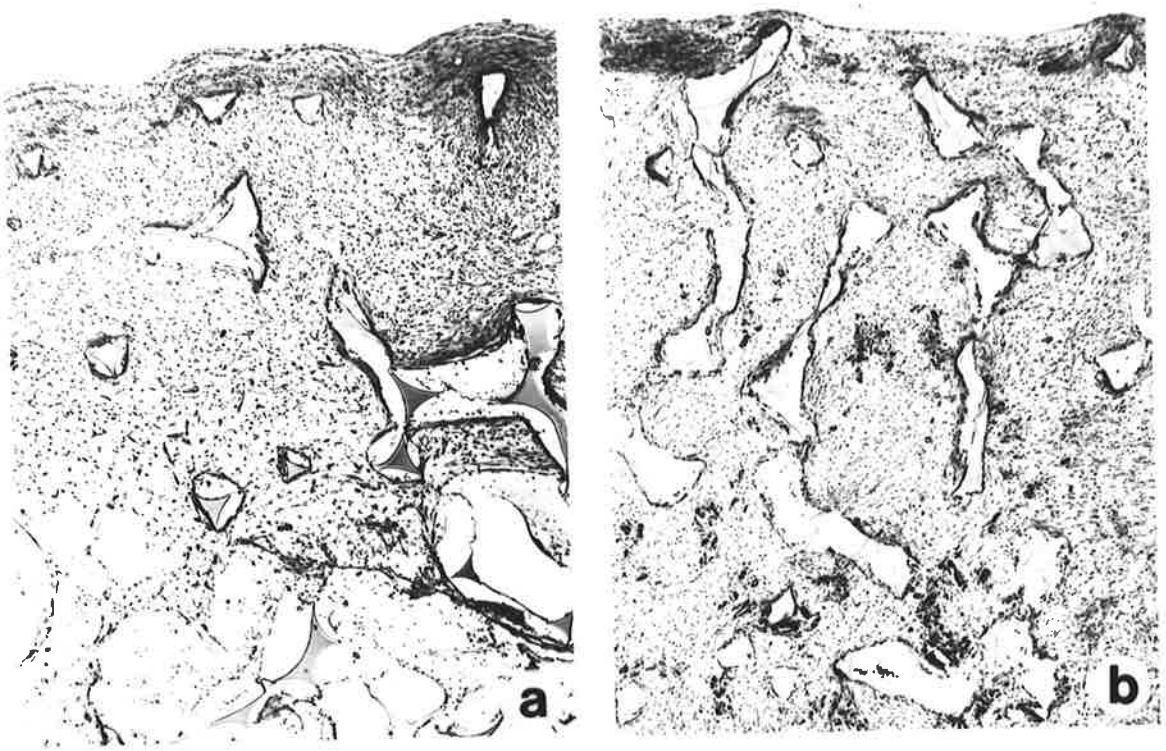


Fig.6.6 Sponges implanted for 14 days in adjuvant treated DA rats. a. Plain sponge infiltrated by a highly cellular granulation tissue. The capsule is prominent. HE VK x35 b. HAC impregnated sponge infiltrated by fibrotic granulation tissue. HE VK x35

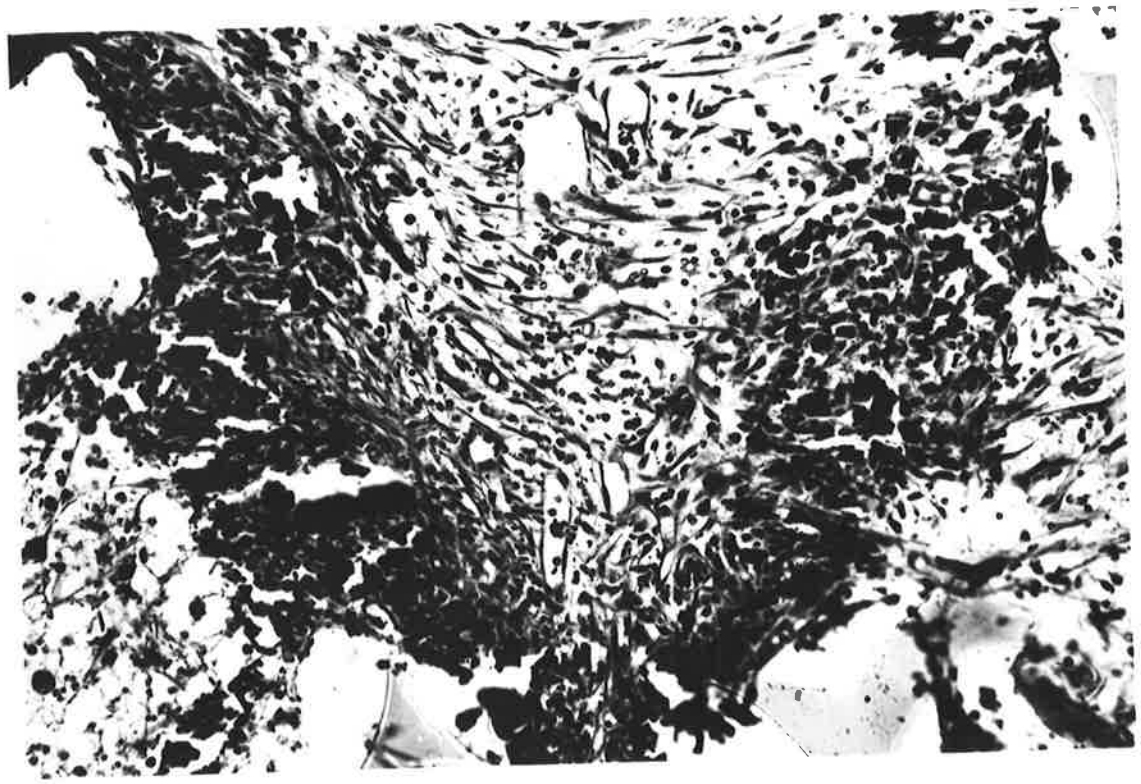


Fig.6.7 Sponge impregnated with HAC implanted for 14 days in adjuvant treated rats. Macrophage, MNGC and lymphocyte response to aggregates of HAC. Fibrosis is also conspicuous. HE VK x190

HAC sponges (fig.6.5b) showed the formation of a well-established granulation tissue with prominent fibroblastic proliferation and focal sclerosis. Fat cells were seen in the periphery of the sponges. The HAC aggregates were surrounded by accumulations of macrophages and MNGC (fig.6.5c) as well as by lymphocytes. Condensation of connective tissue was also seen in close association with the HAC (fig.6.5d).

Sponges implanted in adjuvant treated rats

Plain sponges (fig.6.6a) showed the formation of a highly cellular granulation tissue at the sponge periphery and prominent capsule formation.

HAC sponges (figs 6.6b,c) showed conspicuous fibrosis of the granulation tissue. An intense macrophage and MNGC reaction was directed towards the HAC aggregates. Lymphocytes were also found in the vicinity of the crystalline deposits.

EXPERIMENT 7

Aim

To assess the inflammagenic nature of some calcium phosphates and other microcrystals by the implantation of impregnated sponges over 3, 10 and 14 days in Dark Agouti rats.

Method

Polyurethane sponges were impregnated with a variety of microcrystals using the method described previously for HAC sponge impregnation. Male Dark Agouti rats weighing 220-280g had one plain sponge implanted in the left flank and one impregnated sponge implanted in the right flank. Four rats were randomly assigned to each of the following experimental groups at each time interval studied:

Group	Content of Right-Sided Sponges (20mg/sponge)	Timing of Sponge Removal (Days after implantation)		
1	*Small HAC aggregates	3	10	14
2	**Large HAC aggregates	3	10	14
3	***CPPD crystals	3	10	14
4	+Brushite	3	-	14
5	++MSU crystals	3	-	14
6	+++Calcium carbonate	3	-	14

All particles were sterilised by autoclaving.

* Small HAC aggregates were obtained by sedimentation of Sigma HAC in PBS buffer containing 5% heat inactivated rat serum as previously described. The small crystals (fig.4.4) were harvested from the supernate suspension after overnight sedimentation.

** Large HAC aggregates were obtained from the sediment remaining after overnight settling of Sigma HAC in protein containing buffer. The aggregates corresponded to the larger particles seen in figure 3.1.

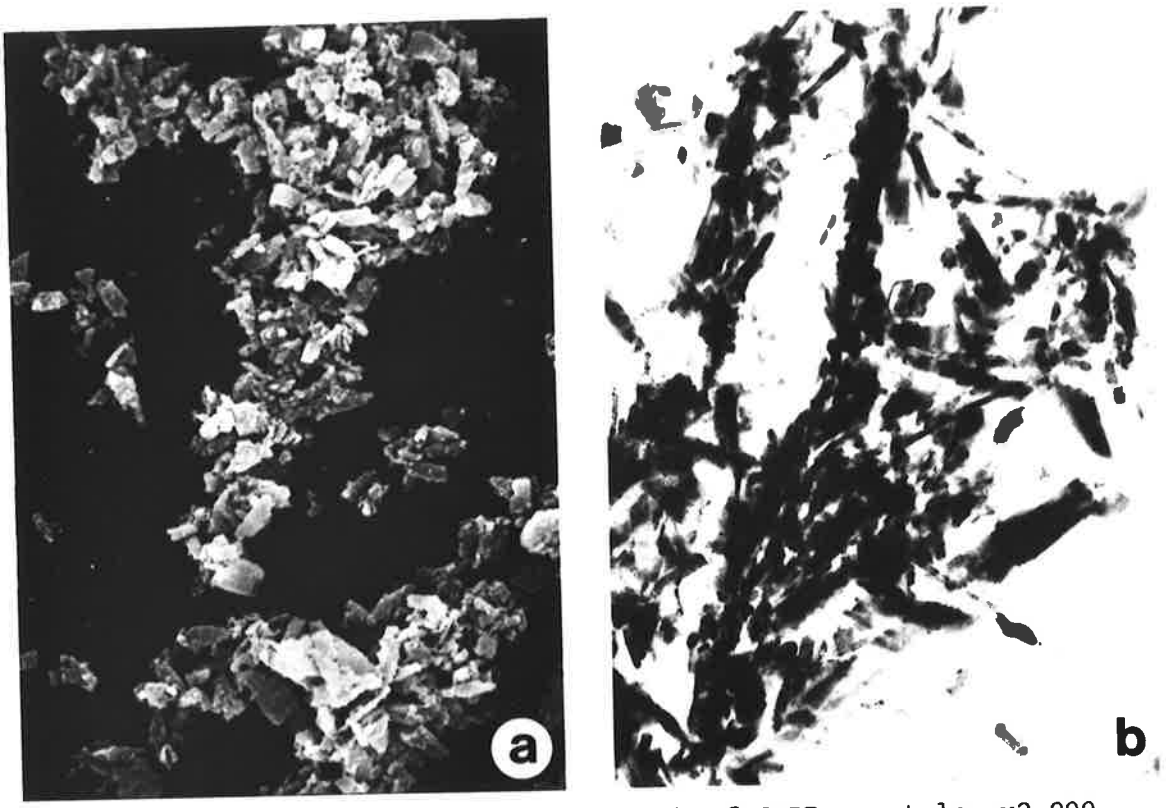


Fig.7.1 a. Scanning electron micrograph of CPPD crystals. x2,000
b. Electron micrograph of CPPD. The rod-shaped crystals are present in transverse and longitudinal section. x79,500

Fig.7.2 Scanning electron micrograph of brushite. xl,000

Fig.7.3 Scanning electron micrograph of MSU crystals. xl,000

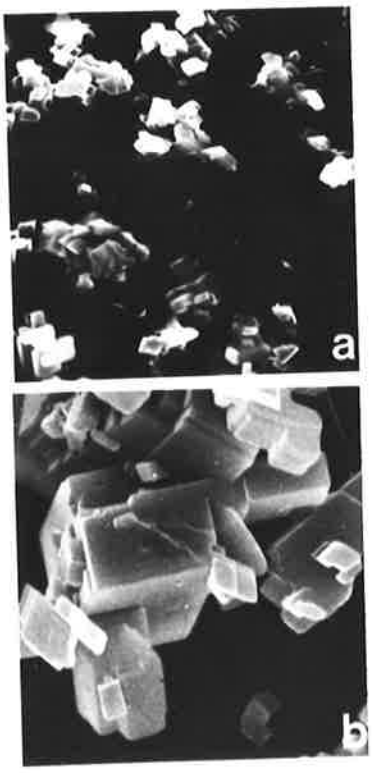


Fig.7.4 Scanning electron micrographs of calcium carbonate particles. a. xl,000 b. x3,000

*** CPPD crystals ($Ca_2P_2O_7.2H_2O$) were a gift from Dr. M.W. Whitehouse, Department of Pathology, University of Adelaide and had been prepared by the method of Denko and Whitehouse (1976). Their physical characteristics are shown in figures 7.1a,b. No attempt was made to modify the size of the crystals.

+ Brushite (calcium orthophosphate dihydrate, $CaHPO_4.2H_2O$, 2-20μ) was obtained as an analytical grade chemical (Analar) and the crystal size was as supplied by the manufacturer. The physical characteristics of these crystals are shown in figure 7.2.

++ MSU crystals ($NaC_5H_4N_4O_3.H_2O$) 3-40μ, were a gift from Dr. T. Gordon, Department of Cinical Immunology, Flinders University, and had been prepared by the method of Denko and Whitehouse (1976). Their physical characteristics are shown in figure 7.3.

+++Calcium carbonate crystals ($CaCO_3$) were obtained as an analytical grade chemical (Analar) and the range of crystal sizes used (1-10μ) was as supplied by the manufacturer. The physical characteristics of these crystals are shown in figures 7.4a,b.

Groups of four rats were sacrificed at 3, 10 and 14 days so that the characteristics of the early and late phases of the inflammatory response could be assessed.

As sponges implanted for 3 days are filled with inflammatory exudate only (no tissue ingrowth at this stage) the following technique was used to avoid compression of the sponges and the consequent loss of fluid during their bisection for dry weight gain measurement. After their excision, the sponges were immersed in liquid nitrogen and each sponge was sectioned with a No.15 scalpel blade while frozen. After thawing, the two portions were weighed as previously described (Chapter 3,F).

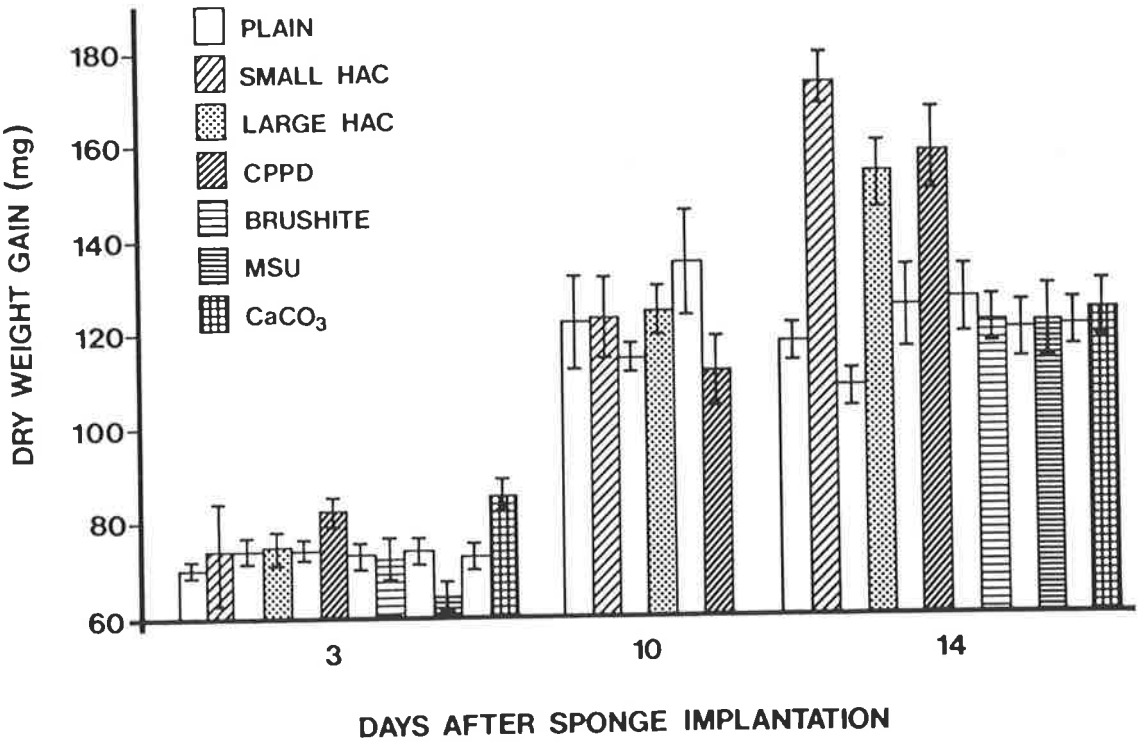
Sponges containing MSU crystals were fixed in 100% ethanol to prevent aqueous dissolution of the crystals. These sponges were subsequently processed for histology by immersion in solutions of xylol/

alcohol (1:1 mixture) xylol and molten paraffin embedding wax for periods of one hour under vacuum.

The parameters measured were sponge dry weight gain, extent of infiltration by granulation tissue (at 10 and 14 days) and comparative cellular response to the different microcrystals.

Results

7a. Sponge Dry Weight Gain (Table 7a and Histogram 7a)



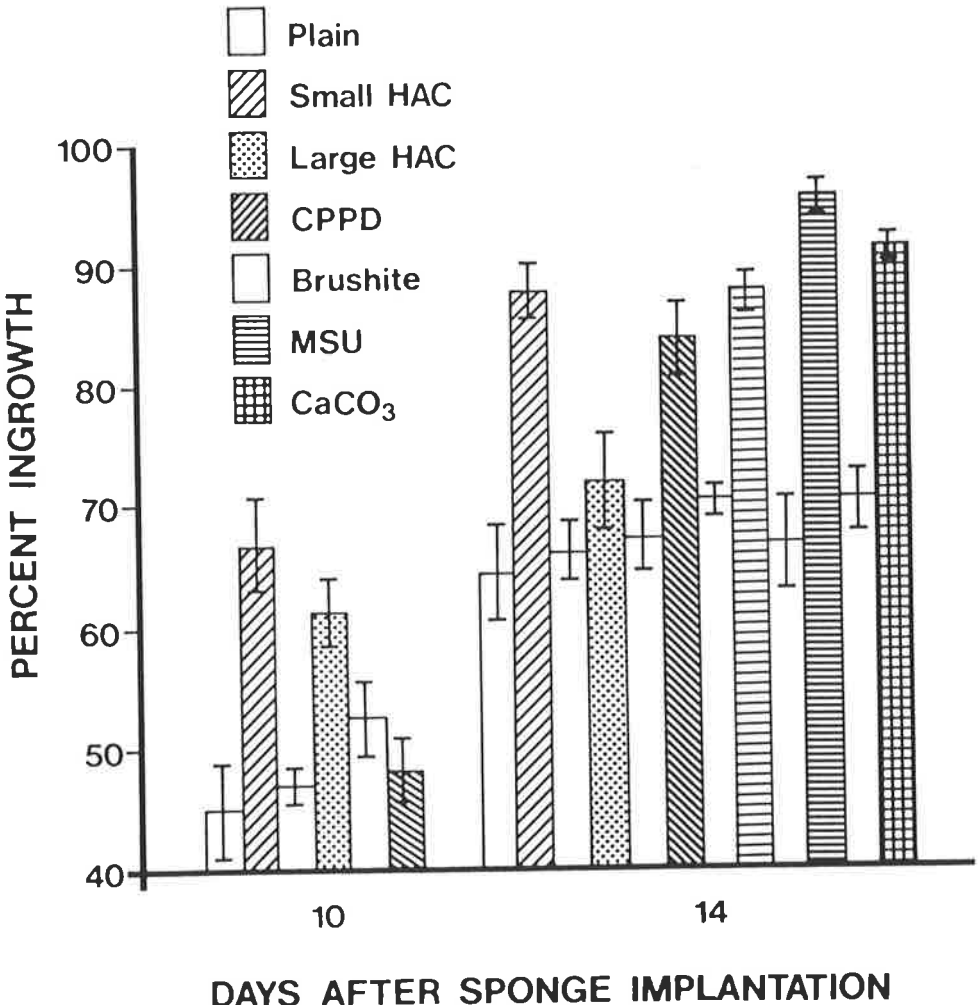
Histogram 7a
Dry weight gain of plain sponges and sponges impregnated with different microcrystals at 3, 10 and 14 days after implantation in Dark Agouti rats.

After 3 days, all sponges other than those impregnated with MSU had similar dry weight gain. MSU containing sponges gained 14% less weight than plain sponges implanted in the same rats.

After 10 days, sponge weight gain was similar for all groups. There was a statistically significant increase in sponge weights measured between days 3 and 10 ($p<.001$). The average sponge weight increase between 3 and 10 days was 48mg.

After 14 days, small HAC, large HAC and CPPD impregnated sponges gained significantly more weight than plain sponges (55mg, 46mg and 33mg respectively). Sponges impregnated with the other microcrystals gained a similar amount of weight as plain sponges.

7b Ingrowth of Granulation Tissue (Table 7b and Histogram 7b)



Histogram 7b
Ingrowth of granulation tissue into plain sponges and sponges impregnated with different microcrystals at 10 and 14 days after implantation in Dark Agouti rats.

After 10 days, small HAC and large HAC impregnated sponges had significantly increased ingrowth of tissue than plain sponges (22% extra for small HAC and 14% for large HAC). CPPD impregnated sponges had a similar degree of tissue infiltration as plain sponges.

After 14 days, all of the microcrystals had stimulated an increased amount of granulation tissue ingrowth when compared with the plain sponges implanted in the same animals. The increases were 24% for small HAC, 6% for large HAC 17% for CPPD and brushite, 29% for MSU and 20% for calcium carbonate. Granulation tissue occupied 95.7% of sponges impregnated with MSU, the highest amount of all particulate materials tested.

7c Histology

Three Days after Implantation

Plain sponges (figs.7.5a,b) contained a proteinaceous exudate with moderate numbers of PMN.

Sponges impregnated with small HAC were filled with a proteinaceous exudate that had precipitated in a lace-like pattern. The HAC had formed large, irregularly shaped clumps which were randomly distributed throughout the sponges (fig.7.6a). A dense infiltrate of PMN was seen in close proximity to the clumps of HAC (fig 7.6b). Most of the PMN seemed to be viable.

Sponges impregnated with large HAC (fig.7.7a) were filled with a dense lacework of precipitated protein. Most of the HAC was found in large clumps surrouned by dense infiltrates of viable PMN (fig.7.7b).

Fig.7.5
Plain sponge, 3 days after implantation
a. Proteinaceous exudate in sponge interstices. HE VK x45
b. Moderate PMN infiltrate in central zone. HE VK x230

Fig.7.6
Sponge impregnated with small HAC, 3 days after implantation.
a. Clumping of HAC particles on the sponge matrix. HE VK x45
b. Dense PMN infiltrate around the clumps of HAC. HE VK x145

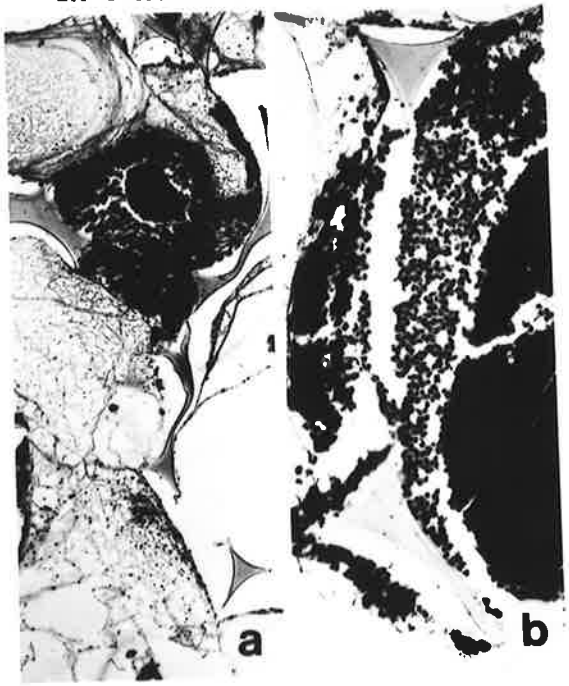


Fig.7.7
Sponge impregnated with large HAC, 3 days after implantation.
a. Dense proteinaceous exudate and clumping of HAC. HE VK x45
b. Intense PMN infiltrate around clumps of HAC. HE VK x145

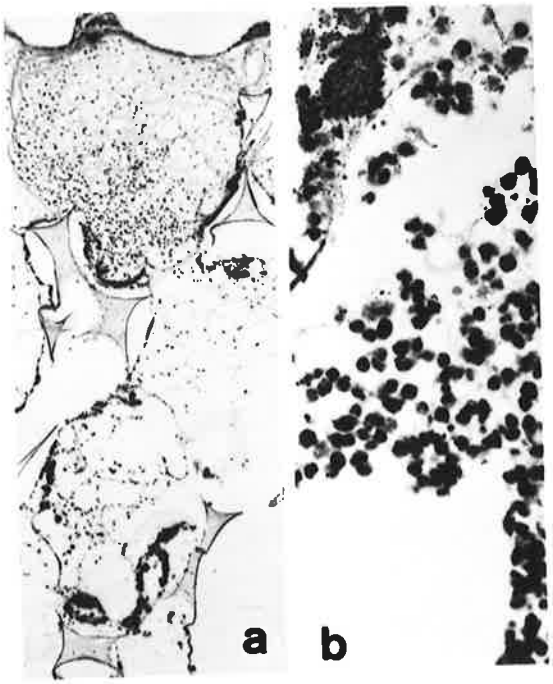


Fig.7.8
Sponge impregnated with CPPD, 3 days after implantation.
a. Light proteinaceous exudate in sponge. HE VK x45
b. Viable PMN infiltrate around granular clumps of CPPD. HE VK x370

Sponges impregnated with CPPD (fig.7.8a) showed a light lace-like pattern of precipitated proteinaceous exudate. The CPPD had formed into small aggregates and were associated with a mild infiltrate of viable PMN (fig.7.8b).

Sponges impregnated with brushite (fig.7.9a) showed signs of extensive acute inflammatory activity with dense lace-like patterns of protein precipitate. Minor clumping of the material had occurred although small individual particles were still present. There was focal infiltration of PMN around the brushite and many of these cells were non-viable, judging by the presence of nuclear fragmentation (nuclear dust) (fig.7.9b).

Sponges impregnated with MSU (fig.7.10a) showed a predominantly fibrinous response. The material was confirmed to be fibrin by the MSB stain. Positive staining for fibrin was not observed in relation to the other microcrystallites implanted. Dense focal masses of PMN were present (fig.7.10b) (presumably located around MSU particles). Clumping was not observed although a special histochemical technique for demonstrating MSU was not used.

Sponges impregnated with calcium carbonate (fig.7.11a) also showed evidence of extensive acute inflammatory activity with dense protein precipitates and focal PMN infiltrates (fig 7.11b). Clumping was not prominent. The alizarin red S stain (fig.7.11c) confirmed the presence of calcium ions but the dense, extensive nature of the precipitate covered the cellular response. The calcium carbonate particles were stained light brown by the von Kossa stain.

Fig.7.9
Sponge impregnated with brushite, 3 days after implantation.
a. Focal areas of acute inflammation around brushite. HE VK x45
b. Infiltrate of PMN around small clumps of brushite. HE VK x175

Fig.7.10
Sponge impregnated with MSU, 3 days after implantation.
a. Dense fibrinous exudate throughout. MSB x45
b. Intense PMN infiltrate within fibrin framework. HE VK x145

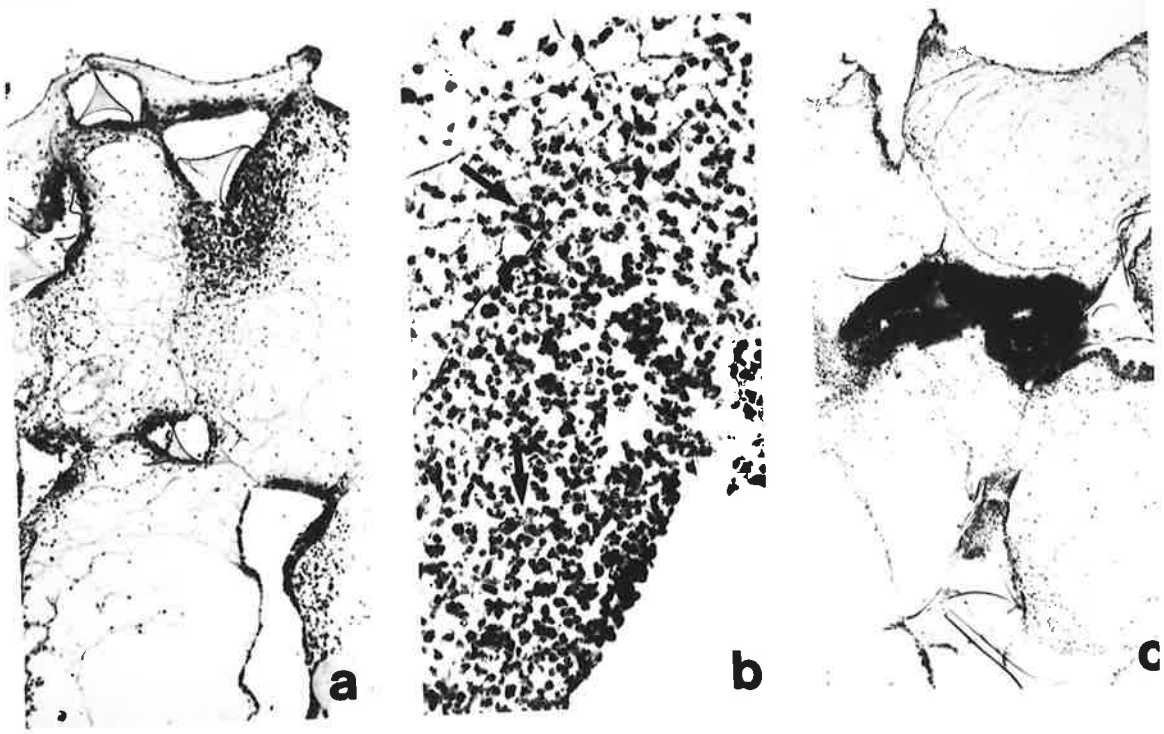


Fig.7.11
Sponge impregnated with calcium carbonate, 3 days after implantation.
a. Focal areas of acute inflammation around deposits of calcium carbonate. HE VK x45.
b. Dense PMN infiltrate around calcium carbonate (arrows). HE VK x230
c. Precipitation of stain around calcium carbonate. Alizarin red S x45

Ten days after implantation

The granulation tissue in plain sponges was identical to that reported in Experiment 1 at 10 days after implantation.

Sponges impregnated with small HAC showed persistent clumping of the HAC and the beginning of a macrophage and MNGC reaction to it (fig 7.12a). Few PMN were found around the HAC in the central part of the sponges.

Sponges impregnated with large HAC (fig.7.12b) also showed the development of a macrophage and MNGC infiltrate around the clumped HAC. Many necrotic PMN were clustered around the HAC in the central sponge zone (fig.7.12c).

Sponges impregnated with CPPD (fig.7.12d) showed a minimal macrophage infiltrate around the clumps of material. MNGC were not prominent. A persistent PMN response was seen in the sponge centres.

Fourteen days after implantation

Sponges impregnated with small HAC (fig.7.13) showed prominent MNGC formation and intracellular uptake of large clumps of HAC. Lymphocytes were scattered in the granulation tissue.

Sponges impregnated with large HAC (fig.7.14) had well established macrophage and MNGC infiltrates that were confined to the vicinity of the clumps of HAC.

Sponges impregnated with CPPD (fig.7.15a) had a predominant macro-

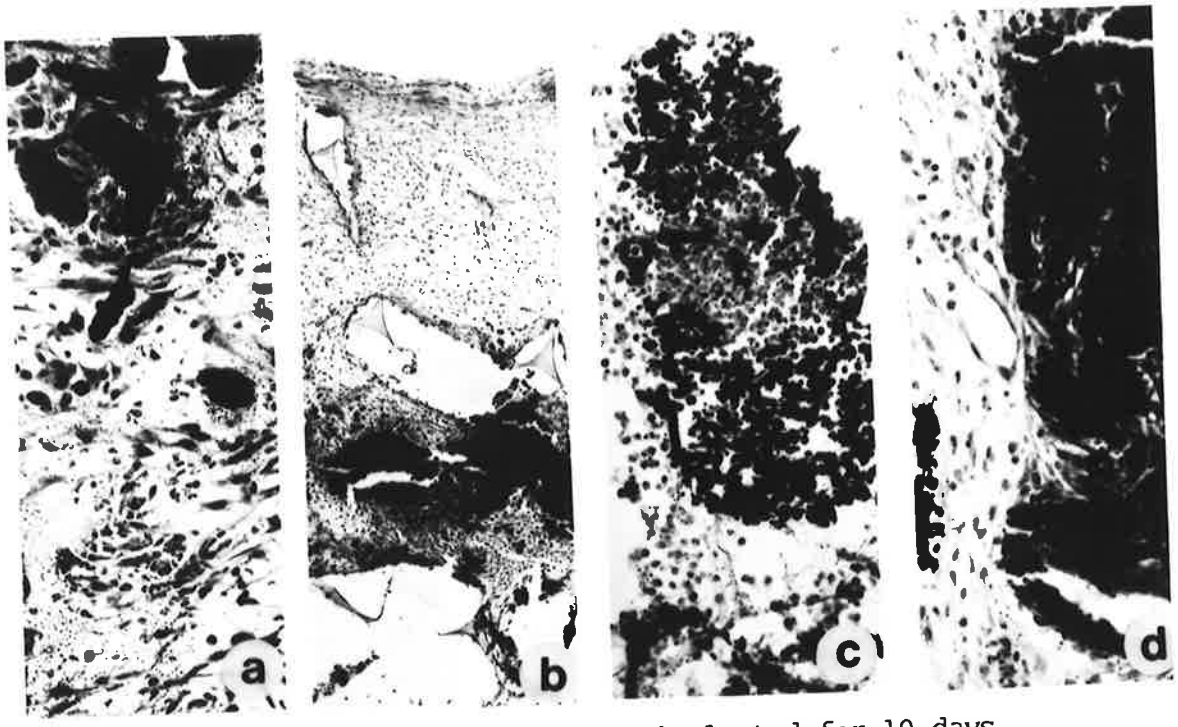


Fig.7.12 Impregnated sponges implanted for 10 days.
a. Small HAC with macrophage and MNGC reaction. HE VK x230
b. Large HAC showing extensive clumping and dense cellular reaction around the clumps. HE VK x45
c. Large HAC with necrotic PMN around the clumps in the central sponge zone. HE VK x175
d. CPPD with minimal macrophage response. HE VK x230

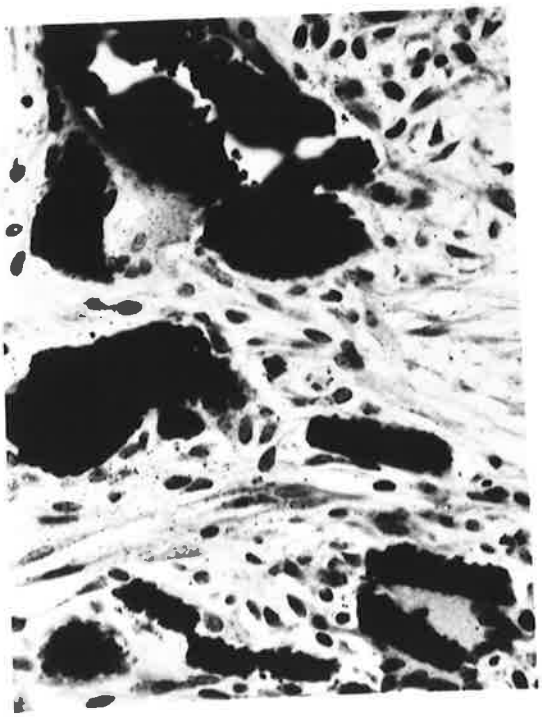


Fig.7.13 Sponge impregnated with small HAC, 14 days after implantation showing uptake of HAC clumps by MNGC. HE VK x370

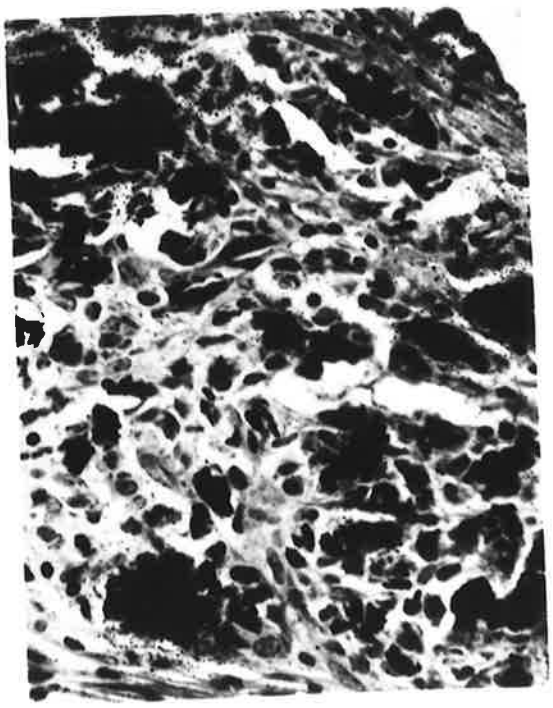


Fig.7.14 Sponge impregnated with large HAC, 14 days after implantation with well established macrophage and MNGC response. HE VK x370

phage response to the CPPD clumps; a certain degree of fragmentation of the clumps into smaller aggregates was observed.

Sponges impregnated with brushite (fig.7.15b) showed sparse distribution of von Kossa positive material. Some particles had attracted an extensive macrophage response (fig.7.15c). A few MNGC were present adjacent to the larger rounded deposits of brushite that were sparsely scattered throughout the sponges (fig.7.15d).

Sponges impregnated with MSU (fig.7.16a) were also infiltrated by a highly cellular and fibrotic granulation tissue. Collagen fibres were well orientated and were generally parallel to the sponge surface. Large numbers of necrotic cells were present near the central zone of the sponges (fig.7.16b).

Sponges impregnated with calcium carbonate (fig.7.17a) had developed a granulation tissue with an apparent increased fibroblastic component. A PMN response persisted towards the $CaCO_3$ in the central part of the sponges (fig.7.17b). Both necrotic and viable PMN were present. The cellular reaction around the particulate material in the granulation tissue consisted predominantly of macrophages with a few scattered lymphocytes.

EXPERIMENT 8

Aim

To induce the deposition of HAC by lead acetate injection into sponges infiltrated by granulation tissue and to study the tissue response over a 21 day period.

Method

Plain polyurethane sponges were implanted subcutaneously in 44 young adult male Porton rats weighing 200-250g. The rats were randomly distributed into eleven groups (4 per group). Each rat had one left- and one right-sided sponge implanted.

Fourteen days after implantation and under general anaesthesia, 0.4ml of a sterilised 5 mg/ml solution of lead acetate (PbAc),

$$Pb(C_2H_2O_2) \cdot 3H_2O$$

obtained from Ajax Chemicals, Sydney, Australia, was injected into the centre of each right-sided sponge using a 26 gauge needle. The same amount of lead acetate solution was injected subcutaneously on the right side near the base of the back of each animal. On the left side, 0.4ml of sterilised normal saline was injected into the sponge and subcutaneously near the base of the back in an identical manner. The dose of calcergen used and the time of injection in relation to sponge implantation were determined from data obtained from an initial pilot study.

Groups of four animals were killed by intracardiac injection of sodium pentothal at the following times after sponge injection: 10 minutes, 5 hours, 1, 2, 3, 4, 5, 6, 7, 14 and 21 days. Sponges were dissected carefully from surrounding tissues and subcutaneous injection

sites were removed *in toto* .

The parameters assessed were : sponge dry weight gain, extent of granulation tissue ingrowth into the sponges and histology. Sections were stained with (i) alizarin red S for calcium, (ii) VK/HE for companion anions and cellular and tissue detail and (iii) neutral red/haematoxylin to demonstrate mast cells. Specimens 2-21 days after PbAc were also fixed and processed for TEM examination (see Experiment 1 for method). TEM sections were used for Xray dispersive analysis.

Sponge samples for EDAX examination were not fixed in osmium and ultrathin sections were not stained. An EDAX Xray detector and display unit was linked to a 707B multichannel analyser which computed elemental ratios of the targets. A live-time of 100-200 seconds was used with the spot size reduced to cover individual crystals or groups of crystals. Background correction for the copper grid was made by normalising on the copper Kα peak.

Results

8a Sponge Dry Weight Gain (Table 8a and Figure 8a)

Significant differences between sponges injected with PbAc and saline were apparent as early as 10 minutes after injection. Up to 7 days post-injection, PbAc injected sponges continued to increase in weight while control sponges showed no weight change. By 14 days, PbAc injected sponges showed a peak dry weight increase which was greatly in excess of that noted in control sponges three weeks after injection.

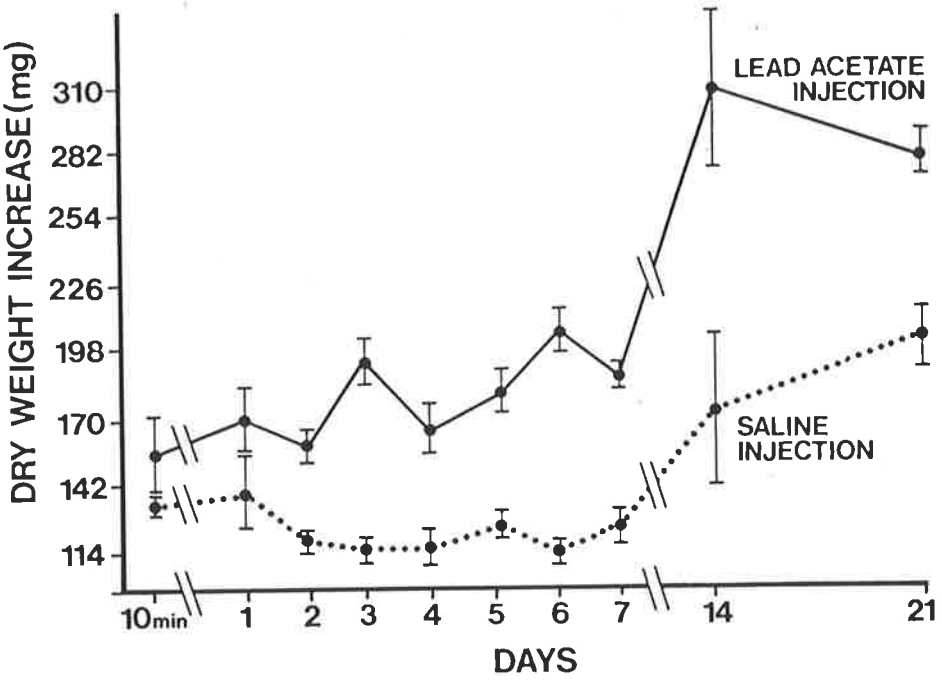


Figure 8a
Dry weight increase of sponges during the 21 days after saline or PbAc injection.

8b Ingrowth of granulation tissue (Table 8b and Figure 8b)

Prior to injection, the sponges which had been implanted for 14 days showed considerable variability in the percentage area of sponge infiltrated by granulation tissue. There was an increase in the area occupied by granulation tissue in saline-injected sponges up to 2 days post-injection. From 3 days post-injection onwards, the extent of ingrowth of granulation tissue was similar in test and control sponges.

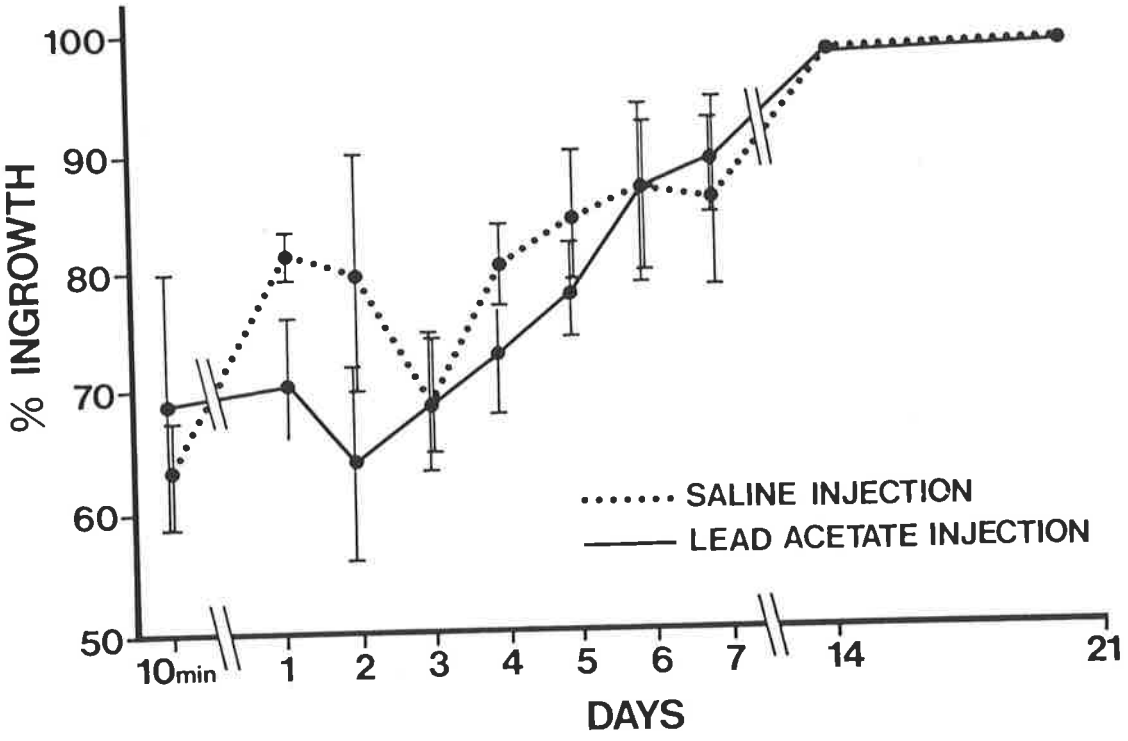


Figure 8b
Ingrowth of granulation tissue into sponges during the 21 days after saline or PbAc injection.

8c Histology

Fourteen days after implantation the sponges were infiltrated by moderately vascular granulation tissue which occupied all but a small central zone. The histological appearance was identical to that described in Experiment 1 for plain sponges implanted for 14 days (figs. 1.6, 1.7 and 1.8).

Ten minutes after the injection of PbAc some small rod-shaped VK positive crystals were found in the central tissue free portion of the sponges (fig.8.1a) as well as in the granulation tissue adjacent to this area (fig.8.1b).

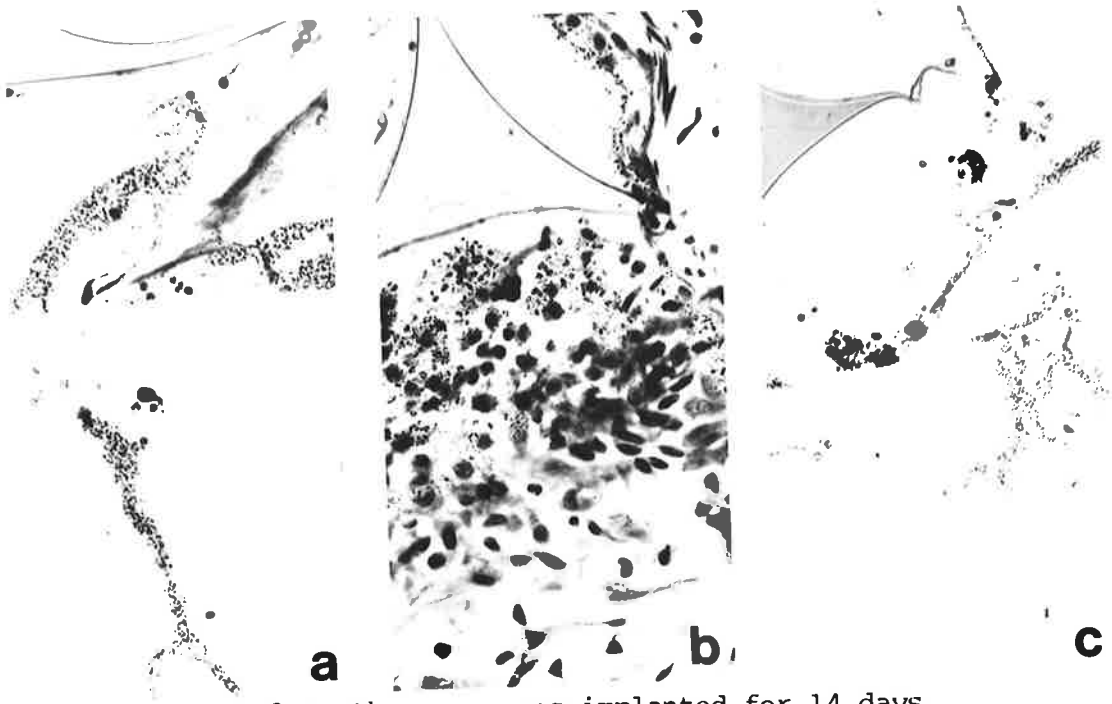


Fig.8.1 Polyurethane sponges implanted for 14 days.
a. Ten minutes after PbAc injection with deposition of crystalline material in the sponge centre. HE VK x290
b. Ten minutes after PbAc injection with deposition of VK positive crystalline material in the granulation tissue adjacent to the sponge centre. HE VK x290
c. Five hours after PbAc injection with granular calcification in the central sponge zone. HE VK x200

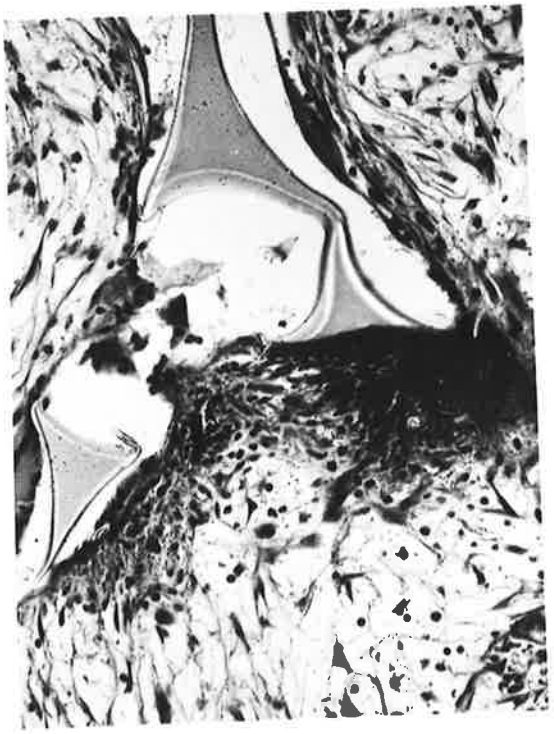


Fig.8.2 Calcification along collagen fibres in granulation tissue, 1 day after PbAc injection. HE VK x290

Fig.8.3 Dense aggregates of calcified material, 1 day after PbAc injection. HE VK x290

Five hours after injection of PbAc, small amounts of von Kossa and alizarin red S positive material were present as clumps in the central zone (fig.8.1c) and deposited as fine granules on the collagen fibres of the granulation tissue. PMN were not noted, and the small numbers of lymphocytes an macrophages were similar in quantity to those present in the control sponges.

One day after injection of PbAc, focal deposits of calcific material were present in the central and peripheral zones of the sponges. Usually, they were deposited along collagen fibres as a finely granular material (fig.8.2). In some zones, the deposits were rounded and were up to 5μ in diameter (fig.8.3). Some PMN were seen in the central part of the sponges and there were increased numbers of macrophages and lymphocytes in comparison to control sponges.

Two days after injection of PbAc, the calcific material was present in greater quantity. Condensation of the innermost zone of the granulation tissue in PbAc injected sponges (fig.8.4b) which contrasted with the persistence of a loosely organized and irregular arrangement of the innermost zone of granulation tissue in saline-injected sponges (fig.8.4a). There was a fine granular deposition on collagen fibres free rounded structures similar to those described previously but now exhibiting concentric lamination (fig.8.5). There were increased numbers of macrophages and some MNGC were seen in relation to the calcific material. A small number of PMN were present in the central zone of the sponge.

Three days after injection of PbAc, the calcific material was more widespread and was present not only in the sponge but also in the surr-

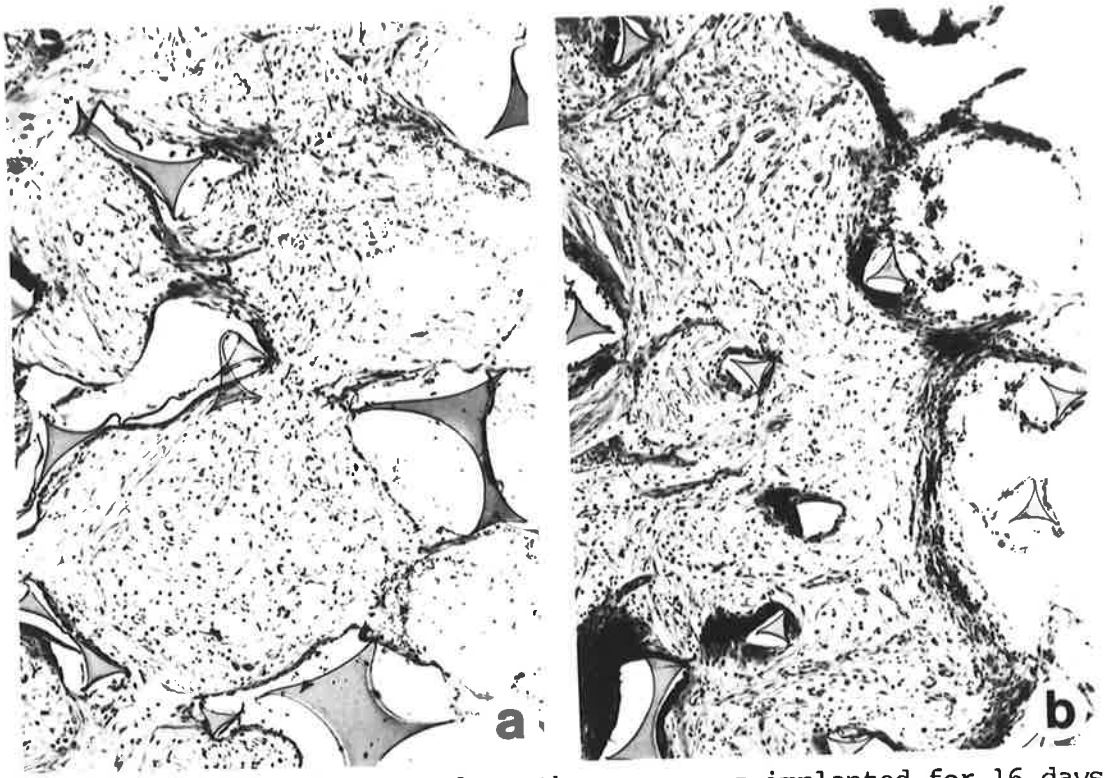


Fig.8.4 Central region of polyurethane sponges implanted for 16 days.
a. Two days after saline injection showing loose arrangement and irregularity of the inner border of granulation tissue. HE VK x60
b. Two days after PbAc injection showing condensation and calcification of the inner border of granulation tissue. HE VK x56

Fig.8.5 Extensive deposition of calcified material along collagen fibres and associated macrophage response 2 days after injection with PbAc. HE VK x290

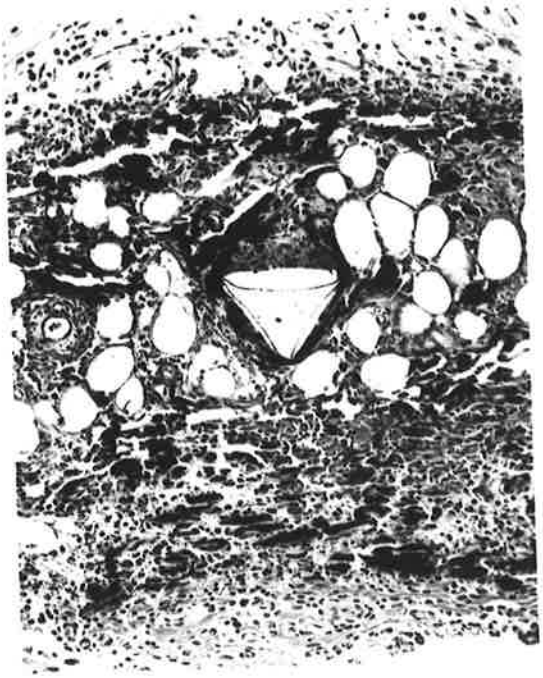


Fig.8.6 Extensive calcification and macrophage response around the peripheral sponge margin, 3 days after injection with PbAc. Fat cells are prominent within the sponge. HE VK x120

ounding fibrous connective tissue and adipose tissue (Fig 8.6). The reaction products were disposed as fine deposits on collagen fibres and as irregular plates and concentrically laminated structures. Increased numbers of macrophages and MNGC were related to the deposits. PMN were absent.

In sponges examined 4 to 21 days after the injection of PbAc, the calcific depositions were equal in amount and similar in appearance, with the major component continuing to be the granular depositions in relation to collagen fibres. Macrophages and MNGC continued to be prominent (fig.8.7).

Control sponges injected with normal saline showed no evidence of calcium salt deposition. Mast cells were not observed in the granulation tissue of either test or control sponges.

Subcutaneous tissue histology

At the sites of subcutaneous injection of PbAc, there was degranulation of mast cells (MC) and vascular dilatation after the injection of PbAc. After one day, VK and alizarin red S positive calcific material was present as fine granular deposits on collagen fibres and around blood vessels and nerves. There was some persistence of degranulation of MCs around the dilated blood vessels. The calcific deposits were granular and similar in size to MC granules. There was a mild inflammatory infiltrate composed predominantly of PMN Around the calcific depositions.

Two days after PbAc injection there was an extensive calcific deposition in the dorsal fascia. An inflammatory reaction surrounding the

Fig.8.7 Dense calcification in granulation tissue 21 days after PbAc injection. Macrophages and MNGC are present around the calcified zones. HE VK x120

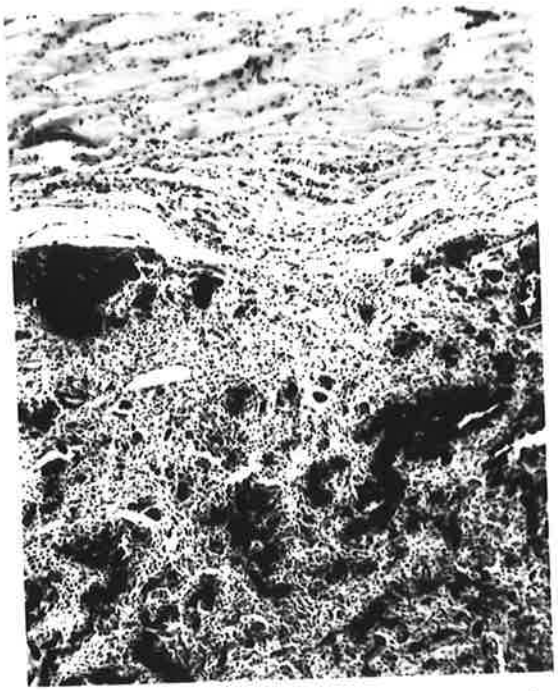


Fig.8.8 Extensive calcification and cellular infiltration in the subcutaneous tissues, 21 days after injection of PbAc into rat skin. A fibrous capsule has formed around the calcified zone. HE VK x50

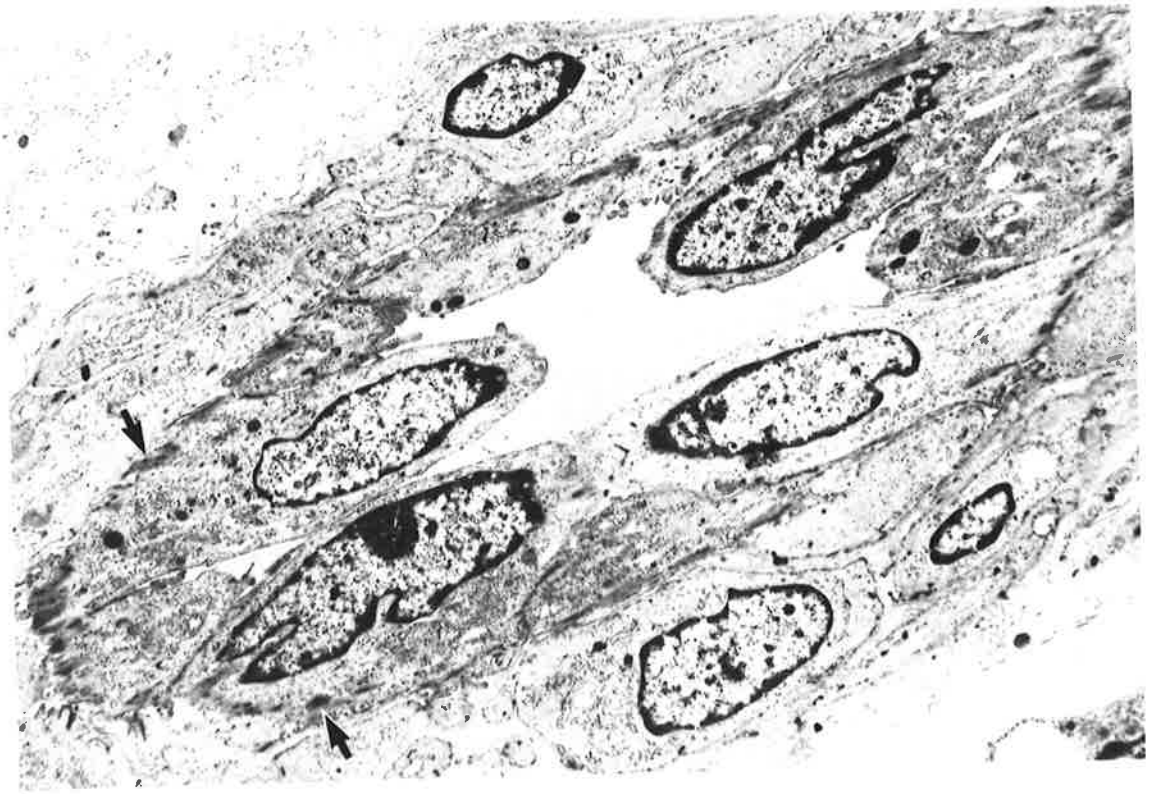


Fig.8.9 Electron micrograph of granulation tissue 3 days after injection of PbAc showing myofibroblasts with crenated nuclei and focal densities on cytoplasmic fibrils (arrows). x5,700

deposits was composed predominantly of macrophages with occasional MNGC closely related to the calcific deposits. PMN were present in much reduced numbers.

From three to seven days after injection of PbAc the calcific deposition increased in extent. The calcific material was disposed as a fine deposit on collagen fibres and as large rounded structures. The numbers of macrophages and MNGC were increased around the calcific zone, and PMN had disappeared by the sixth day. External to this cellular zone there was increasing fibroblastic proliferation and, by 21 days (fig.8.8), there was a well-defined fibrous tissue capsule around the lesion.

8d Transmission Electron Microscopy

TEM of sponges removed two days after injection of PbAc, showed myofibroblasts with crenated nuclei and intracellular myofibrils within the granulation tissue (fig.8.9). Crystals having the morphological needle- shaped characteristics of HAC (fig.8.10) were found within cells and also at extracellular locations (fig.8.11). The aggregates of crystals were associated with rounded electron dense structures (fig.8.12). Endocytosis of these conglomerates by macrophages (fig. 8.13) was evident.

At 3 days after PbAc injection, the material resembling HAC was found deposited along collagen fibres (figs.8.14a,b). After 4 days, masses of electron dense material was deposited extracellularly (fig. 8.15a) and uptake of smaller dense particles occurred at other sites

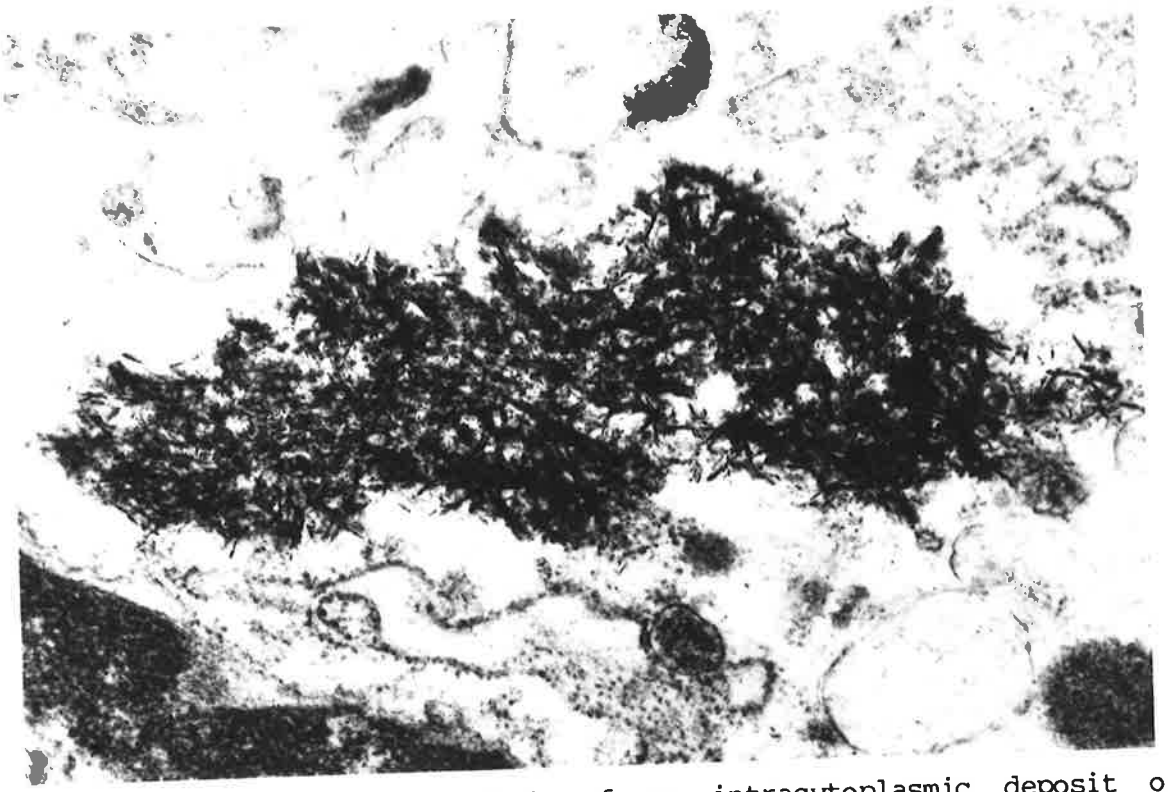


Fig.8.10 Electron micrograph of an intracytoplasmic deposit of calcific material 2 days after injection of PbAc. The crystalline appearance of the deposit is characteristic of HAC. x51,300

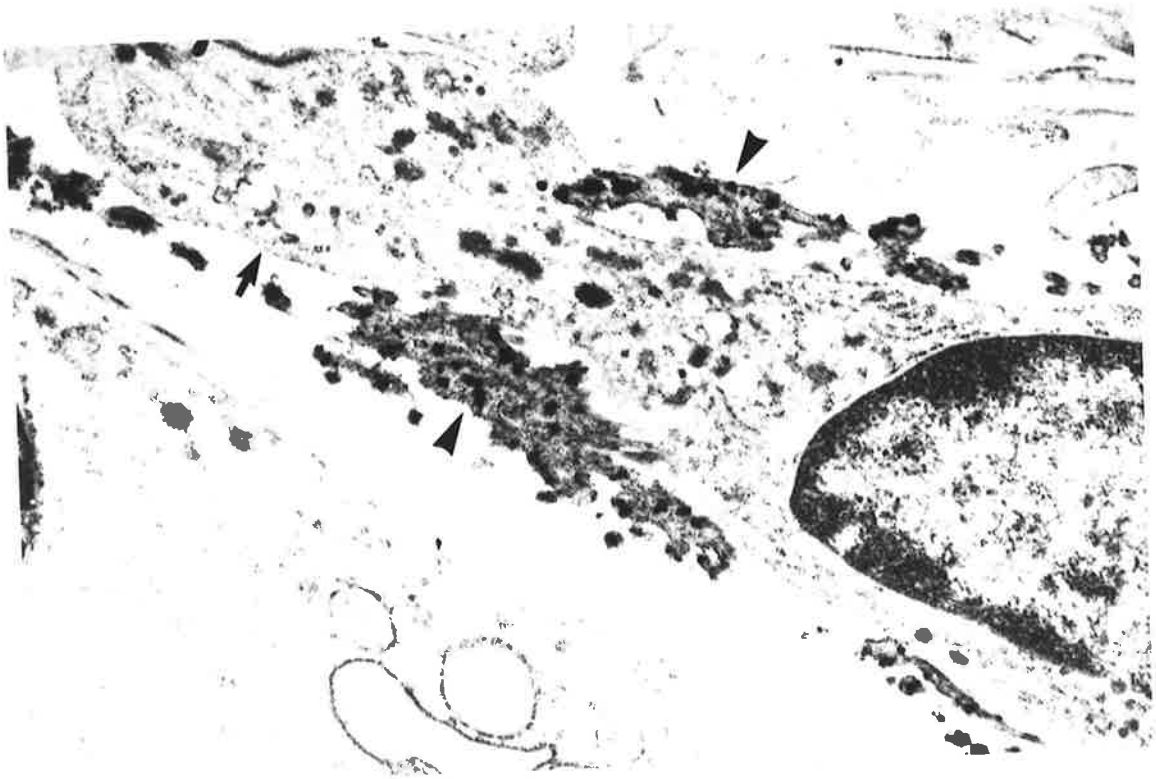


Fig.8.11 Electron micrograph of aggregates of crystalline material (arrowheads) close to a fibrocyte cell membrane (arrow) two days after injection of PbAc. x20,600

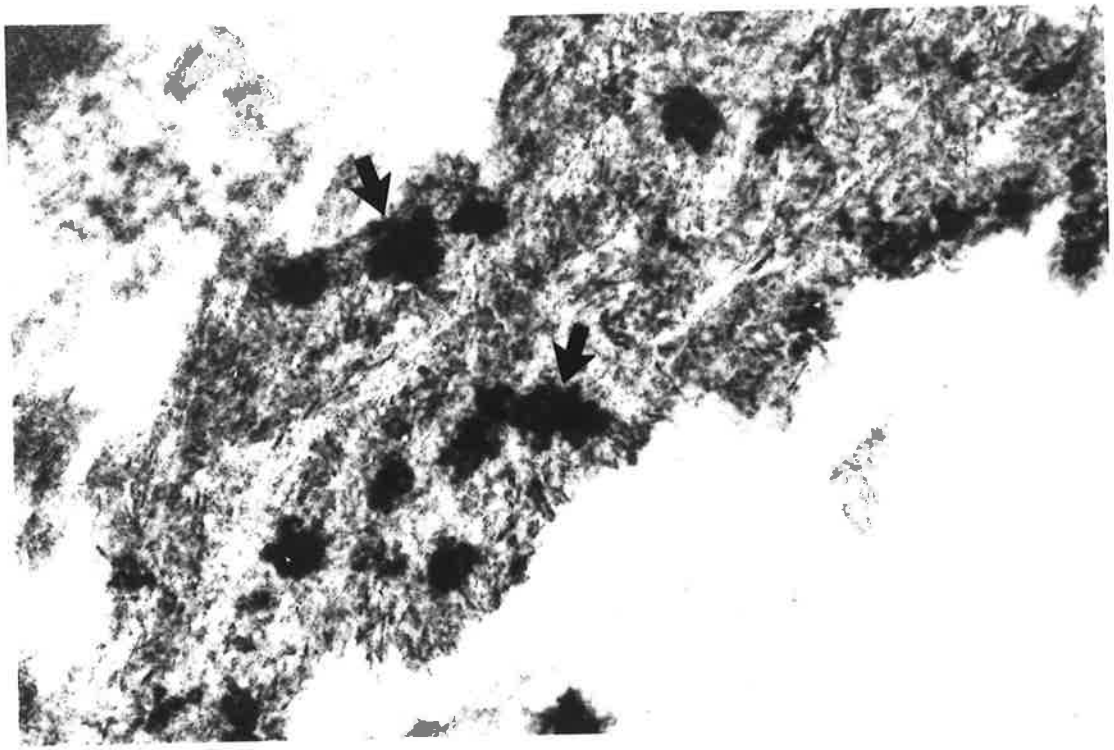


Fig.8.12 Higher magnification of portion of crystalline aggregate in fig.8.11. The calcific material contains rounded electron-dense areas (arrows) surrounded by needle-shaped crystalline material. x102,000

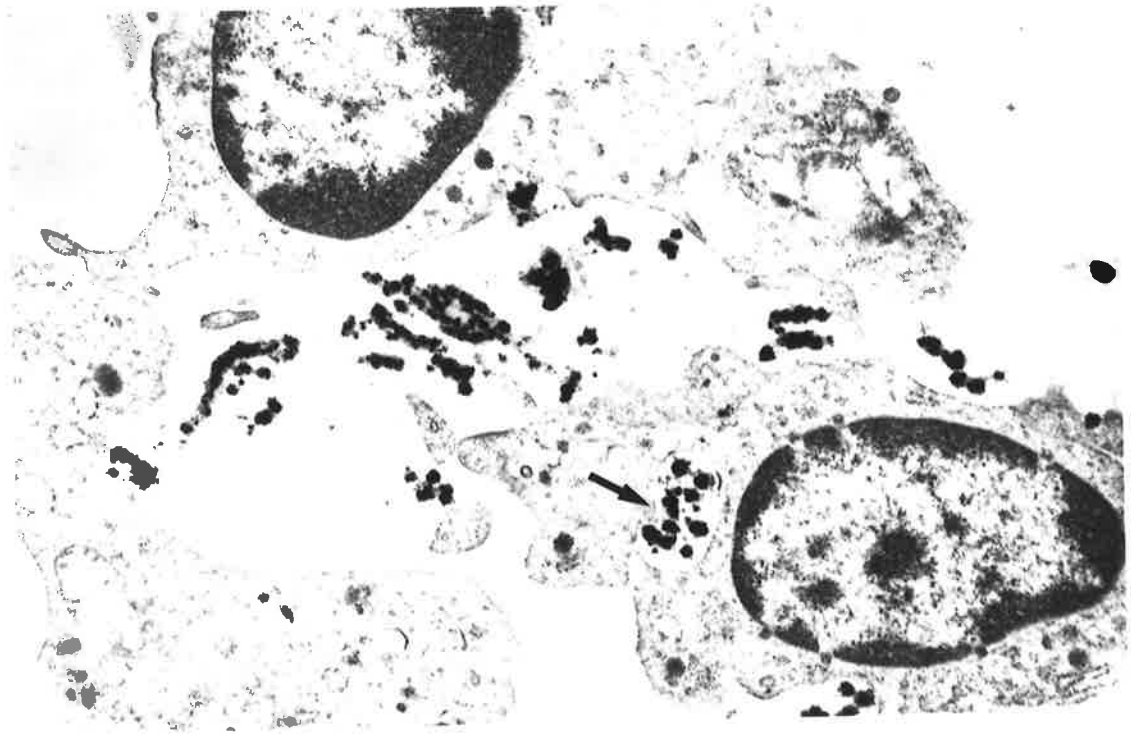


Fig.8.13 Electron micrograph of macrophages in a sponge 2 days after PbAc injection. Crystalline material is seen outside the cells and in the process of being endocytosed (arrow). x13,200

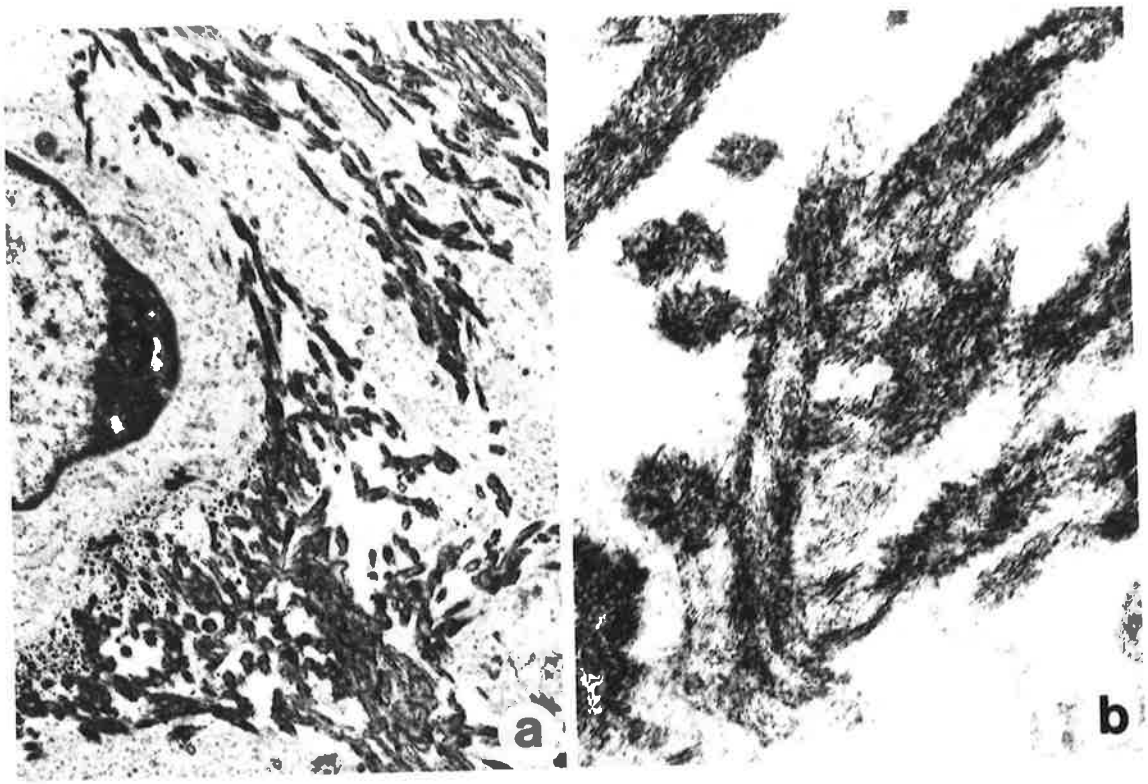


Fig.8.14 Electron micrographs of sponges 3 days after PbAc injection.
a. Deposits of calcific material on collagen fibres. x12,300
b. Higher magnification showing needle-shaped crystals arranged along the length of the collagen fibres. x70,560

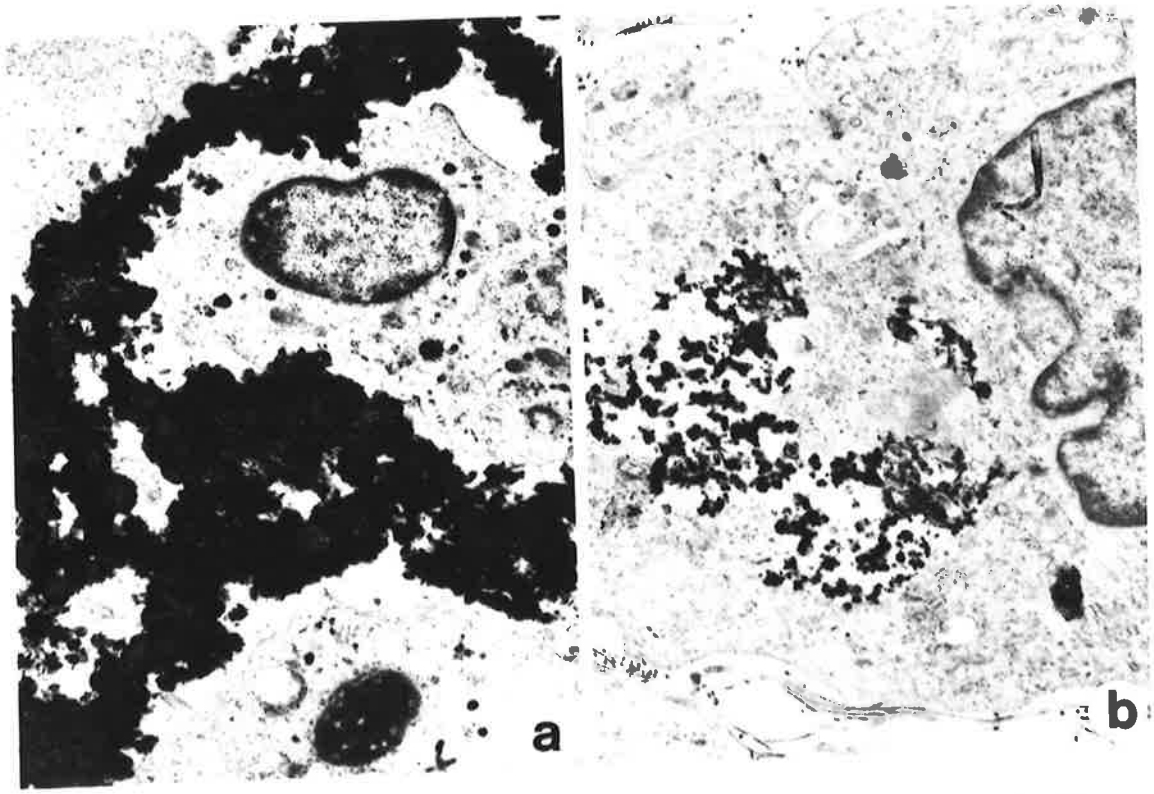


Fig.8.15 Electron micrographs of sponges 4 days after PbAc injection.
a. Dense deposits of crystalline material between macrophages. x7,300
b. Small aggregates of crystalline material endocytosed by a macrophage. x10,000

(fig.8.15b) by macrophages. After 6 days, sheets of electron dense material were found in intercellular deposits. At 7 days (fig.8.16) intracellular aggregates of needle-shaped crystals were present in membrane bound phagosomes within macrophages 7 days after PbAc injection (fig.8.17) showed the deposition of electron dense material along collagen fibres.

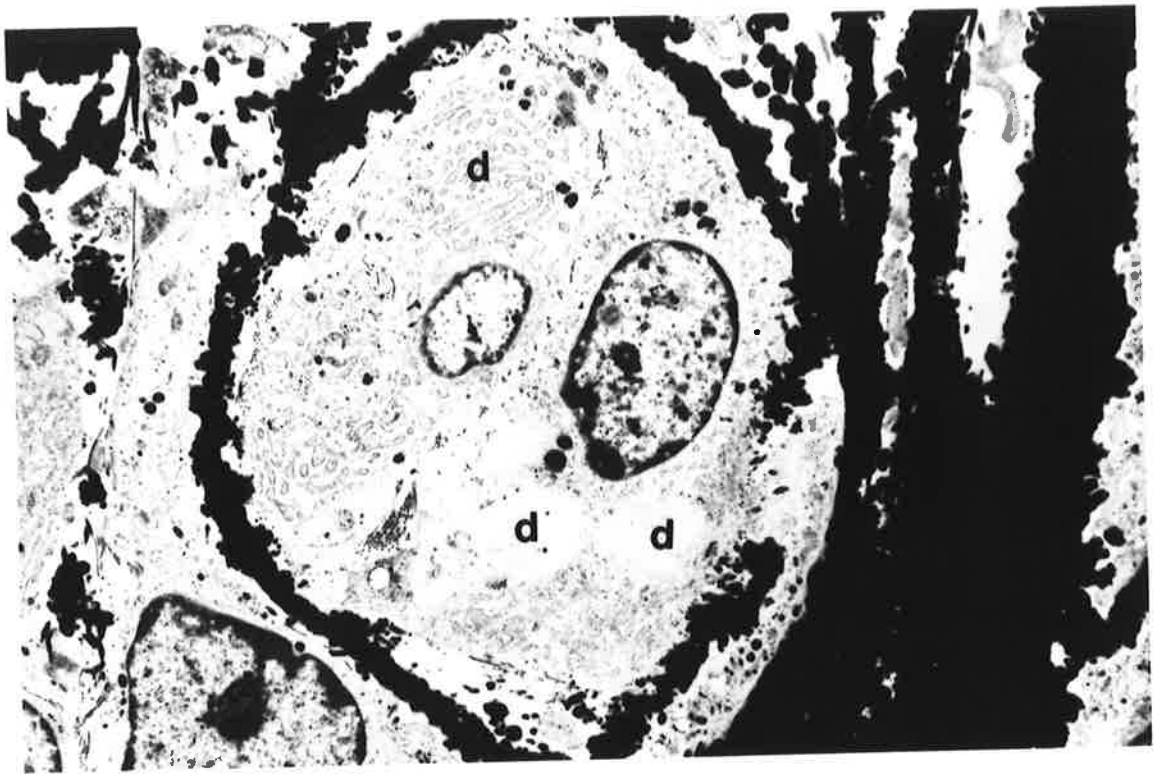


Fig.8.16 Electron micrograph of sponges 6 days after injection of PbAc. Macrophages are surrounded by extensive, very dense intercellular deposits (containing lead as determined by EDAX) with evidence of cytoplasmic degeneration (d) and dilated endoplasmic reticulum. x4,700

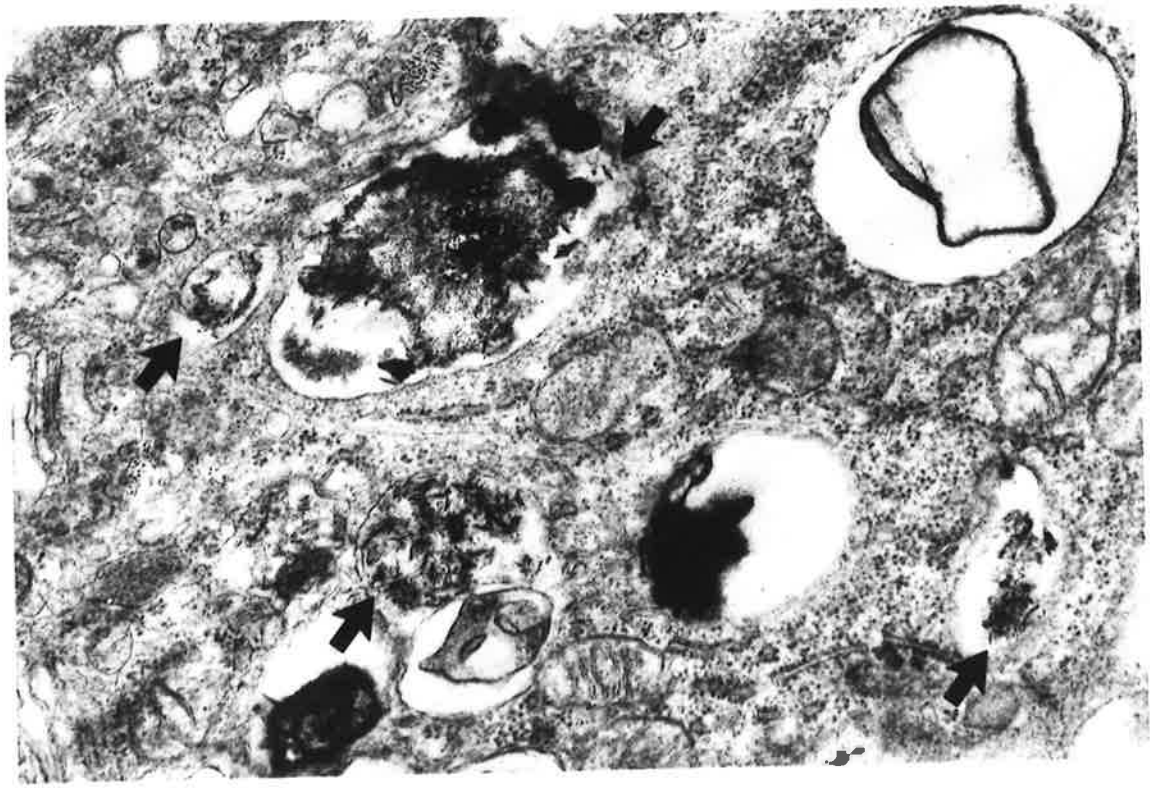


Fig.8.17 Electron micrograph of portion of a macrophage cytoplasm with membrane bound phagosomes containing needle-shaped crystals (arrows), 7 days after injection of PbAc. x35,300

EXPERIMENT 9

Aim

To induce the deposition of HAC in granulation tissue by the implantation of sponges soaked in lead acetate (PbAc) and to study the tissue response over a 140 day period.

Method

Adult male Lewis rats weighing 300-350g were randomly distributed into 11 groups of four rats per group. Each rat had one plain sponge implanted in the left flank and one sponge that had been soaked in lead acetate solution implanted in the right flank. The PbAc soaked sponges were prepared as follows. A 5mg/ml solution of lead acetate in double distilled water was made and then autoclaved. Polyurethane sponges were compressed between the beaks of a pair of tweezers and immersed in the PbAc solution where they were released. Sponges were carefully placed on a sterile stainless steel tray and dried at 60°C. The sponges were turned over every few minutes to ensure that the PbAc evenly coated the sponge matrix as the water evaporated. The dried sponges were weighed to check that they contained similar amounts of PbAc. Since the sponges could absorb 1.5ml of fluid, each sponge contained approximately 7.5mg PbAc. The concentration of PbAc used was determined from data obtained from an initial pilot study.

Groups of four animals were killed by intracardiac injection of sodium pentothal at the following times after sponge injection: 5 hours, 1, 5, 7, 14, 21, 28, 35, 42, 49 and 140 days. The parameters assessed were: sponge dry weight gain (excluding the sponges retrieved

5h after implantation), extent of ingrowth of granulation tissue into the sponges (excluding the sponges retrieved 5h and 1 day after implantation), histology (same stains as used in Experiment 8) and TEM together with Xray dispersive analysis.

Results

9a Sponge Dry Weight Gain (Table 9a and Figure 9a)

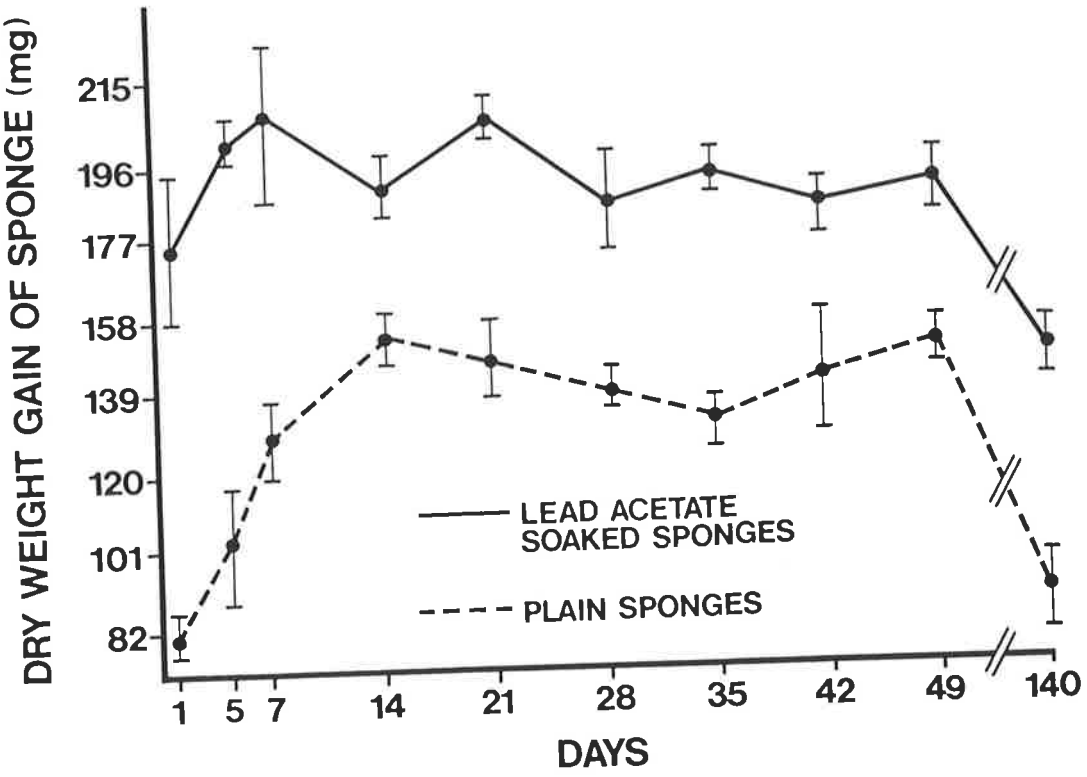


Figure 9a
Dry weight increase of plain sponges and sponges soaked in PbAc during the 140 days after implantation.

Plain sponges rapidly gained weight during the first 14 days after implantation after which a slight decrease to Day 35 occurred. At Day 49, the sponges had regained the same amount of weight as at Day 14. Between 49 and 140 days after implantation, the sponge dry weight gain decreased by 42%.

PbAc soaked sponges had gained significantly more weight at all

times measured than plain sponges ($p<.001$). After one day, PbAc sponges had gained more than twice as much weight as plain sponges. Dry weight gain increased until Day 7. From this time until Day 49 no significant changes in dry weight gain occurred. However, between Days 49 and 140, sponge dry weight gain decreased by 23%.

There was a statistically significant difference in sponge dry weight gain of plain and PbAc soaked sponges at the 10 time periods studied ($p<.001$).

9b Ingrowth of Granulation Tissue (Table 9b and Figure 9b)

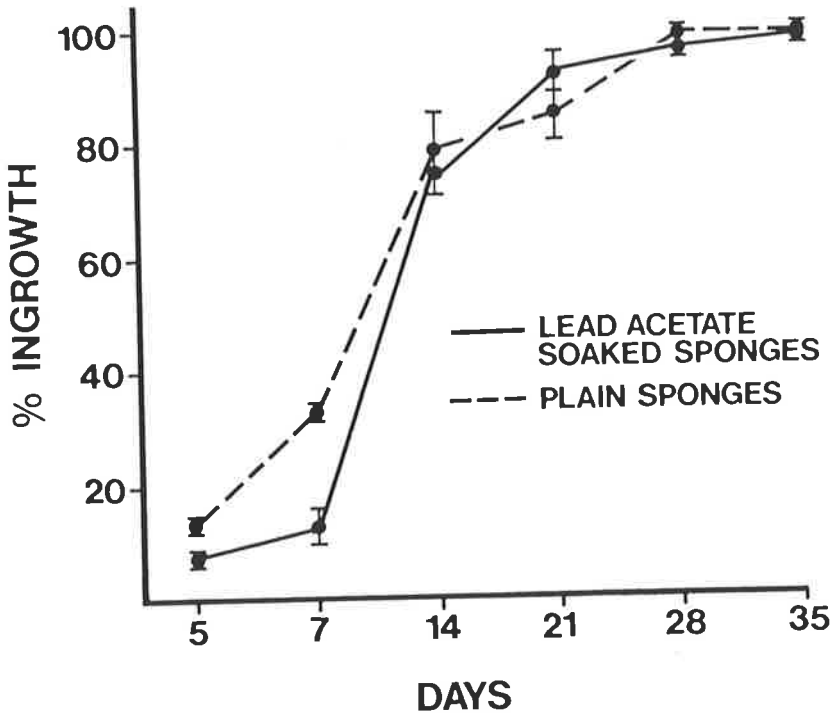


Figure 9b
Ingrowth of granulation tissue into plain sponges and sponges soaked in PbAc during 35 days after implantation.

The amount of granulation tissue occupying plain sponges increased by 68% between 5 and 14 days after implantation. After this time, the

rate of tissue ingrowth slowed 14% of the sponges by Day 28. Most sponges were fully occupied by granulation tissue at this time.

In PbAc soaked sponges, tissue ingrowth was significantly retarded relative to plain sponges at 5 and 7 days after implantation. At Day 7, this difference was 21%. By 14 days and at all times thereafter the amount of tissue ingrowth paralleled that occurring within plain sponges.

There was a statistically significant difference in tissue ingrowth between plain and PbAc soaked sponges at the 9 time periods studied ($p<.001$). There was an interaction between the ingrowth into plain and PbAc sponges and the time periods at which the ingrowth was measured ($p<.001$).

9c Total Sectional Areas of Plain and PbAc Soaked Sponges

(Table 9c and Figure 9c)

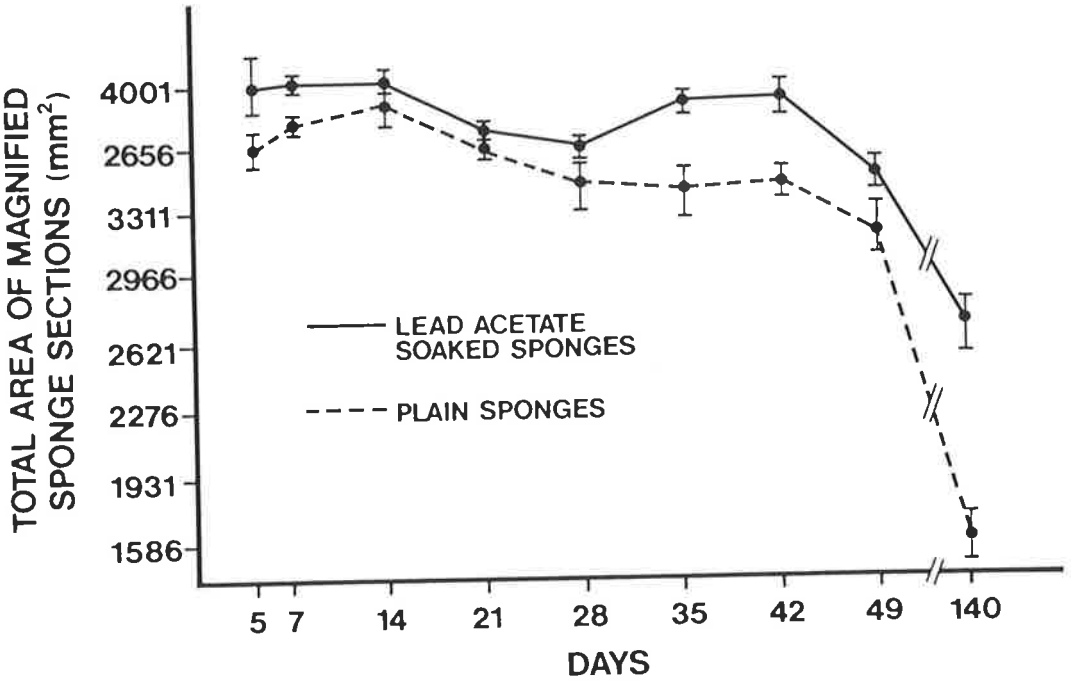


Figure 9c Changes in the areas of histological sections of plain sponges and sponges soaked in PbAc during the 140 day implantation period.

The total section area of plain sponges slowly increased after implantation and the value peaked on Day 14. The area then gradually declined so that at Day 49, the sponges were 17.5% smaller than at Day 14. Between Days 49 and 140 the sponges shrank and were 59% smaller than at Day 14.

PbAc soaked sponges were statistically significantly larger than plain sponges at all time periods studied ($p<.001$). No significant changes in area occurred until Day 21 when there was a slight decrease (6.5%). The sponges had regained their original (Day 5) areas by Day 42 after which the sponge areas decreased so that at Day 140 they were 33% smaller than at Day 5.

There was a statistically significant difference in total section areas between plain and PbAc soaked sponges at the 9 time periods studied ($p<.001$). There was an interaction between the areas of plain and PbAc soaked sponges and the time periods at which the areas were measured ($p<.001$).

9d Histology

At five hours , plain sponges were devoid of cells (fig.9.1a). PbAc sponges had dense deposits of VK positive material in close relationship to the sponge matrix (fig.9.1b). Closer examination revealed the presence of large crystals (approximately 50-60μ) in addition to rounded dense amorphous masses (fig.9.1c). A few PMN were scattered throughout the sponges. Alizarin red S staining was positive for calcium at this time.

At one day , the peripheral part of the plain sponges were infil-

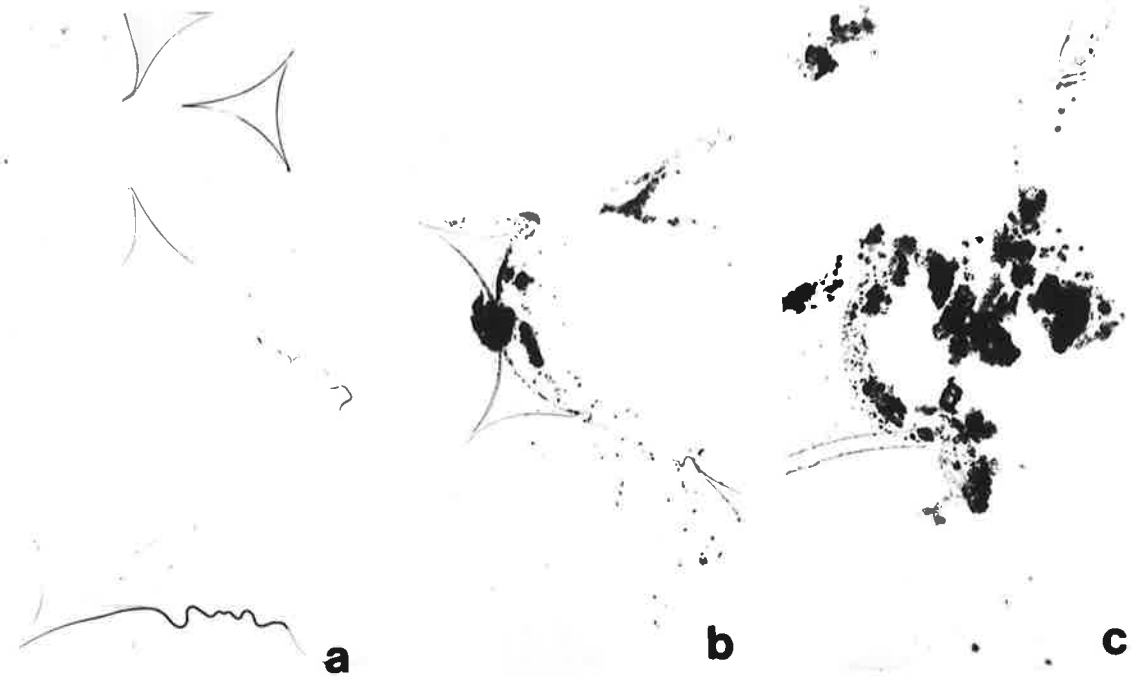


Fig.9.1 Sponges implanted for 5 hours.
a. Plain sponge, devoid of cells and exudate. HE VK x90
b. PbAc soaked sponge with dense deposits of VK positive material near sponge matrix. HE VK x90
c. Higher magnification of PbAc soaked sponge demonstrating the crystalline nature of the deposits. HE VK x175

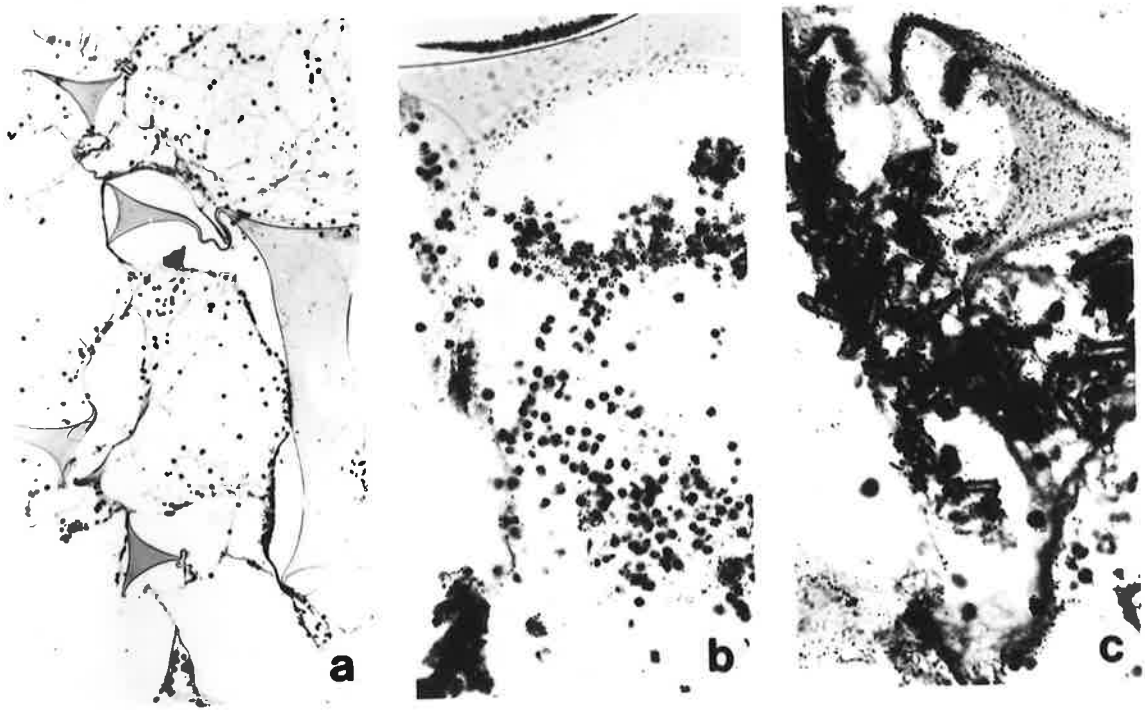


Fig.9.2
a. Plain sponge implanted for 1 day with light PMN infiltrate and proteinaceous exudate. HE VK x90
b. PbAc soaked sponge implanted for 1 day with PMN around calcergic deposits. Note granular material on sponge surface. HE VK x230
c. PbAc soaked sponge implanted for 5 days. Large elongated crystals have been deposited close to the sponge matrix in the central part of the sponges. HE VK x370

trated by PMN and a proteinaceous exudate (fig.9.2a). In PbAc sponges (fig.9.2b), foci of PMN were adjacent to the dense VK positive deposits found throughout the sponges.

At five days , plain sponges showed early granulation tissue ingrowth. Little tissue ingrowth was seen in PbAc sponges. Many large elongated crystals were seen throughout (fig.9.2c). The crystals were clumped in loose arrangements near the sponge matrix which was covered in a finely granular VK positive deposit. An extensive PMN infiltration and proteinaceous exudate was present in the sponges (fig.9.3a). At the junction of the sponge and surrounding connective tissue, dense focal deposits of VK positive material were orientated in strips parallel to the surface of the sponge (fig.9.3b). An intense macrophage reaction to the deposited material was present in the sponge capsule.

At seven days , granulation tissue ingrowth was not prominent in PbAc sponges. The PMN response had subsided. Dense VK positive deposits were scattered throughout the sponges (fig.9.3c) and calcergic material in the sponge capsule elicited an intense macrophage and MNGC response (fig.9.4a).

At 14 days , plain sponges were almost fully occupied by granulation tissue with the characteristics previously described. PbAc sponges showed scattered deposits of calcergic material (fig.9.4b) that attracted only a sparse macrophage reaction. The capsular macrophage and MNGC response continued.

At 21 days , collagen fibres became more prominent in the granulation tissue of plain sponges. In PbAc sponges, isolated calcergic

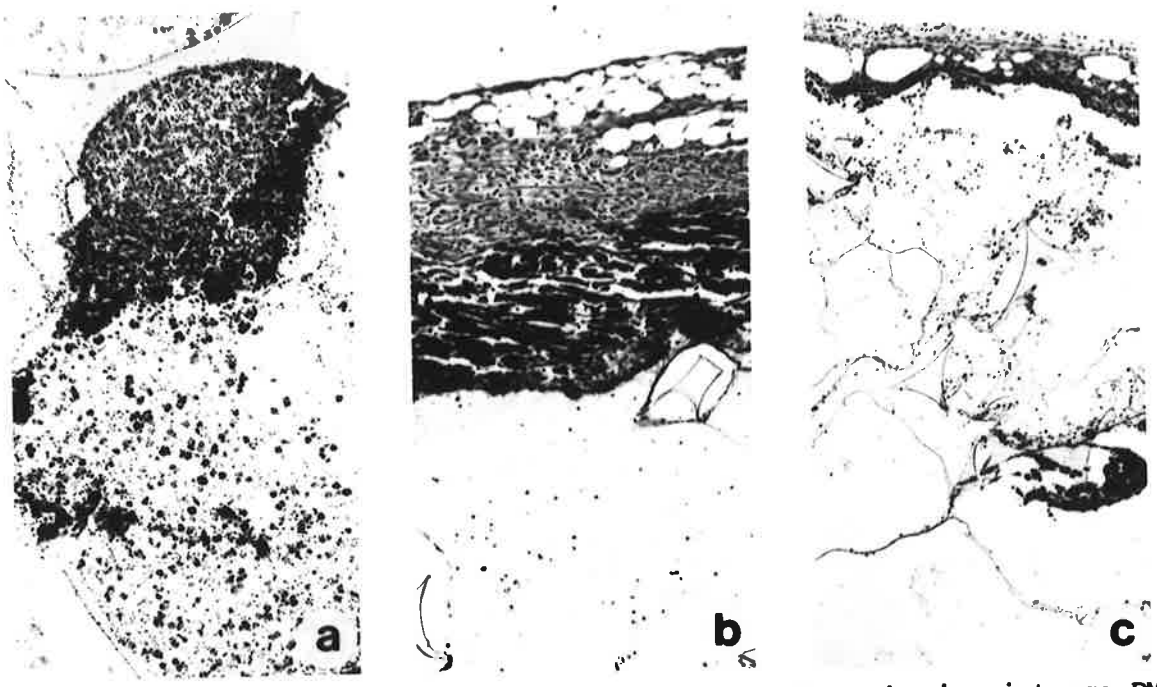


Fig.9.3 a. PbAc soaked sponge implanted for 5 days showing intense PMN infiltrate. Most PMN are necrotic. HE VK x175
b. Periphery of PbAc soaked sponge implanted for 5 days showing deposition of dense deposits of VK positive material in strips parallel to the sponge surface. An intense macrophage and MNGC response is seen in the sponge capsule. HE VK x65
c. PbAc soaked sponge implanted for 7 days. There is no evidence of granulation tissue ingrowth. HE VK x45

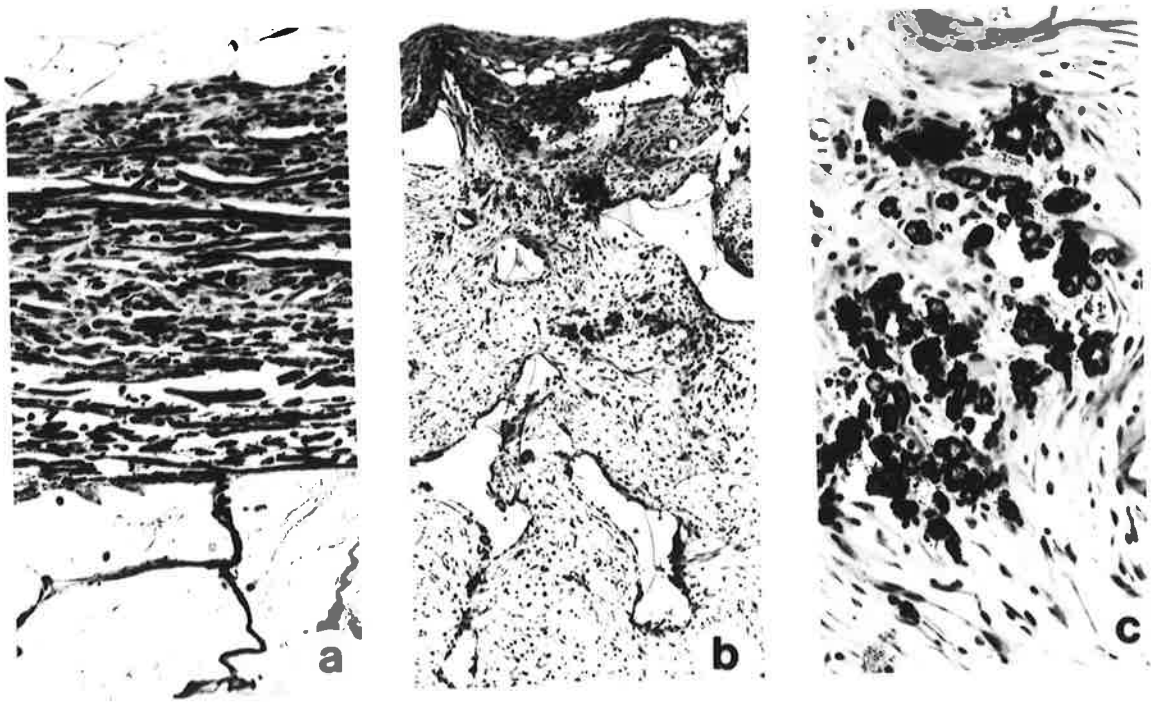


Fig.9.4 a. Periphery of PbAc soaked sponge implanted for 7 days. There is an intense macrophage and MNGC response to the calcergic material deposited in the sponge capsule. HE VK x200
b. PbAc soaked sponge implanted for 14 days showing scattered calcergic deposits in the granulation tissue. HE VK x45
c. PbAc soaked sponge implanted for 21 days. The crystalline nature of the calcergic deposits is evident and the cellular response to it is minimal. HE VK x175

deposits were predominantly crystalline (fig.9.4c) and had not elicited a prominent macrophage response. MNGC were rare.

At 28, 35 and 42 days , granulation tissue in plain sponges became progressively more collagenous with definite orientation of the collagen fibres. The macrophages and MNGC surrounding the sponge matrix became more prominent with increasing time of implantation. In PbAc sponges, focal calcergic deposits were surrounded by macrophages (fig. 9.5a) and the VK positive deposits were becoming more granular. A few MNGC were seen after 42 days (fig.9.5b). There was less fibrosis in PbAc sponges than in plain sponges implanted for the same time periods. The quantity of deposited material in the capsular tissues and the cellular response to it gradually decreased between 28 and 42 days after implantation.

At 49 days , plain sponges showed prominent collagenization of granulation tissue of decreased vascularity. Masses of macrophages and MNGC had formed around the sponge matrix (fig.9.5c). These cells had apparently caused splitting of the sponge material (fig.9.6a). PbAc sponges had persistent focal calcergic deposits (fig.9.6b) which were associated with an intense macrophage and sparse MNGC response (fig. 9.6c). The collagen fibres were not as definitively orientated as in plain sponges where they encircled the sponge matrix.

At 140 days , the histological appearance of plain sponges had changed dramatically. Extensive fatty infiltration had replaced up to approximately 40% of the sponges, leaving macrophages and MNGC surrounding the sponge matrix. The fatty infiltration occurred from the superficial aspect of the sponges which was adjacent to the subcutaneous tissues (fig.9.7a). The remaining granulation tissue in the

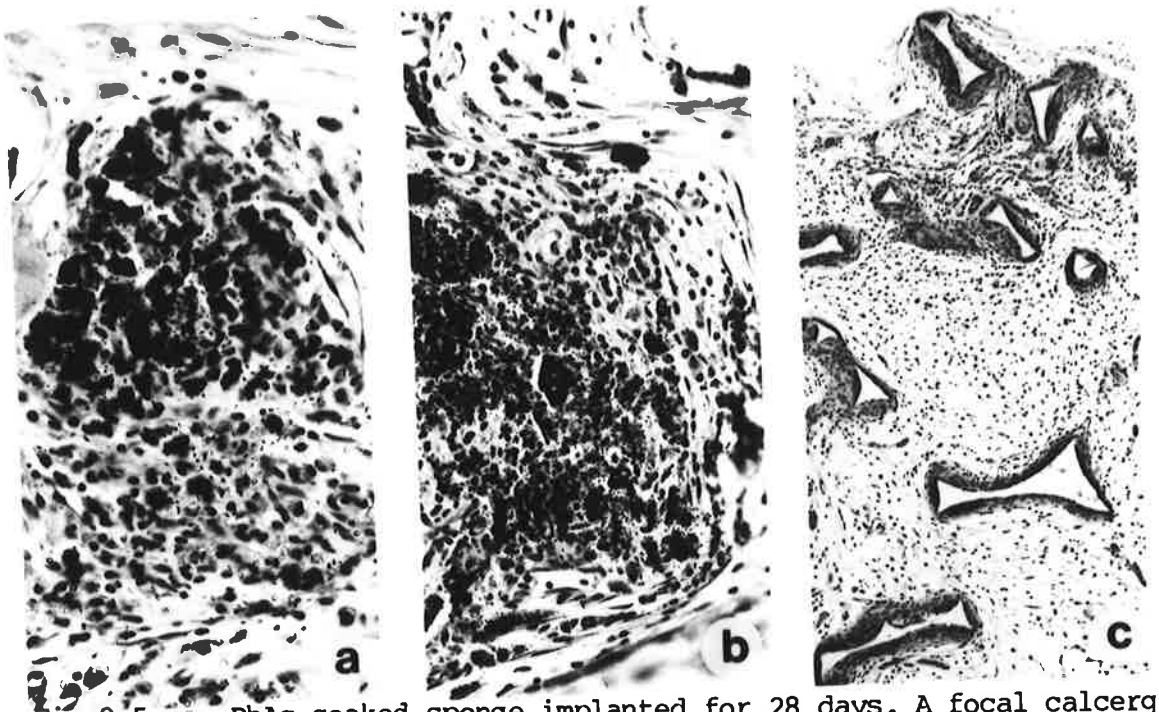


Fig.9.5 a. PbAc soaked sponge implanted for 28 days. A focal calcergic deposit is surrounded by macrophages. HE VK x230
b. PbAc soaked sponge implanted for 42 days. The calcergic material has been disaggregated and a few MNGC are seen in addition to macrophages. HE VK x145
c. Plain sponge implanted for 49 days showing prominent collagenization of granulation tissue and the formation of masses of MNGC around the sponge matrix. HE VK x45

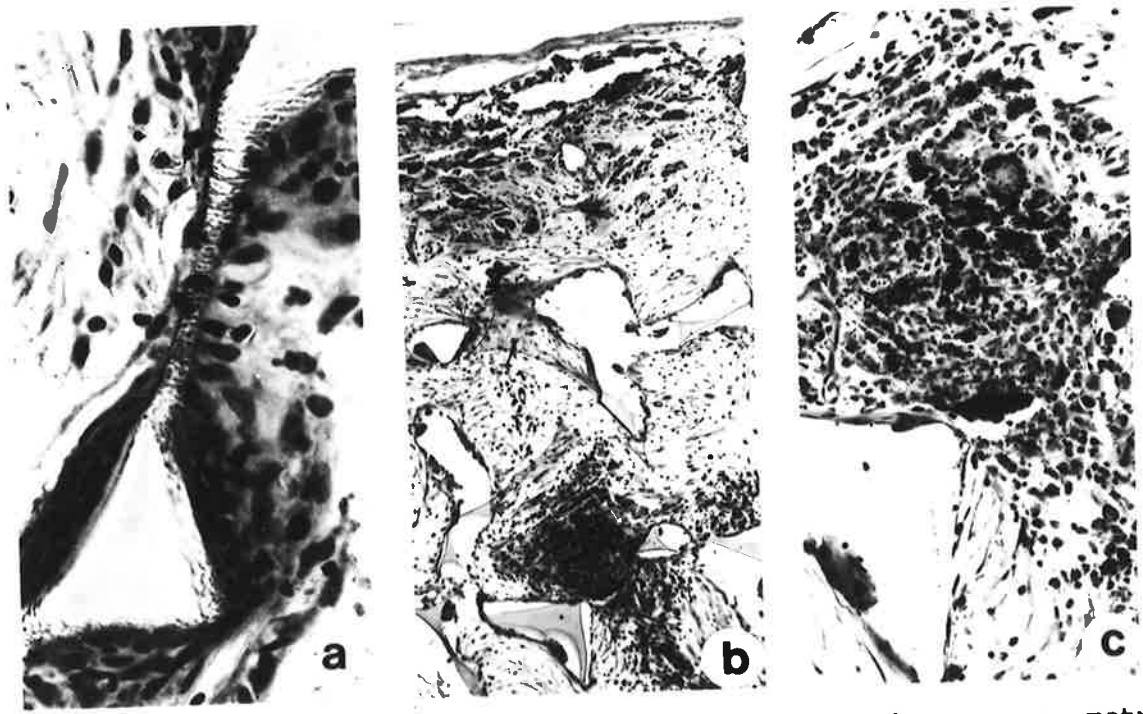


Fig.9.6 a. Higher magnification of MNGC response to the sponge matrix in Fig.9.5c. Splitting of the sponge matrix is evident. HE VK x370
b. PbAc soaked sponge implanted for 49 days with the persistence of focal calcergic deposits throughout. HE VK x45
c. Higher magnification of b. showing MNGC and macrophage response to the granular calcergic deposits. HE VK x145

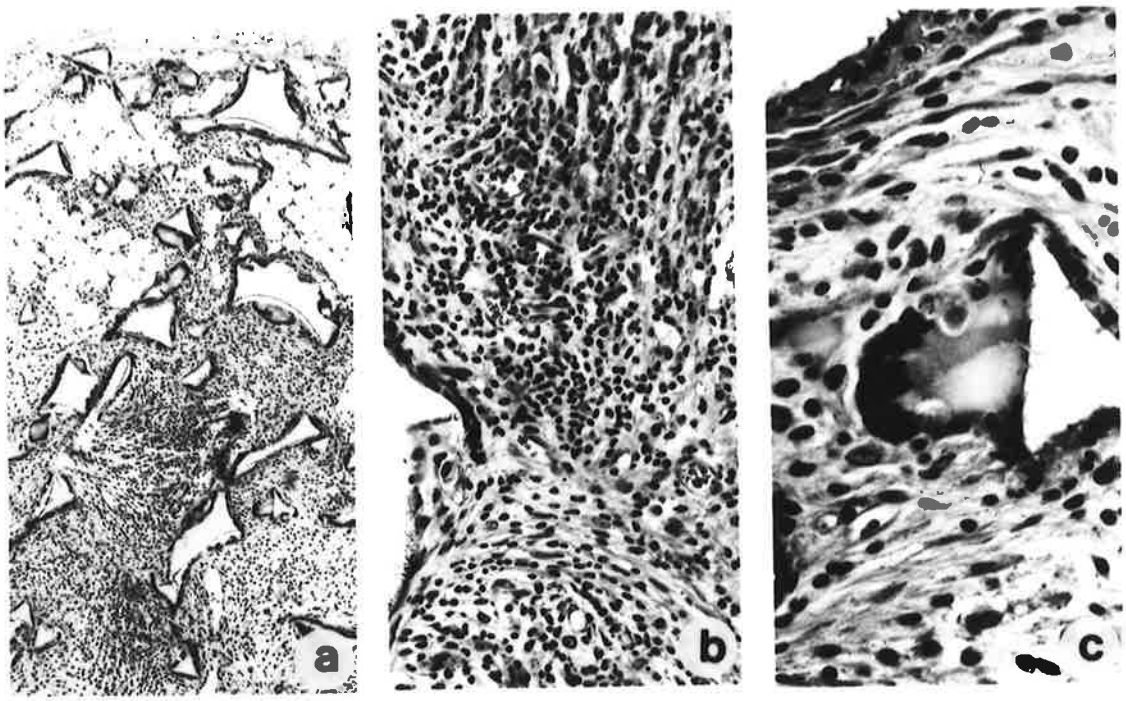


Fig.9.7 Plain sponge 140 days after implantation.
a. Extensive fatty infiltration extending from the periphery of the sponge. Islands of MNGC remain around the sponge matrix. HE x45
b. Dense macrophage and MNGC infiltrate. HE x175
c. MNGC with pale cytoplasmic inclusions attached to the split sponge matrix. HE x370

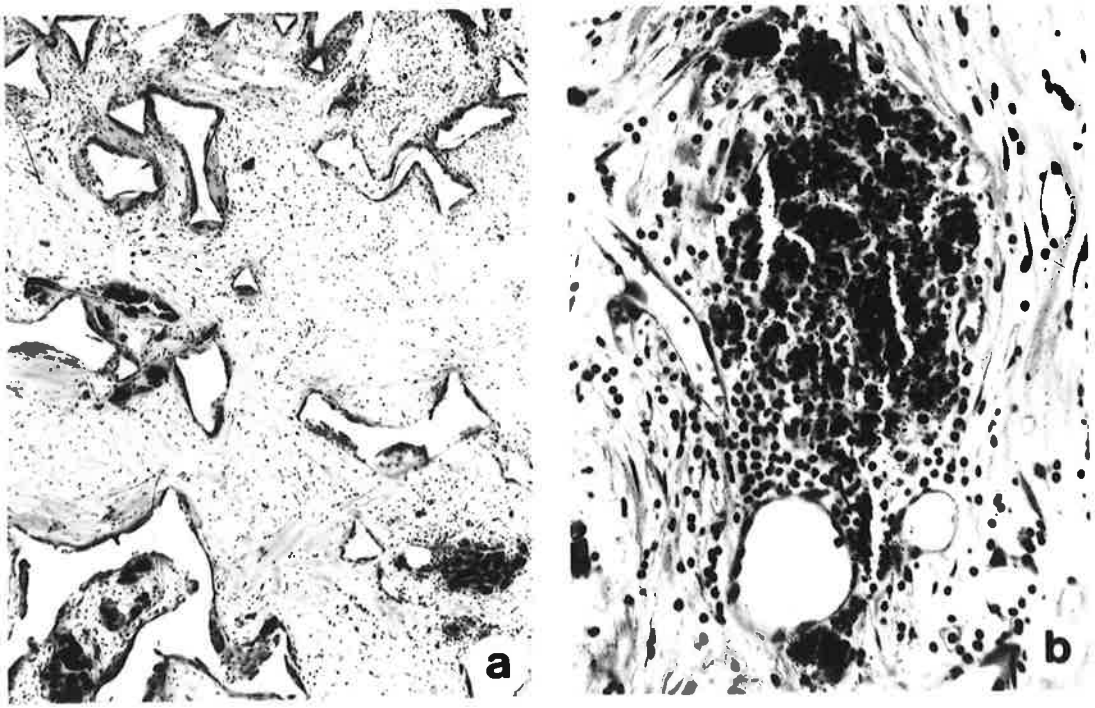


Fig.9.8 PbAc soaked sponge 140 days after implantation.
a. Prominent collagen bundles and sparsely cellular granulation tissue. Scattered calcergic deposits are present. HE VK x45
b. Macrophage and lymphocyte response to calcergic deposit. HE VK x230

sponges was densely infiltrated by lymphocytes and macrophages (fig. 9.7b). Capillaries were prominent but fibrocytic cells were scarce. MNGC were attached to the sponge matrix (fig.9.7c) which showed splitting and dissolution. Pale staining amorphous masses (presumably sponge or sponge breakdown products) were seen in the cytoplasm of the MNGC. Collagen fibres were not prominent.

PbAc sponges had less fatty infiltration than plain sponges. The granulation tissue was similar to that at 49 days after implantation of PbAc sponges. Collagen fibres and bundles were prominent (fig.9.8a) although definitive orientation of the collagen was not evident. Calcergic deposits persisted in the granulation tissue and at isolated areas of the capsule. The cellular response was minimal and consisted mainly of macrophages. A few lymphocytes were also scattered around the deposits (fig.9.8b). The macrophage and MNGC response to the sponge matrix was less intense than in plain sponges. There were more artefactual spaces around the sponge matrix in PbAc soaked sponges than around plain sponges at this time.

9e Transmission Electron Microscopy

At five hours after implantation, small dense rounded deposits were present on the surface of the sponge matrix (fig.9.9a) as well as in deposits of fibrous protein which was attached to and extended away from the sponge. Higher magnification (fig.9.9b) showed that the deposits were composed of aggregates of smaller particles. The fibrous nature of the protein deposit was more apparent at this magnification. The deposits consisted of lead and phosphate according to EDAX analysis.

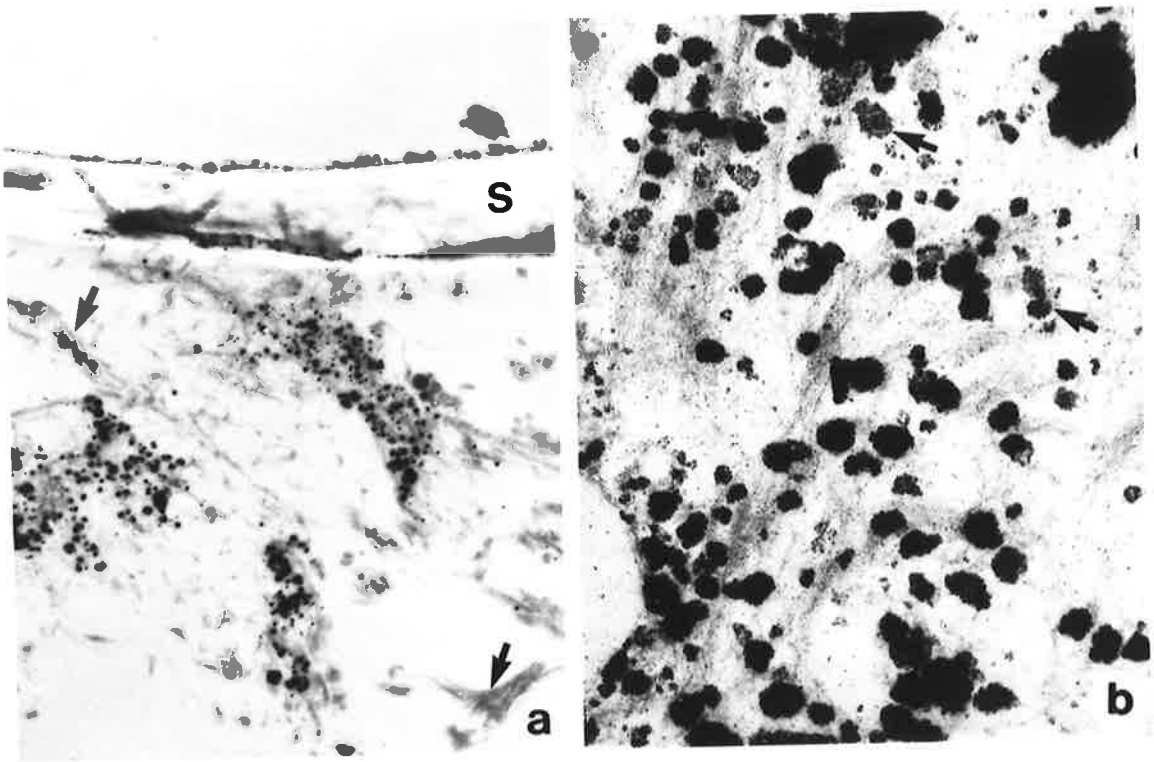


Fig.9.9
Electron micrographs of PbAc soaked sponge removed after 5 hours.
a. Dense rounded deposits on sponge matrix (S) and in the fibrous protein (arrows) extending from the sponge. x9,950
b. Higher magnification showing the distribution of the dense deposits amongst the fibrous protein. The deposits are composed of aggregates of smaller particles (arrows). x62,500

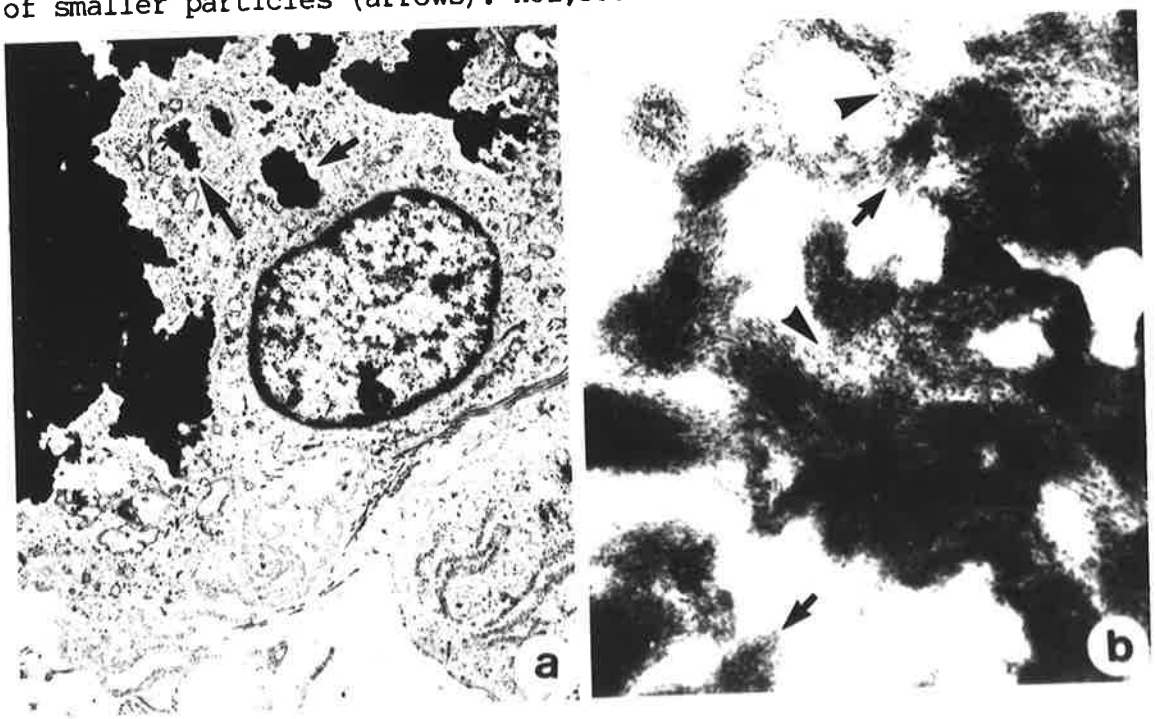


Fig.9.10
Electron micrographs of PbAc soaked sponges removed after 2 days.
a. Macrophage that has endocytosed irregularly shaped masses of dense material (arrows). A large calcergic deposit is present between the cells. x6,200
b. Higher magnification of calcergic deposit showing needle-shaped crystals in longitudinal section (arrows) and transverse section (arrowheads). x70,000

At two days uptake of conglomerates of dense material by macrophages had occurred (fig.9.10a). Large deposits were also present between the cells which appeared to be intact. At higher magnification (fig.9.10b), some of the material was composed of needle shaped crystals, seen in both longitudinal and transverse section. EDAX analysis confirmed the presence of calcium, phosphate and lead. The Ca:P ratio was slightly less than that of synthetic HAC.

At 14 days calcific deposits on collagen fibres were found in the sponge granulation tissue (fig.9.11a).

At 28 days macrophages that had endocytosed large quantities of dense rounded deposits (consisting predominantly of lead as determined by EDAX) showed degenerative changes including breakdown of the cell membrane (fig.9.11b) causing release of the calcergic deposits. Other aggregates of dense rounded structures had less electron dense, needle shaped outgrowths which had the morphological and EDAX characteristics of HAC.

At 140 days there was convincing evidence of cellular breakdown of the sponge matrix of plain sponges. Macrophages and MNGC had endocytosed pieces of sponge of varying shape and size (fig.9.12a). Large phagolysosomes containing smaller fragments of sponge were present in the cytoplasm (figs.9.12b and 9.13).

In PbAc soaked sponges masses of calcergic material with a fibrous pattern were found in the extracellular space (fig.9.14a) and was also deposited on collagen fibres, which were seen in transverse and longitudinal sections (fig.9.14b). The calcergic material was not uniformly dense.

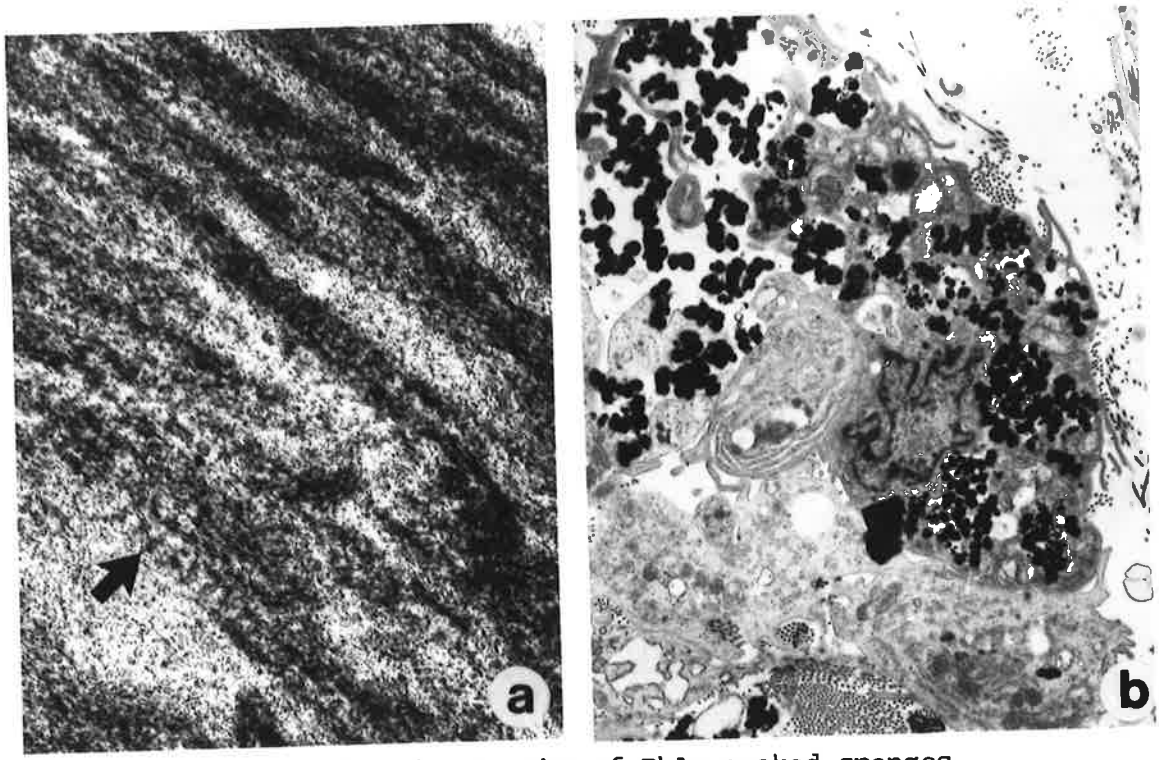


Fig.9.11 Electron micrographs of PbAc soaked sponges.
a. 14 days after implantation with calcific deposits covering remnants of collagen fibres (periodicity of banding at arrow = 70nm). x39,000
b. 28 days after implantation showing macrophages in a collagen matrix. The central cell has endocytosed many dense rounded structures and its organelles show degenerative changes. x6,000

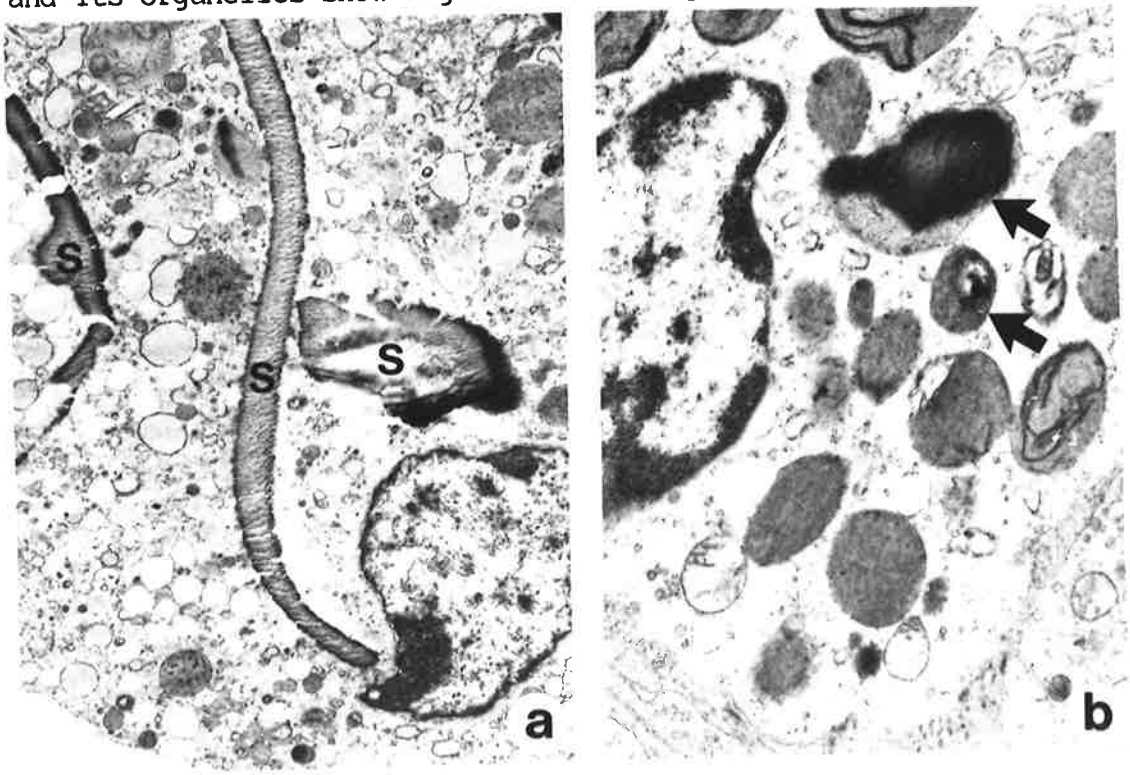


Fig.9.12 Electron micrographs of plain sponges implanted for 140 days.
a. Portion of a MNGC which has endocytosed pieces of sponge matrix (S). All of the organelles have a swollen appearance suggestive of necrotic changes. x6,125
b. Portion of a MNGC with phagosomes containing pieces of sponge matrix (arrows). Other phagosomes contain membraneous material that may be the remnants of sponge or cells. x17,700

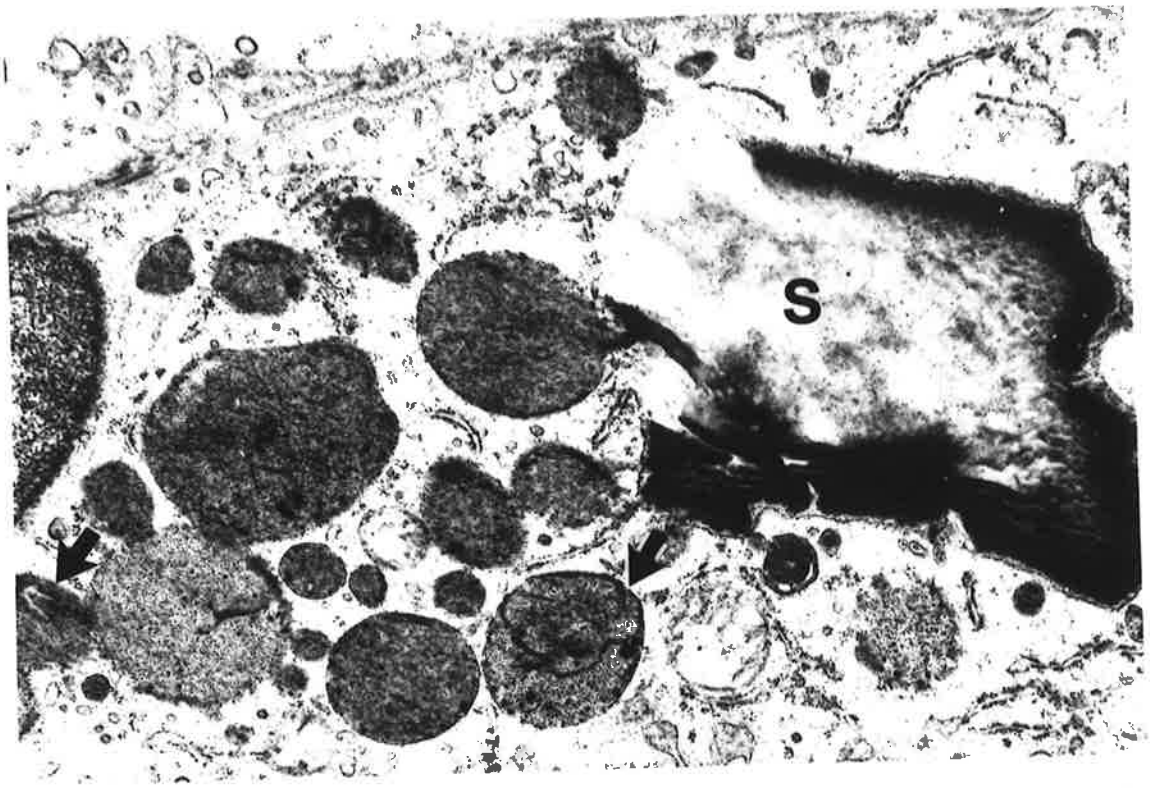


Fig.9.13 Electron micrograph of a plain sponge implanted for 140 days featuring a portion of a MNGC with a membrane bound piece of sponge (S) in the cytoplasm. Other membrane bound bodies (arrows) contained material similar to sponge matrix. x14,100

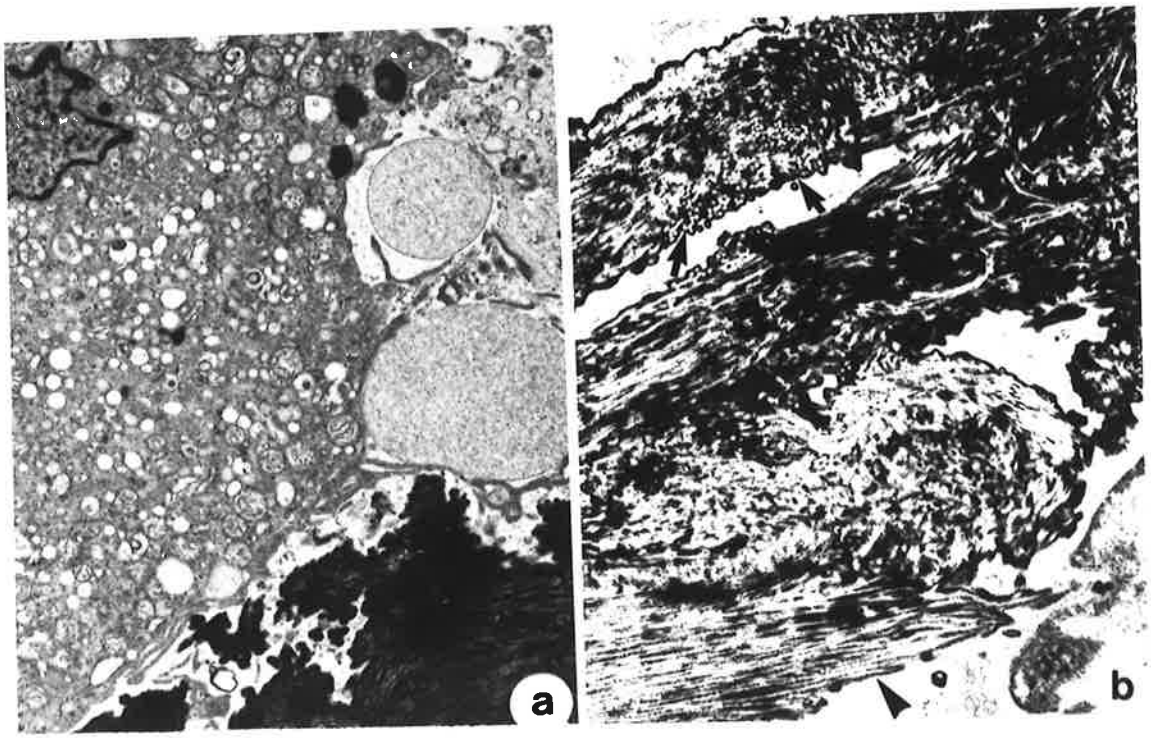


Fig. 9.14
Electron micrographs of PbAc soaked sponges implanted for 140 days.
a. Portion of a MNGC with an extracellular dense deposit which has a fibrous pattern. x5,500
b. Extensive deposits of calcergic material on collagen fibres seen in longitudinal section (arrowhead) and transverse section (arrows). x3,650

EXPERIMENT 10

Aim

To study the effect of intraarticular injection of hydroxyapatite and brushite into rat stifle (knee) joints by histological examination.

Method

Male Lewis rats weighing 280-340g were carefully shaved while under general anaesthesia so that the areas around both stifle joints were free of hair. The rats were shaved one day prior to intraarticular injection to circumvent any traumatic release of inflammatory mediators that could have complicated interpretation of the response to the crystals.

A sterile technique was used during the injection of the joints. Under general anaesthesia, the skin overlying the knees was swabbed with 70% alcohol. The material to be injected was aspirated into a tuberculin syringe fitted with a 27.5 gauge needle.

The patella was located by palpation and the knee joint slightly flexed by placing a finger under the joint. Entry of the needle into the intraarticular space was achieved by its insertion from a lateral aspect under the patella, the bevelled edge being guided by contact with the femur. Once inside the joint space, the required amount of crystal suspension was slowly injected. Swelling of the skin overlying the filling bursae indicated that the suspension was being injected into the joint space.

Experimental Design

Group	Material Injected (0.1ml/300g rat)		Time of Sacrifice (days after injection)
	Left Stifle	Right Stifle	
1	Saline	Small HAC (5mg/ml)	1
	"	"	4
	"	"	7
	"	"	14
2	Saline	Brushite (5mg/ml)	1
	"	"	4
	"	"	7
	"	"	14
3	Saline	PbAc (5mg/ml)	14
	"	"	28

The sterilized microcrystals were suspended in sterile saline. The suspensions were agitated prior to aspiration into the syringes and immediately prior to injection. The concentration of microcrystals used and the volume of suspensions to be injected were determined by reference to McCarty et al (1966) and Schumacher et al (1977). Other work being undertaken concurrently in these laboratories involved the injection of cobalt-chrome alloy wear particles from joint prostheses into rat stifle joints and a similar injection regime was used.

Rats were sacrificed by intracardiac injection of sodium pentothal while under general anaesthesia. A 27.5 gauge needle was inserted intraarticularly into each knee joint and joint fluid aspirated. Fluid was smeared on to an albuminized glass slide and placed in a Coplins jar containing a 1:1 mixture of ether and absolute alcochol and stained with HE.

The skin overlying the knee joints was dissected and the muscles trimmed. Bone forcepts were used to cut through the femur and tibia at points 2-3cm distal to the knee joints. The joints were then placed on

glass slides and held in an extended position by rubber bands around each end of the dissection. The tissues were fixed in 10% formal saline for 4 days. Undecalcified sections of the specimens were prepared as follows. Fixed knee joints were sectioned in a sagittal plane with a fine-bladed band saw. The sections were made approximately one third of the way through the joint to allow for the penetration of the processing and embedding fluids. The remaining two thirds of the joints were processed by being dehydrated through graded alcohols, cleared with acetone and placed in a 1:1 acetone : Araldite solution. Blocks were prepared by setting the joints in Araldite D expoxy solution in rubber moulds in an oven at 56°C. Sections were cut from the blocks at a thickness of 7μ using a Jung K microtome. Sections were mounted on gelatinised slides and dried in a hot oven overnight. Araldite was removed with an alcohol/potassium hydroxide solution.

Undecalcified sections were stained with HE/VK. The synovial fluid smears and undecalcified knee sections were examined by light microscopy.

Results

10a. Light Microscopy of Undecalcified Stifles

A majority of the sections cut were of little value for examination by light microscopy because of artefactual tearing of the soft tissues within and surrounding the joints.

The few intact sections of HAC injected joints showed that the HAC had been endocytosed by synovial cells, particularly those lining the bursal recesses of the joints.

Figure 10.1 shows the uptake of HAC by synovial cells 4 days after injection and considerable synovial cell hyperplasia. At 14 days, MNGC were prominent around the clumps of HAC that had been taken up by the synovial cells (figs.10.2 and 10.3). Figure 10.4 shows a MNGC with a Touton nuclear pattern in apposition to a clump of HAC. Synovial cell hyperplasia was a feature of the response to HAC injection after 14 days.

Only one block of PbAc injected stifle joints had not been subjected to artefactual tearing during processing. In this section (fig. 10.5) a few small rounded dense structures, presumably consisting of calcergic material were taken up by synovial cells in a bursal recess. Synovial cell hyperplasia was apparent around the calcified material.

There was no evidence of synovial hyperplasia in sections of joints injected with saline. None of the sections of joints injected with brushite were suitable for examination.

10b. Synovial Fluid Smears

Smears of synovial fluid taken 24h after injection of saline showed a sparse scattering of macrophages. Smears taken at subsequent intervals were negative for cells.

Macrophages were the predominant inflammatory cell in response to HAC injection at this time. A few PMN were also seen (fig.10.6a). The macrophage cytoplasm had a foamy appearance.

Four days after injection of HAC, the macrophage number had de-

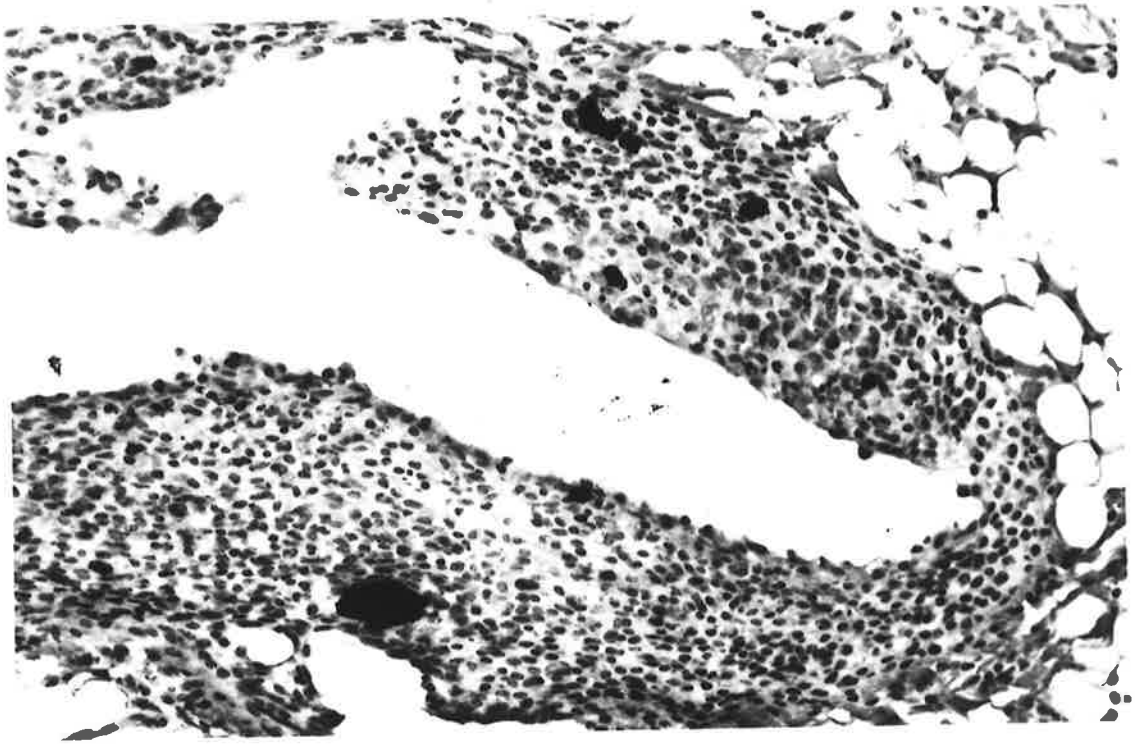


Fig.10.1 Deposits of HAC in the synovium of a rat stifle joint 4 days after intraarticular injection. Synovial cell hyperplasia is prominent. HE VK x200

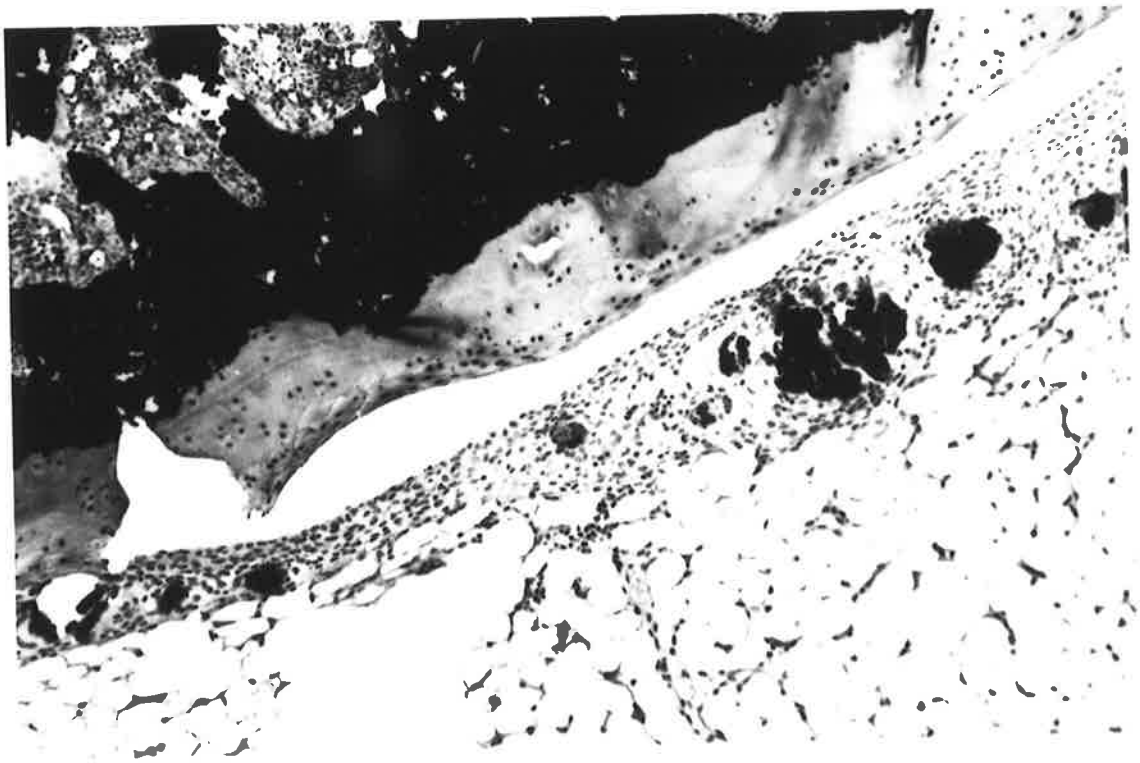


Fig.10.2 Deposits of HAC in the synovium of a rat stifle joint 14 days after intraarticular injection. Clumping of the HAC has occurred and many MNGC have formed around the clumps. HE VK x130

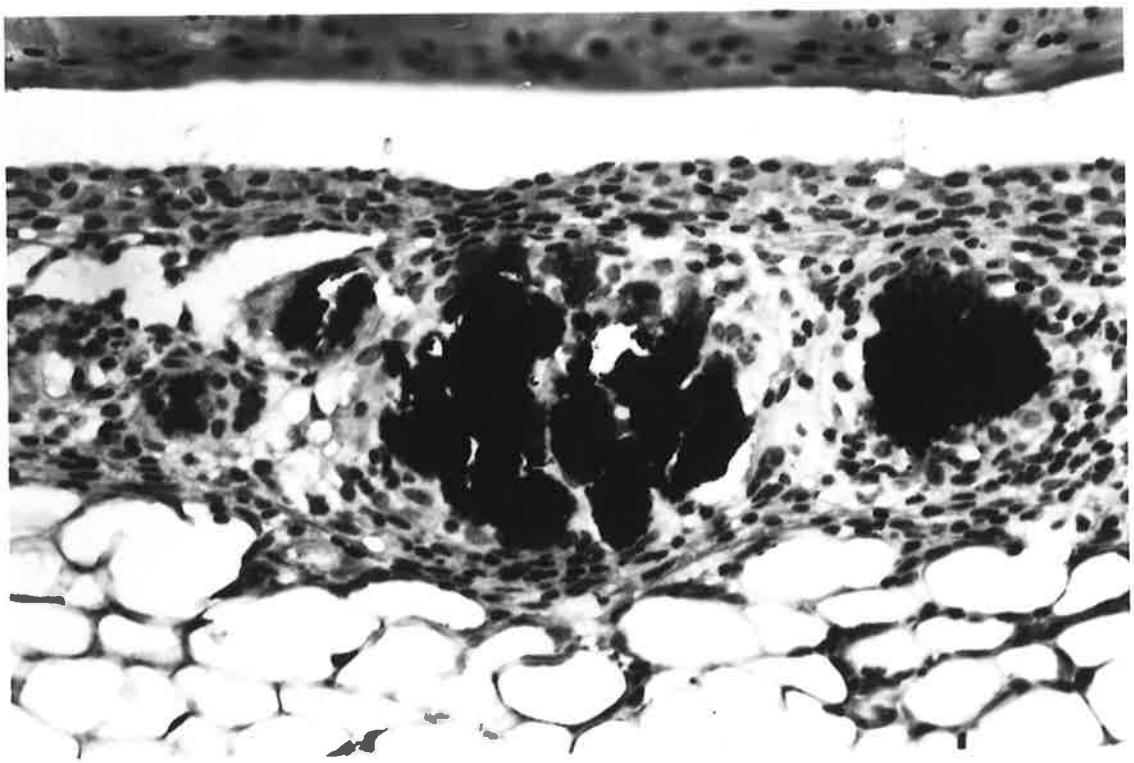


Fig.10.3 Higher magnification of fig.10.2 detailing macrophage and MNGC response to clumps of HAC in the synovium. HE VK x330

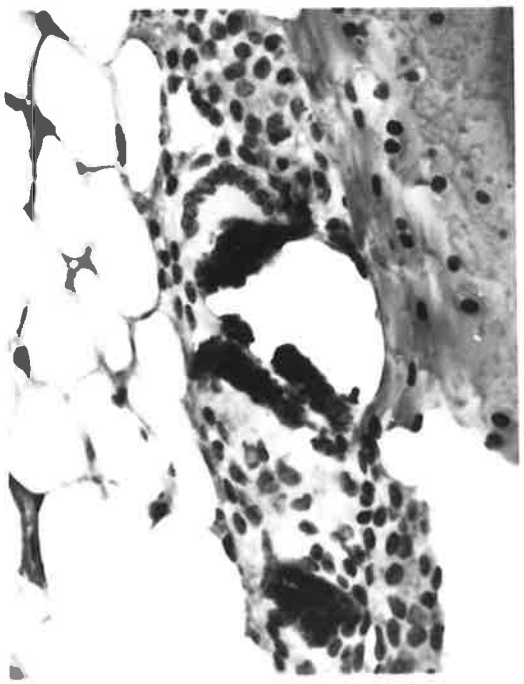


Fig.10.4 Higher magnification of fig.10.2 featuring a MNGC with a Touton nuclear pattern in apposition to a clump of HAC. HE VK x370

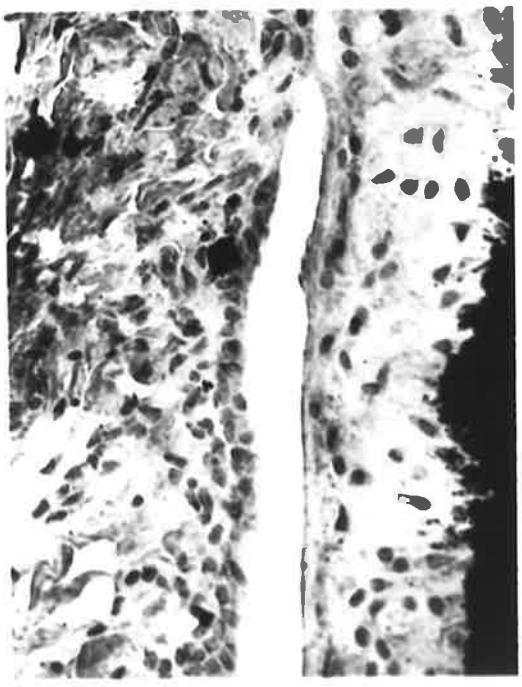


Fig.10.5 Calcergic deposits in the synovium of a rat stifle joint 28 days after intraarticular PbAc injection. Synovial thickening is evident around the deposits. HE VK x175

creased (fig.10.6b). The cells' cytoplasm continued to be prominent and foamy. Macrophage numbers were further decreased at 7 (fig.10.6c) and 14 (fig.10.6d) days after injection. The cytoplasm was less prominent at 14 days than in earlier smears.

Brushite crystals elicited a substantial proteinaceous fluid exudate and a dense macrophage response with a scattering of PMN (fig. 10.7a) 24h after injection. A smear taken after 14 days (fig.10.7b) showed a few scattered macrophages with a background of necrotic cell debris. Seven days after PbAC injection, synovial fluid smears were characterized by a dense proteinaceous exudate and the presence of a moderate number of macrophages and lymphocytes (fig.10.7c). By 14 days, the majority of cells in the smears were macrophages (fig.10.7d) and a protein rich exudate was not seen.

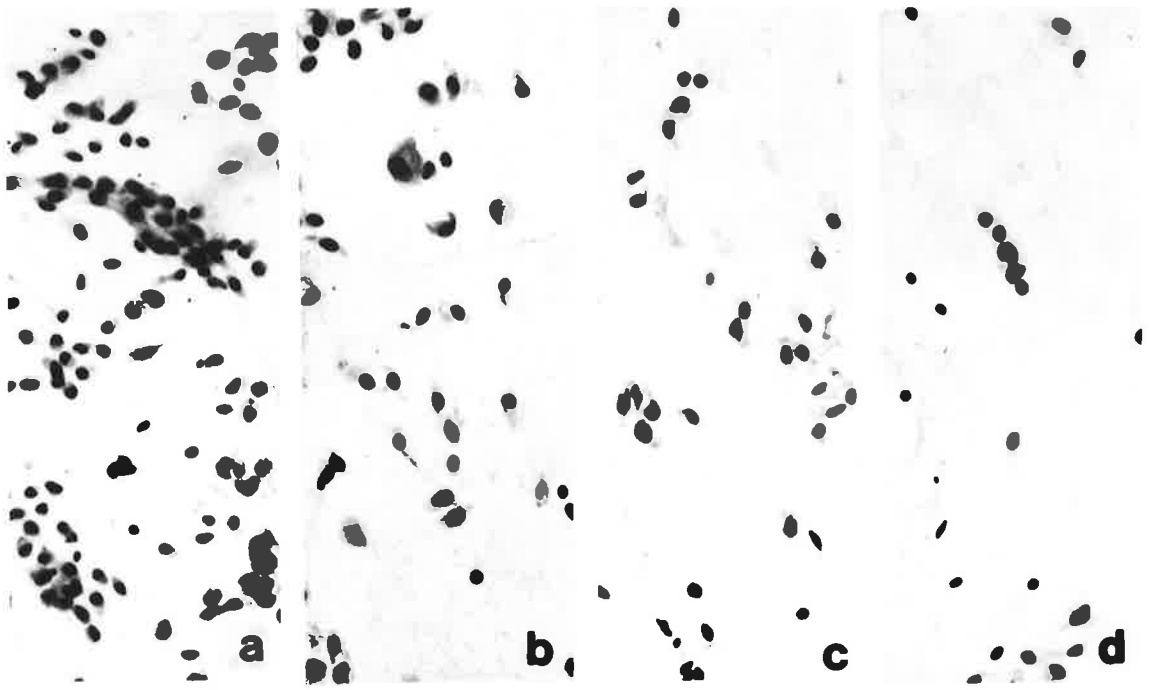


Fig.10.6
Synovial fluid smears from rat stifle joints injected with small HAC.
a. 24 hours after injection.
b. 4 days after injection.
c. 7 days after injection.
d. 14 days after injection. All HE x290

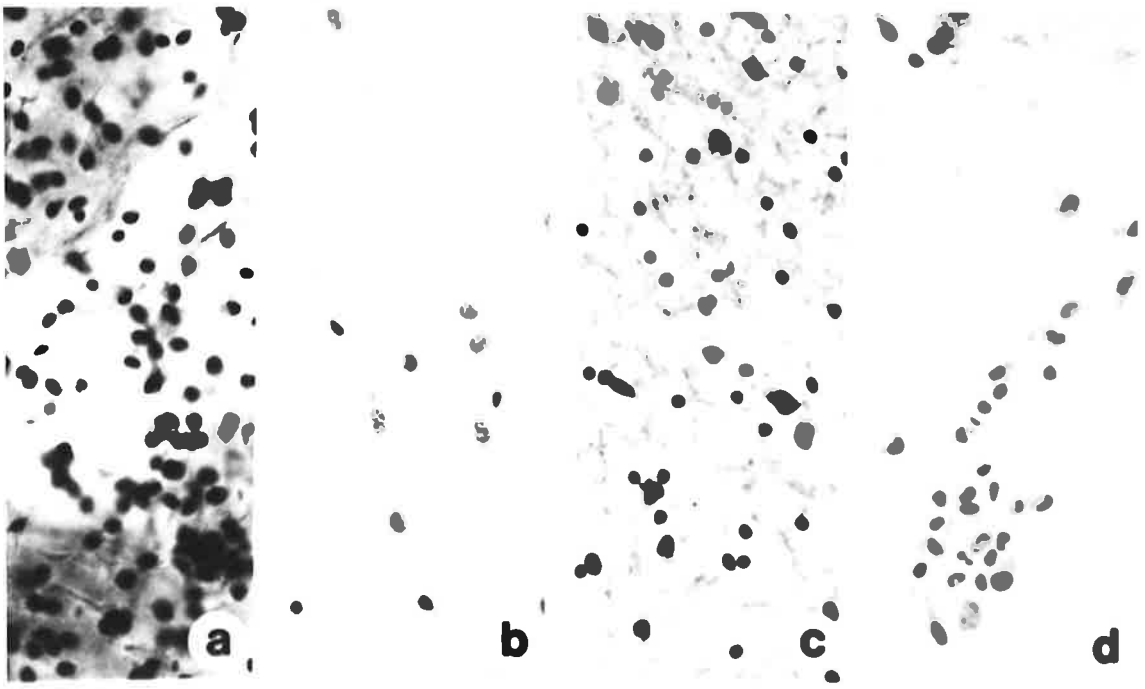


Fig.10.7 Synovial fluid smears from injected rat stifle joints.
a. 24 hours after brushite injection
b. 14 days after brushite injection
c. 7 days after PbAc injection
d. 14 days after PbAc injection. All HE x290

EXPERIMENT 11

Aim

To study the *in vitro* interaction of macrophages with HAC and other microcrystals.

Method

Male C57 mice of average age 12 weeks were the source of macrophages in this experiment. One day prior to harvesting the cells, 1ml of sterile protease peptone, 10% w/v (Difco Detroit, Michigan, USA). was injected into the peritoneal cavity of each mouse. The mice were sacrificed with an overdose of ether and the macrophages were harvested using the method described by Meltzer (1981).

Using a sterile technique, a thin strip of skin 1cm long was cut in the midline of the ventral surface overlying the peritoneal cavity. Care was taken not to pierce the thin mesenteric membrane. The peritoneum was lavaged by insertion of a 23 gauge needle (bevel upwards) and the rapid expression of 2ml of sterile Dulbecco's salt solution. The shaft of the needle was elevated to create a small tent just below the xyphoid process and the animal's flanks were gently massaged for 30 seconds. The fluid contents of the peritoneum were withdrawn into the same syringe. Usually 1-1.5ml of fluid was collected by this method. Fluids contaminated by red blood cells were discarded. The peritoneal washouts were pooled in plastic cenrifuge tubes placed in ice and centrifuged at 800rpm for 20 minutes at $4^{o}C$.

The supernate was discarded and the pellet of cells resuspended in RPMI-1640 tissue cuture medium (Flow Labs. Rockville, Md. USA) suppl-

emented by 10% heat-inactivated fetal calf serum (CSL,Melbourne), L-glutamine (30mg/100ml), penicillin (100 units) and streptomycin 100mg/100ml (hereafter called supplemented RPMI). For each mouse, 1ml of supplemented RPMI was added. A haemacytometer was used to estimate the number of cells/ml and extra medium was added to bring the final count of cells to $2x10^6$/ml. To each 16mm well of a Costar tissue culture plate (Cambridge,Mass. USA), 1ml of the cell suspension was added. In another culture plate, sterile millipore filters (Type HA, 13mm diameter, pore size 0.45μ) obtained from the Millipore Corporation (Bedford,Mass. USA) had been placed in each well. The filters were soaked for 10 minutes in supplemented RPMI prior to the addition of the cell suspension.

The plates were incubated in a humidified atmosphere of 5% carbon dioxide and air at 37°C for one hour. After this time, non-adherent cells were removed by vigorously washing each cell four times with Dulbecco's salt solution. The remaining cells were cultured in supplemented RPMI. These cultures gave a sheet of well spread cells after incubation for 24h. Approximately 60-70% of the number of cells originally plated remained and about 95% were macrophages as determined by light microscopy and nonspecific esterase staining using the method of Koski et al (1976). The culture medium was removed, replaced by fresh medium and the followng microcrystals added:

a) Small HAC aggregates were prepared by sedimentation of synthetic HAC (Sigma) in PBS containing 5% fetal calf serum (CSL). The sedimentation and recovery technique was otherwise identical to that described in Experiment 4.

b) Large HAC aggregates were prepared from the sediment remaining after overnight settling of synthetic HAC as above.

c) CPPD crystals were those used in Experiment 7.

d) MSU crystals were those used in Experiment 7.

e) Calcium carbonate crystals were used as supplied by the manufacturer (Analar).

Suspension of the microcrystals (other than MSU) were made in Dulbecco's salt solution to a concentration of 4mg/ml. The suspensions were autoclaved and then diluted by adding 1ml to 9ml of supplemented RPMI. Suspension was maintained by standing the container in an ultrasonic water bath. A micropipette was used to add 300µl of the diluted microcrystal suspension to each culture well. Approximately 120µg of each suspension was added to each well, similar to the amount of bone particles added by Teitelbaum et al (1979) to their macrophage cultures in wells of the same dimension. The microcrystals carpeted the bottom of the wells.

MSU crystals (120µg/well) were added directly from their storage medium (urate buffer containing 10mg MSU/ml) to the wells in order to avoid their aqueous dissolution.

After addition of the microcrystals, the tissue culture plates were incubated in a humidified atmosphere of 5% carbon dioxide and air at 37°C. The culture medium was carefully aspirated from each well and replenished by fresh supplemented medium every second day.

Duplicate cultures of cells with and without added microcrystals were maintained and examined by phase contrast light microscopy at 4, 20, 24 and 50 hours, 7, 9 and 14 days after addition of the crystals. Photomicrographs were taken at these time intervals using Kodak Technical Pan film.

Cultures of cells on millipore filters were used for TEM and SEM

studies. For TEM, one millipore filter per cell culture was assigned for the examination of small and large HAC-cell interactions in addition to control cell cultures at 4, 10, 60 minutes and 24, and 48 hours after microcrystal addition. MSU and CPPD were examined by TEM at 60 minutes after addition to the monolayer. The filters were fixed and processed as for sponge samples (Experiment 1) and then flat embedded in 2mm of resin. After curing, the resin discs containing the filters were sawn into cubes and glued edgewise on to blank resin stubs with Araldite. This procedure ensured that cross sections of the cells and filters were obtained. The resin blocks were cut and sections were stained as previously described.

For SEM examination, one millipore filter per cell culture was assigned for to study small HAC, MSU, and control cells 24 hours after addition of the crystals. Cell cultures grown on cellulose acetate filters were fixed and dehydrated as for TEM specimens. The specimens were critically point dried using liquid carbon dioxide as the transitional fluid, mounted on copper stubs with silver dag adhesive and carbon and gold coated.

Results

11a. Light Microscopy

11a.1 Control Cell Culture

At the time of addition of microcrystals, the macrophages were present as a dense monolayer (fig.11.1a). Some cells had a rounded appearance, others were elongated.

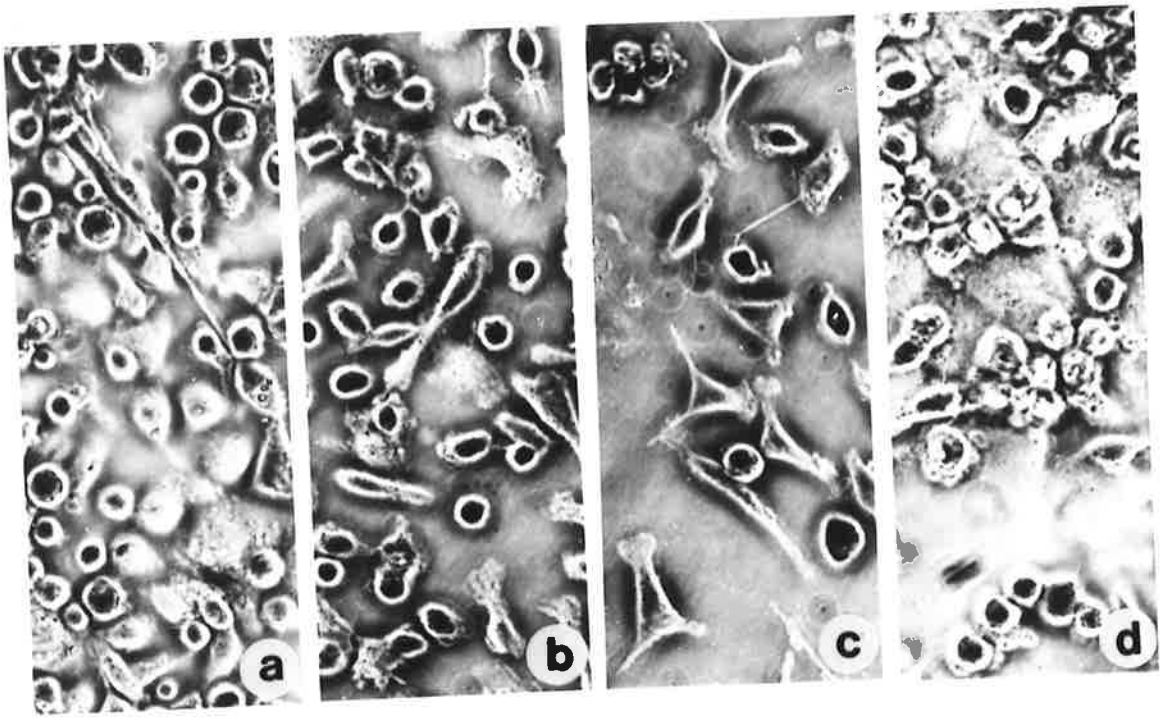


Fig.11.1
Light microscopic appearance of control murine macrophage cultures.
a. at the time of crystal addition to test cultures
b. after 24 hours
c. after 7 days
d. after 14 days.
All phase contrast x380

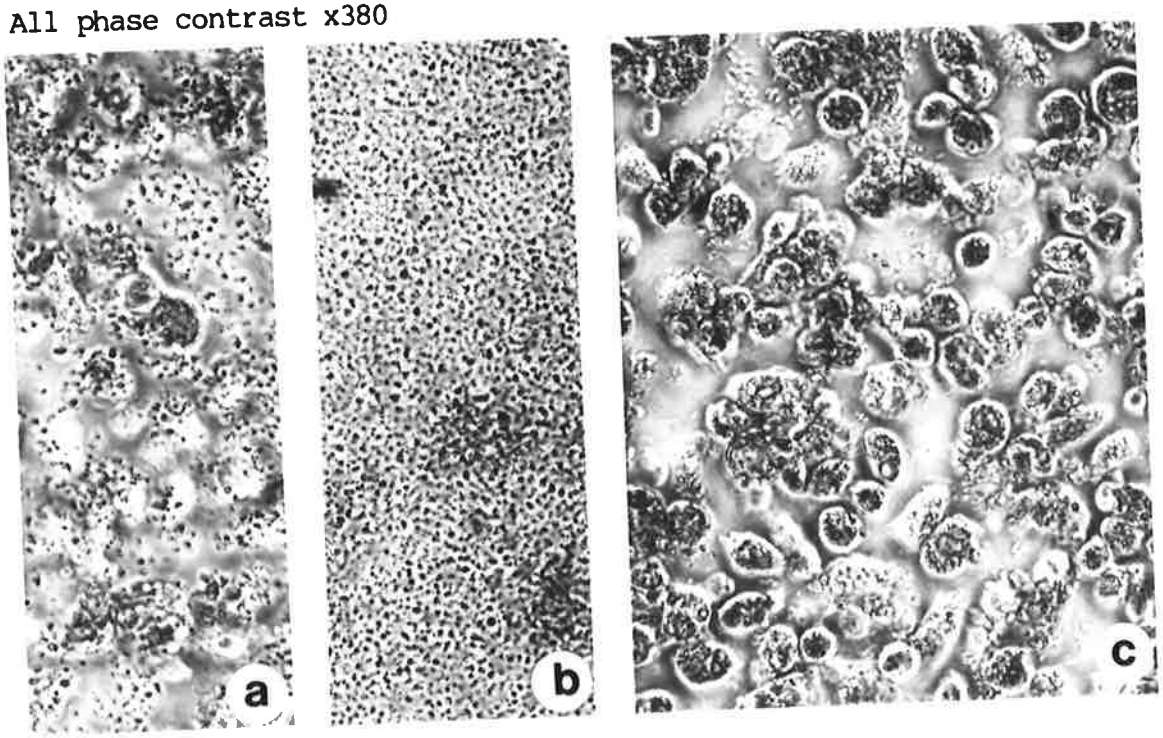


Fig.11.2
Light microscopic appearance of small HAC added to macrophage cultures.
a. several minutes after addition
b. small HAC particles only
c. after 4 hours the cells have become rounded.
All phase contrast x380

After 24h, control macrophages were less numerous and those remaining were well spread (fig.11.1b). The culture had a similar appearance after 50h. After 5 days, the density of cells was further decreased when compared to the cell density at 50h. However, the macrophages were well spread with elongated nuclei and cytoplasm. After 7 days, a further reduction in cell number had occurred and some stellate cells were present (fig.11.1c).

After 9 days, proliferative activity was apparent. Some of the cells were atypical of macrophages. They had extensive cytoplasmic processes filled with small vacuoles. These cells had the morphological features of fibroblasts.

The proliferation of cells had become more evident after 14 days when most of the cell culture consisted of proliferating cells (fig.11.1d).

11a.2 Small HAC Aggregates

Several minutes after addition of small HAC, there was evidence of cellular uptake of the particles (fig.11.2a), seen as small clear zones around the cells. This effect was not seen in culture wells to which small HAC alone had been added (fig.11.2b).

After 4h, most of the small HAC particles had been taken up by the macrophages, which had become rounded (fig.11.2c) and their cytoplasm filled with particles.

After 24h, the cells had become less numerous. Those remaining were engorged with particulate material (fig.11.2d) and it appeared as

though the cells were aggregating in clusters. After 50 hours, ballooning of some cells was seen and all cells contained intracytoplasmic particulate material. Clustering of cells was also a feature (fig.11.2e).

After 5 days, some cells had become more elongated; cell clustering persisted and particulate material was still seen in the cell cytoplasm. After 7 days, cells were clustered around concentrations of particulate material (fig.11.2f). Cells distant to the clusters did not appear to contain particulate material. After 9 days, the cell culture underwent a proliferative phase with many spindle-shaped cells devoid of intracellular particulates. Occasional focal deposits of crystalline material were present and were surrounded by clusters of cells. After 14 days (fig.11.2g) a similar situation was observed, with intense clusters of about 5-10 cells around focal deposits of granular material (presumably remnants of the small HAC particles).

11a.3 Large HAC Aggregates

Aggregates of HAC were concentrated near the cells a few minutes after their addition to the monolayer (fig.11.3b). After 4h this effect had become more pronounced (fig.11.3c) with clustering of both large HAC and cells, some of which had undergone ballooning of the cytoplasm. By 24h (fig.11.3d), this effect was more apparent and further clumping of the HAC had occurred.

After 50h (fig.11.3e) HAC aggregates surrounded by clusters of cells, some with cytoplasmic ballooning, were prominent. Five days from the start of the experiment, cell numbers had decreased and a

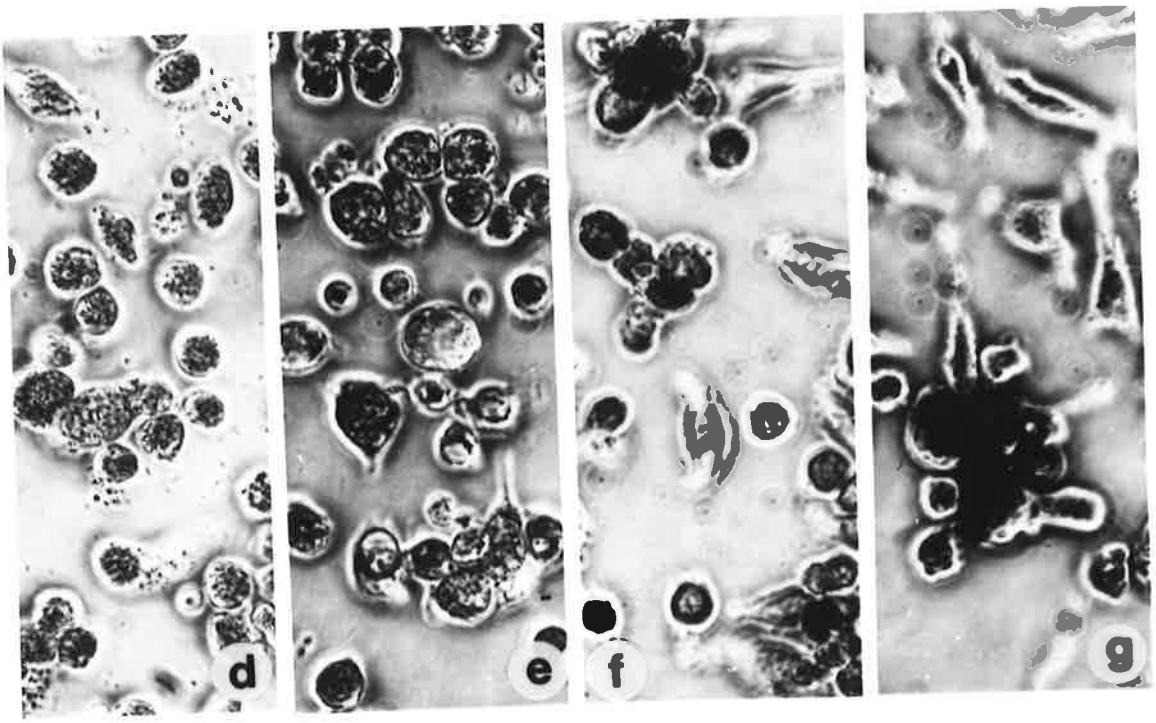


Fig.11.2 (cont.)
d. after 24 hours, the macrophages are engorged with small HAC
e. after 50 hours, clusters of cells and cytoplasmic ballooning are evident
f. after 7 days, cell clusters around clumps of HAC
g. after 14 days.
All phase contrast x380

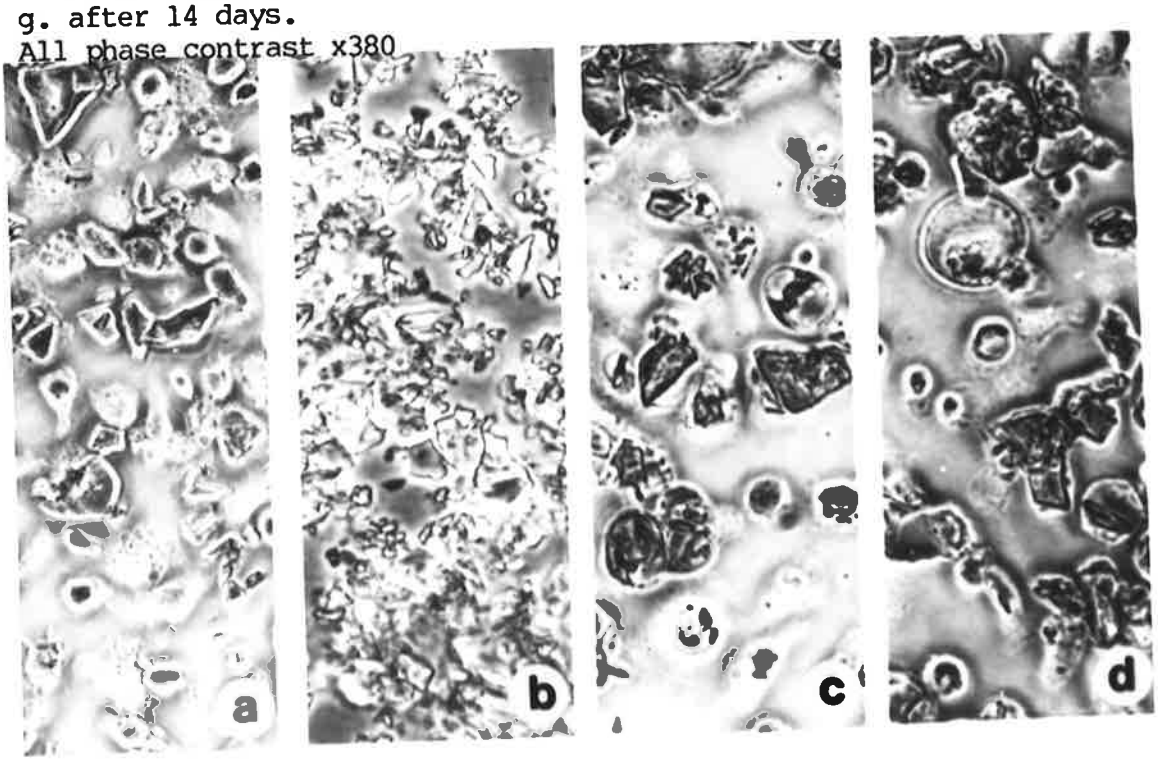


Fig.11.3
Light microscopic appearance of large HAC added to macrophage cultures.
a. several minutes after addition
b. large HAC particles only
c. after 4 hours, note cytoplasmic ballooning
d. after 24 hours with clumping of HAC and cytoplasmic ballooning.
All phase contrast x380

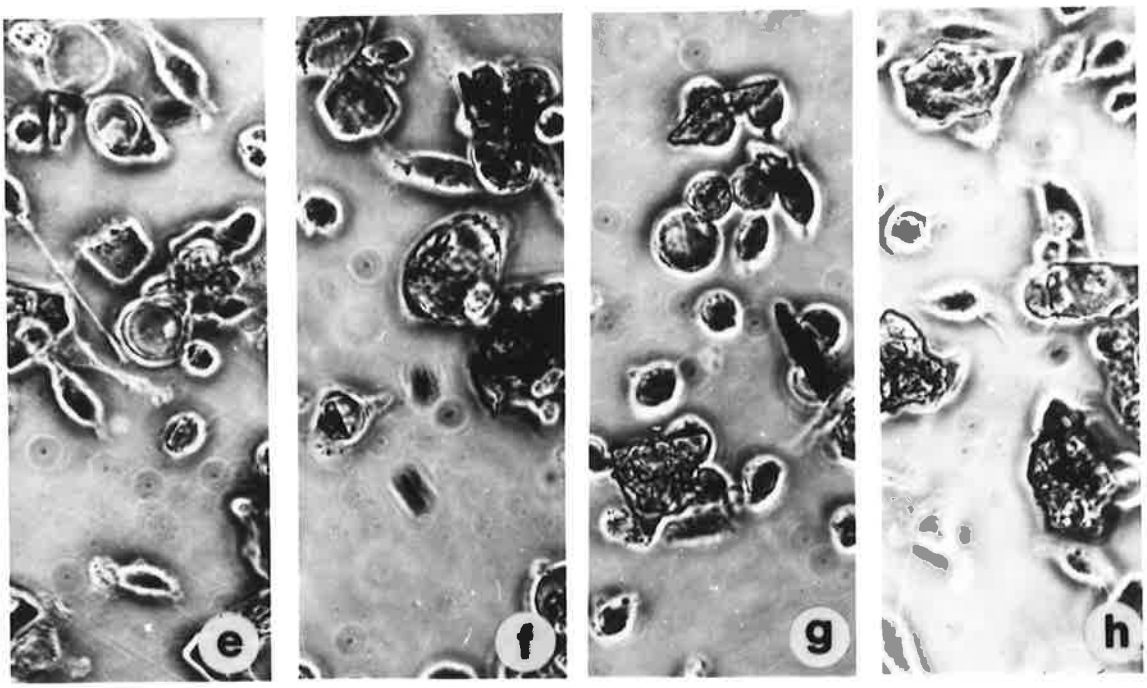


Fig.11.3 (cont.)
e. after 50 hours, cytoplasmic ballooning is prominent
f. after 5 days, reduced cell numbers and clustering of cells
g. after 7 days
h. after 14 days, cells becoming less rounded.
All phase contrast x380

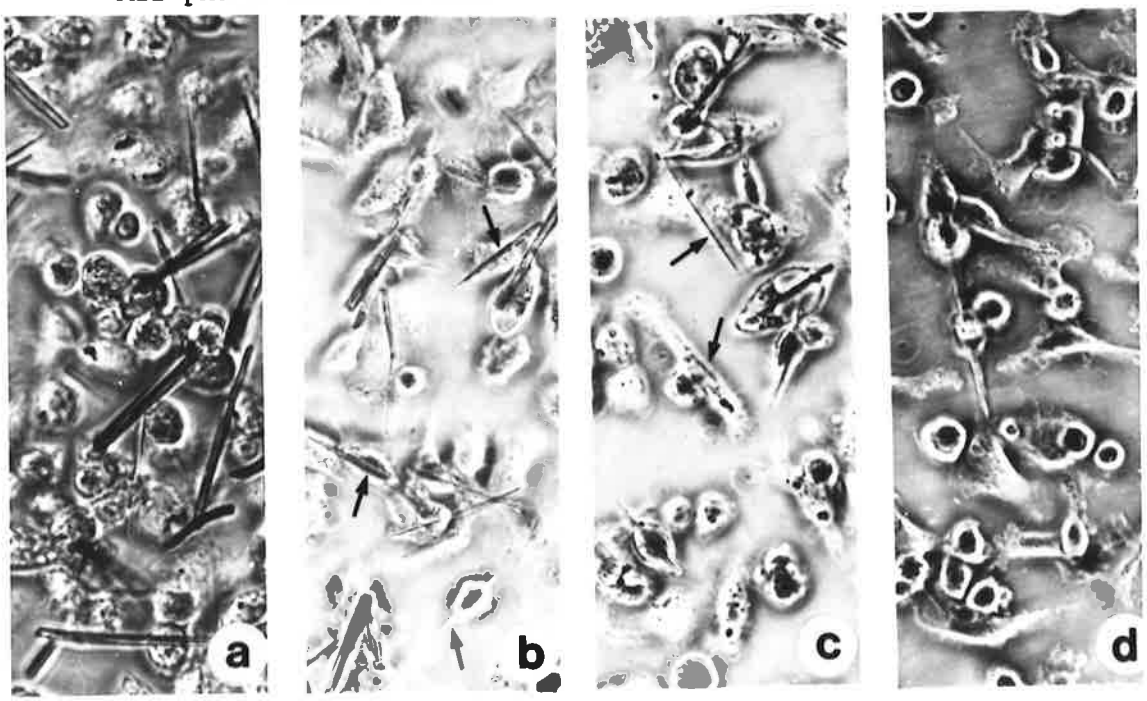


Fig.11.4
Light microscopic appearance of MSU added to macrophage cultures
a. several minutes after addition
b. after 4 hours, endocytosis is evident (arrows)
c. after 24 hours, endocytosis of all crystals has occurred.
Note elongated cytoplasmic extensions (arrows)
d. after 50 hours, few crystals are evident within the cells.
All phase contrast x380

consistent feature was aggregation of the particles around which cells were clustered (fig.11.3f). After 7 days (fig.11.3g), the cellular reaction was similar as that observed at 5 days, although there were less cells with cytoplasmic ballooning. A proliferative phase was apparent after 14 days (fig.11.3h). The cells were less rounded.

11a.4 MSU Crystals

A few minutes after addition, the elongated crystals were randomly distributed on the monolayer (fig.11.4a). The cells appeared similar to those of the control culture. After 4h cytoplasmic elongation of cells had occurred and it appeared that endocytosis of MSU was taking place (fig.11.4b). By 24h, most of the MSU had been endocytosed by cells with markedly elongated cytoplasmic extensions (fig.11.4c). Cytoplasmic ballooning of some cells was also a feature at this time.

After 50h, some MSU crystals were still visible within the elongated cytoplasm of a few cells (fig.11.4d) but by 5 days MSU was no longer evident within or outside the cells.

11a.5 Calcium Carbonate Crystals

A few minutes after addition of calcium carbonate (fig.11.5a), the crystals were randomly scattered in clumps over the monolayer. After 4h, the clumps underwent further aggregation and some smaller particles had been endocytosed (fig.11.5b). By 24h, most of the particles had been taken up by the cells (fig.11.5c) some of which were rounded or had undergone cytoplasmic ballooning.

After 50h, the particles were concentrated in more well-defined

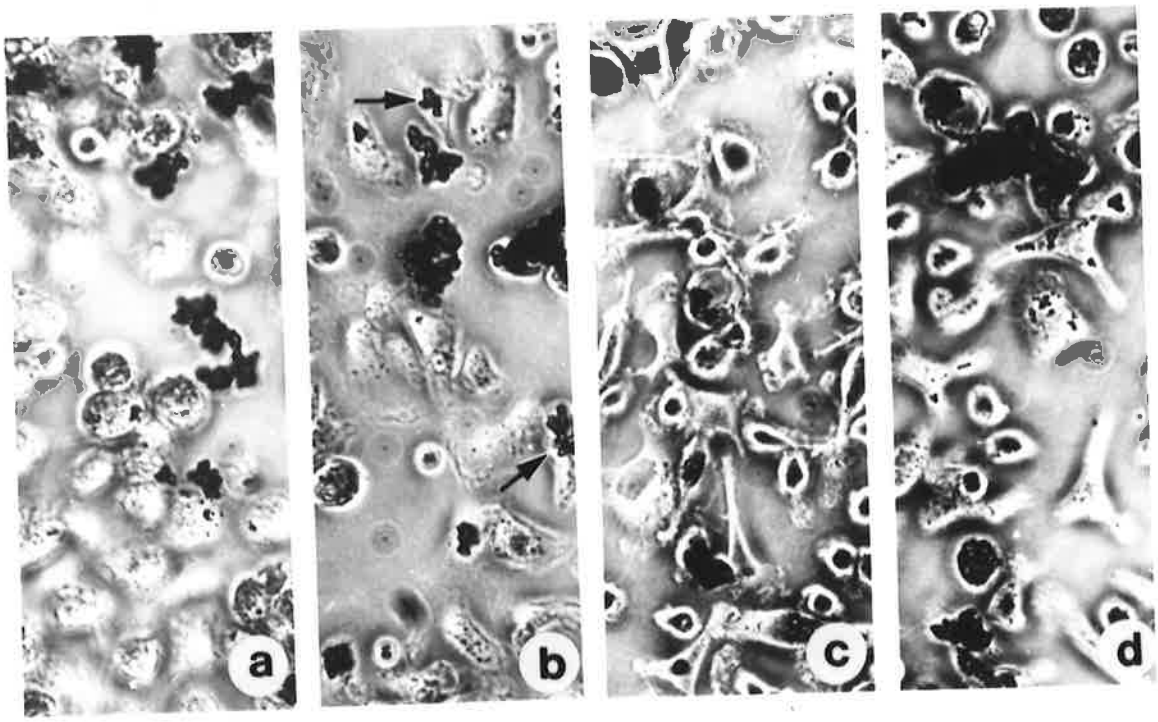


Fig.11.5
Light microscopic appearance of calcium carbonate particles added to macrophage cultures.
a. several minutes after addition, note clumping of the particles
b. after 4 hours, endocytosis is evident (arrows)
c. after 24 hours, most of the particles have been endocytosed. Cytoplasmic ballooning is prominent
d. after 50 hours, clusters of cells have formed around clumps of calcium carbonate. All phase contrast x380

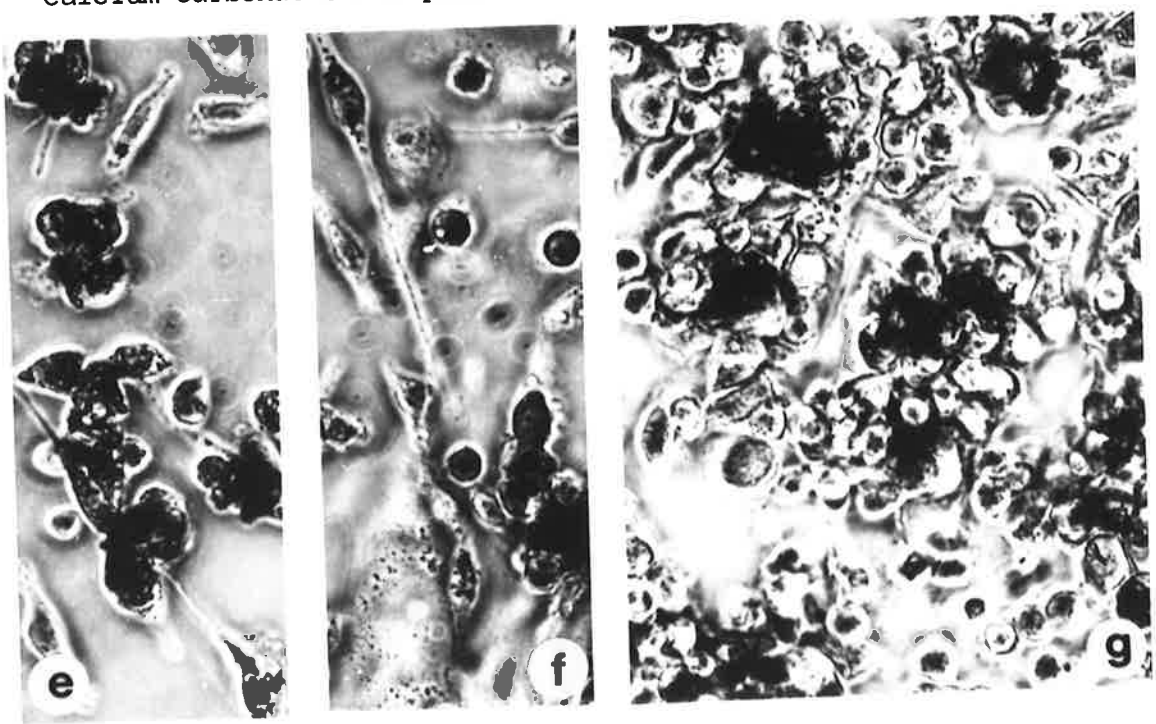


Fig.11.5 (cont.)
e. after 5 days, showing persistence of material in cells
f. after 7 days, with some cells showing cytoplasmic elongation
g. after 14 days, with marked cellular proliferation around the aggregates of calcium carbonate.
All phase contrast x380.

clumps within the cells (fig.11.5d). Fewer cells were seen after 5 and 7 days (figs.11.5e and 11.5f) and the calcium carbonate aggregates were present in clumps within cell clusters. After 14 days there had been an intense proliferation of cells around the calcium carbonate aggregates (fig.11.5g).

11b Transmission Electron Microscopy

11b. 1 Control Cell Culture

At the time of addition of the microcrystals, the surface of the filters was densely populated by macrophages. Their cell processes extended a short distance into the millipore filters. Cells that had direct contact with the filter were elongated, while cells that were attached to the underlying cell layer had a rounded morphology (fig 11.6). Lysosomes were prominent in all cells.

11b.2 Small HAC Aggregates

After 10 minutes (fig.11.7a), endocytosis of small aggregates had occurred. At increased magnification (fig.11.7b), the phagosomal membrane was seen. The HAC had formed irregularly shaped aggregates that were in close proximity to the surfaces of the cells. After 60 minutes, the macrophages were elongated and attached to the filters (fig. 11.8). Phagosomes containing HAC were present but the cells contained few lysosomes relative to the control cells.

After 24 hours, most macrophages had endocytosed massive quantities of HAC so that most of the cytoplasm had become a phagosome (fig. 11.9). The nucleus was confined to the extremity of a cell process. The

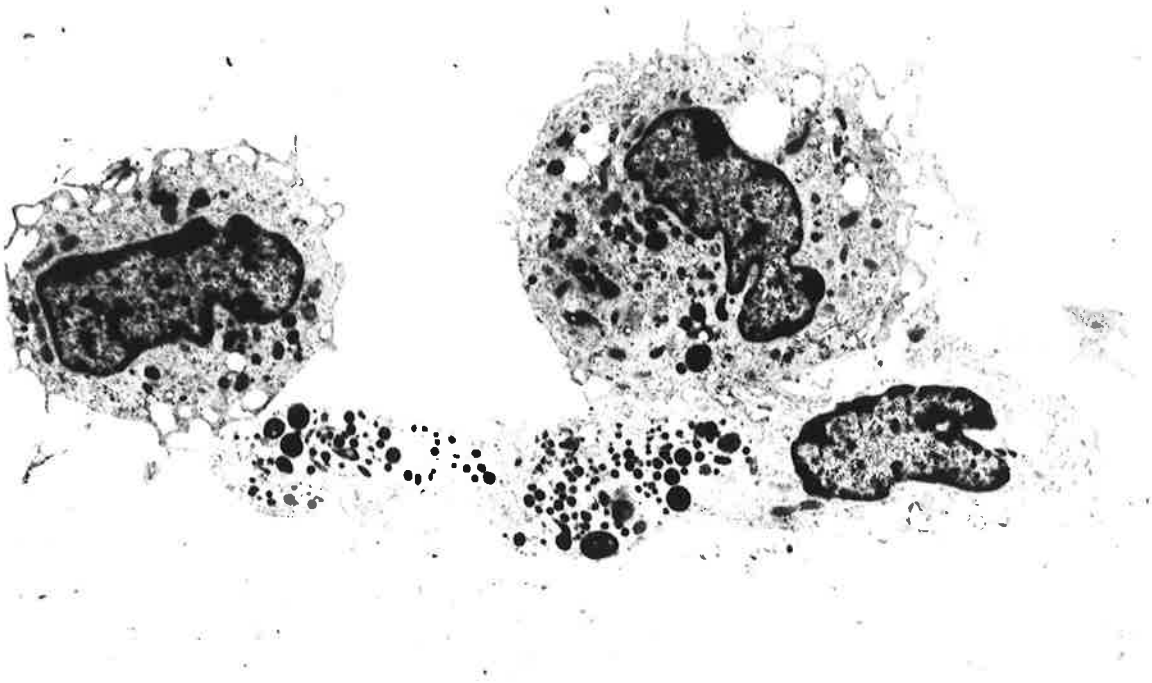


Fig.11.6 Electron micrograph of control macrophage culture. The macrophages show varying morphology and contain numerous lysosomes. The elongated cell is attached to the millipore filter. x5,000

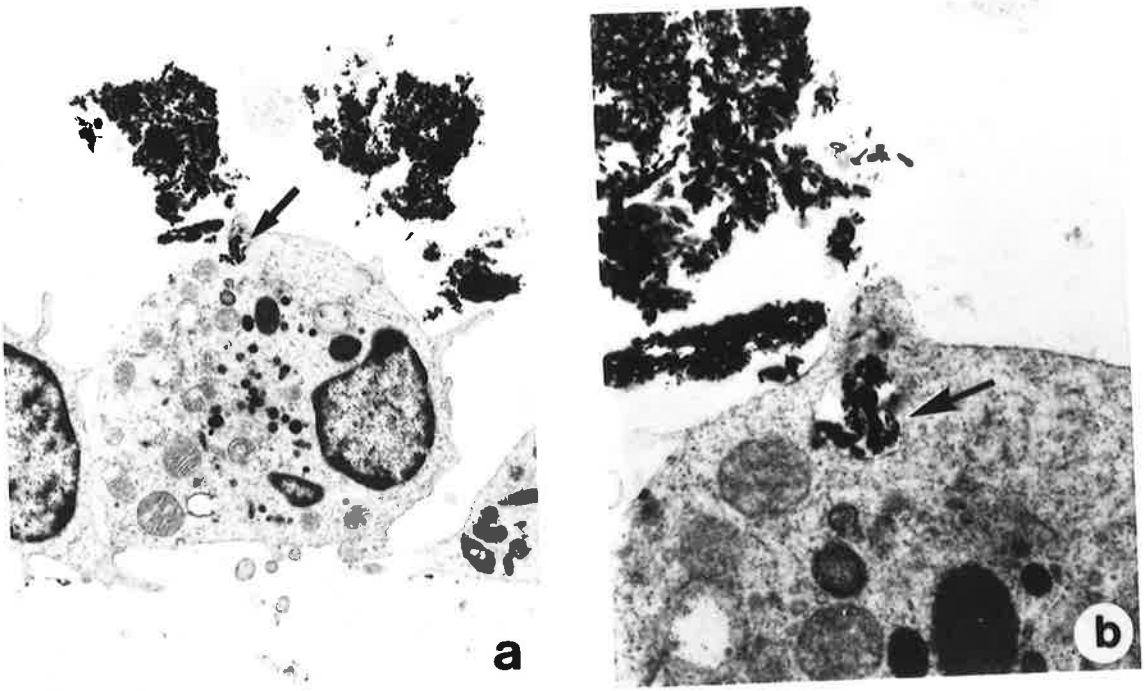


Fig.11.7
Electron micrographs of a macrophage culture, 10 minutes after the addition of small HAC.
a. Aggregates of small HAC in the proximity of the cells. Endocytosis of HAC has begun (arrow) x7,500
b. Higher magnification of a. showing HAC in a phagosome (arrow) near the cell surface. x26,100

Fig.11.8 Electron micrograph of a macrophage 60 minutes after the addition of small HAC. Phagosomes (arrows) contain HAC. There are less cell processes and lysosomes than in control cells. Droplets of lipid (L) and a large amorphous inclusion are also present. x6,850

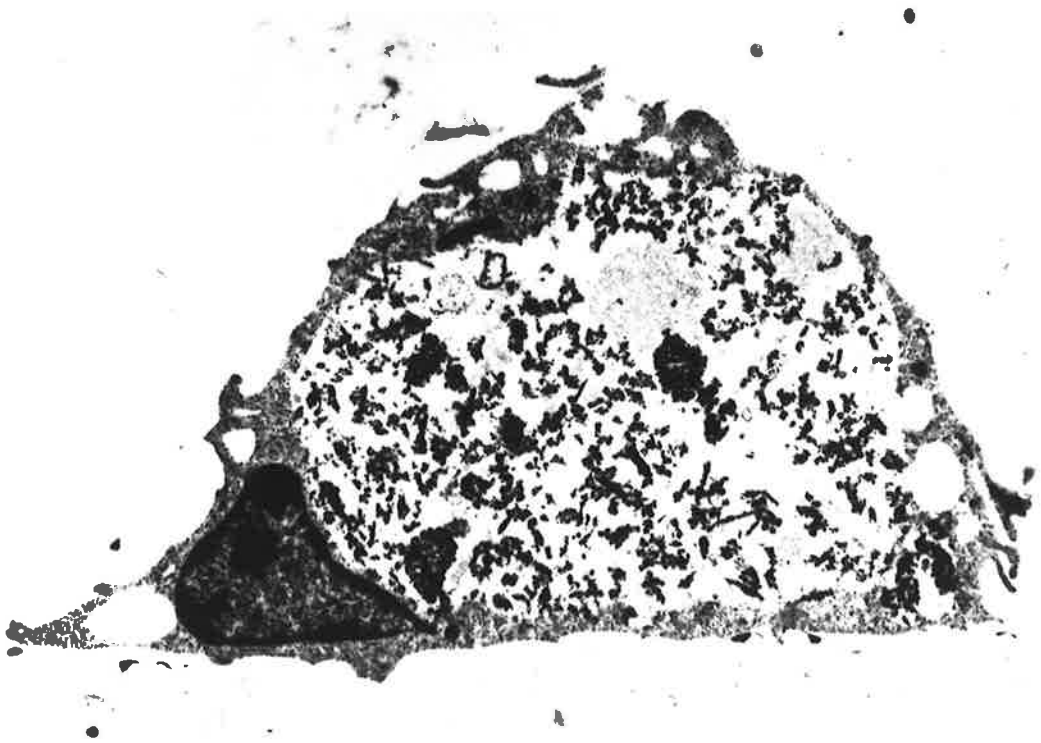


Fig.11.9 Electron micrograph of a macrophage, 24 hours after the addition of small HAC. Large amounts of disaggregated HAC occupy the bulk of the cytoplasm. The cell has become rounded and has less intact organelles and cell processes than control cultures. x8,750

cell was still attached to the filter and presented a rounded upper surface. Fibrous proteins were present amongst the HAC which had become disaggregated. After 2 days, cells containing HAC were intact (fig.11.10). Disaggregation of the HAC was evident and small deposits of crystals were found scattered throughout the cytoplasm of the cells.

11b.3 Large HAC Aggregates

After 4 minutes, a few macrophages had already formed large phagosomes containing crystalline material (fig.11.11). After 10 minutes, some macrophages were extending cell processes around the HAC (fig. 11.12), while others had already endocytosed a large quantity (fig. 11.13). Cells that were engaged in endocytosis had less prominent lysosomes than control cells.

After 60 minutes, many cells had multiple HAC containing phagosomes and prominent localisation of lysosomes in their vicinity (figs. 11.14 and 11.15).

After 2 days, macrophages that had endocytosed large HAC aggregates remained intact (fig.11.16). Disaggregation of the crystals was not prominent. Some cells had taken up vast quantities of HAC so that the cell nucleus was confined to a small cytoplasmic extension (fig. 11.17).

11b.4 MSU Crystals

After 60 minutes, evidence of uptake of MSU was disclosed by the presence of membrane-bound intracytoplasmic spaces (fig.11.18). The MSU

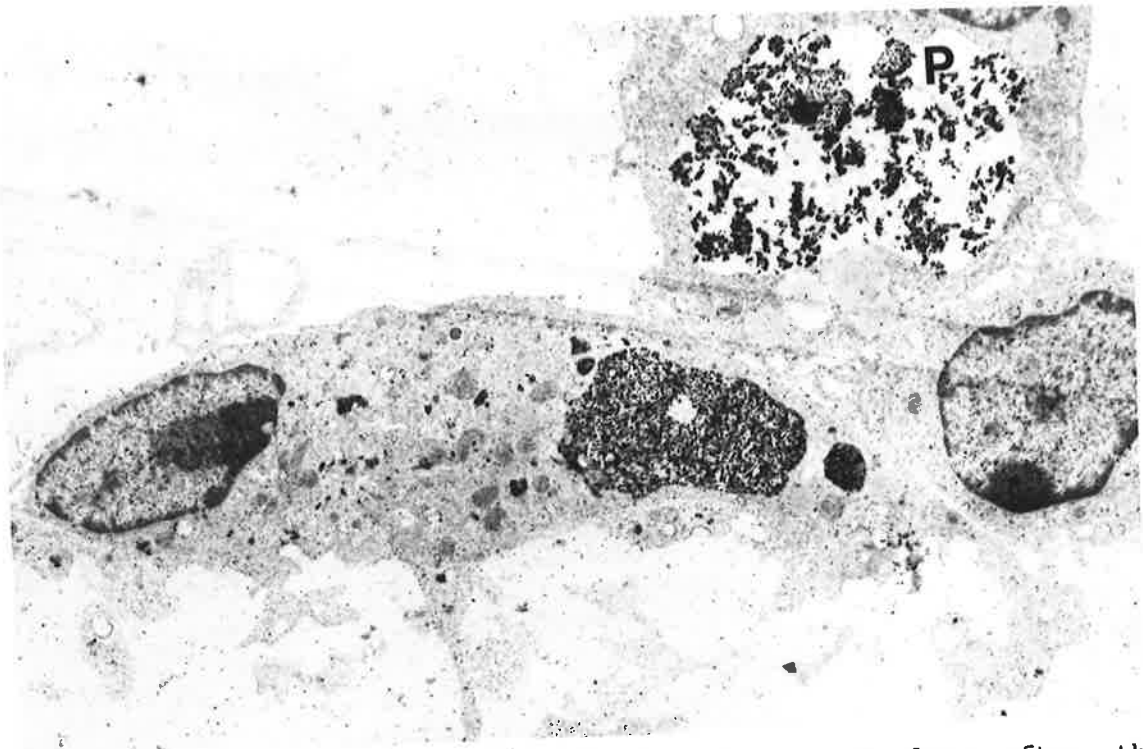


Fig.11.10 Electron micrograph of macrophages, 2 days after the addition of small HAC. Disaggregation of the HAC is evident in the large phagosome (P). The cell attached to the millipore filter contains numerous HAC-containing phagosomes. x6,600

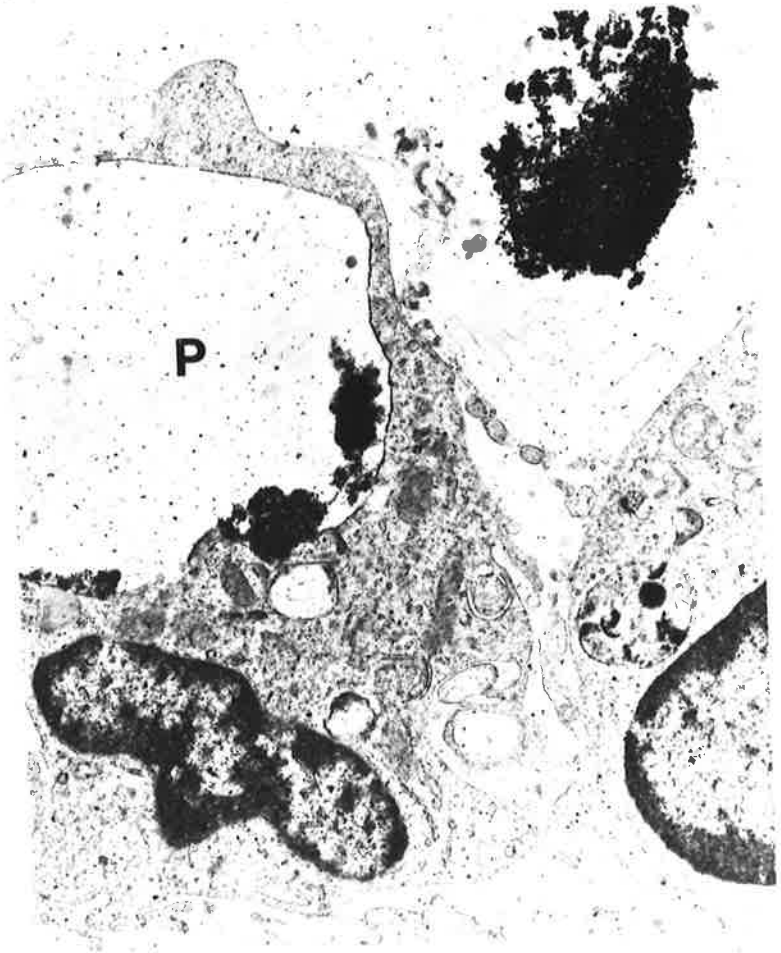


Fig.11.11 Electron micrograph of macrophages, 4 minutes after the addition of large HAC. An extensive phagosome (P) containing HAC has formed. x10,500

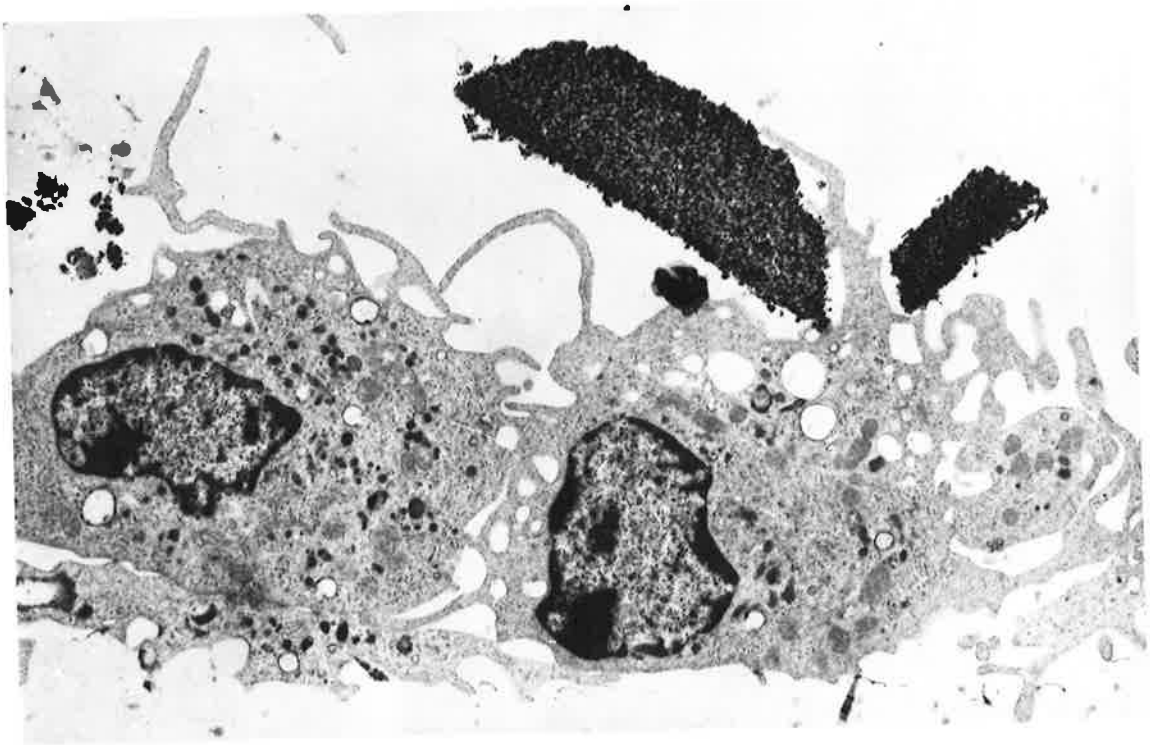


Fig.11.12 Electron micrograph of macrophages, 10 minutes after the addition of large HAC. Elongated cell processes extend around the crystal aggregates. x7,400

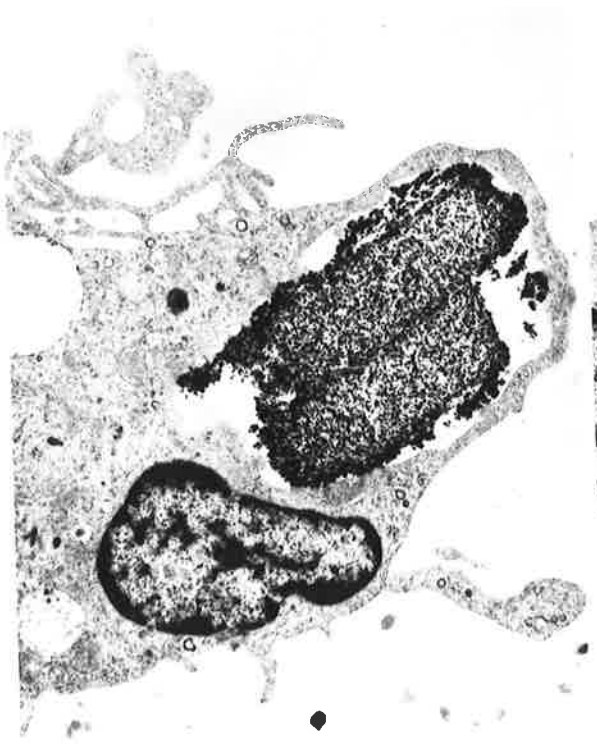


Fig.11.13 Electron micrograph of a macrophage, 10 minutes after addition of large HAC. The cell has endocytosed a large amount of HAC. x7,850

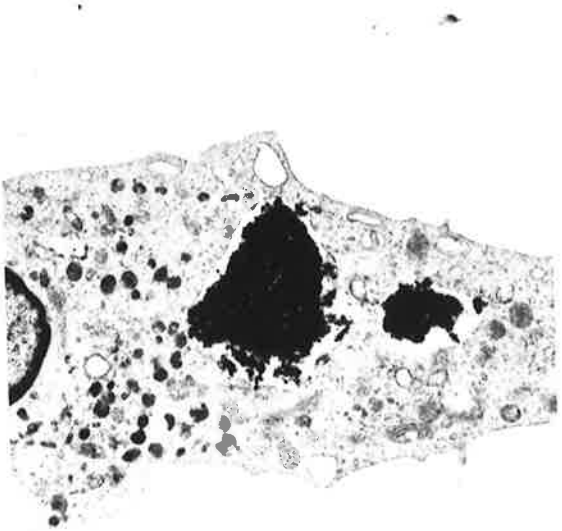


Fig.11.14 Electron micrograph of portion of a macrophage, 60 minutes after addition of large HAC. Several HAC containing phagosomes are present with concentrations of lysosomes nearby. x8,430

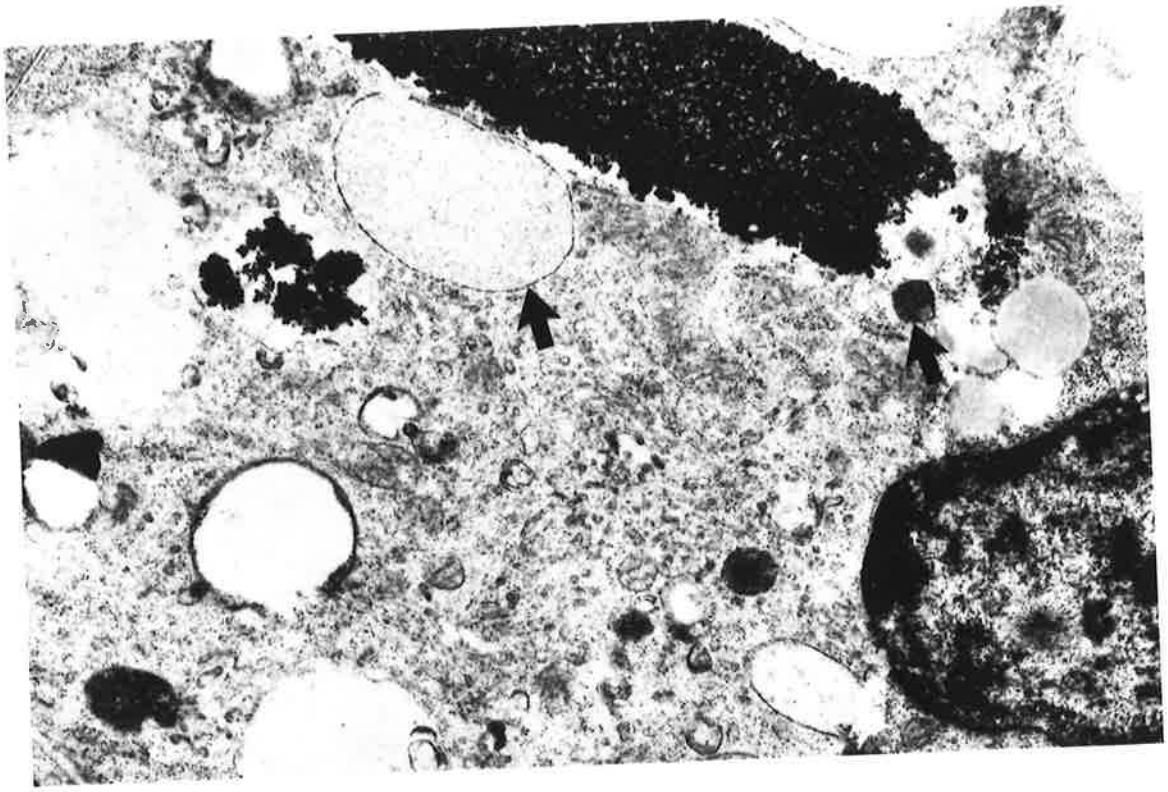


Fig.11.15 Electron micrograph of portion of a macrophage, 60 minutes after addition of large HAC. An aggregate of HAC is present in a phagosome with lysosomes (arrows) directly adjacent. There is evidence of disaggregation of the HAC. x17,100

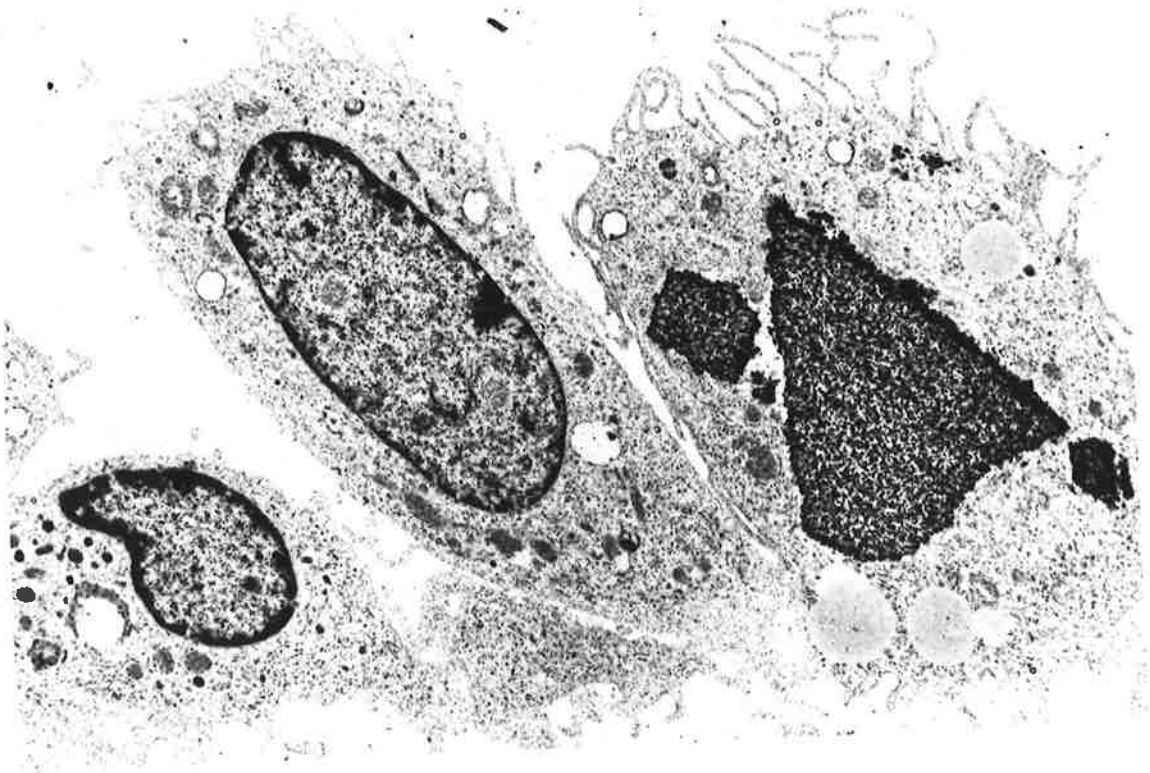


Fig.11.16 Electron micrograph of macrophages, 2 days after the addition of large HAC. The cells remain intact and attached to the millipore filter even when containing a large amount of HAC. x5,900

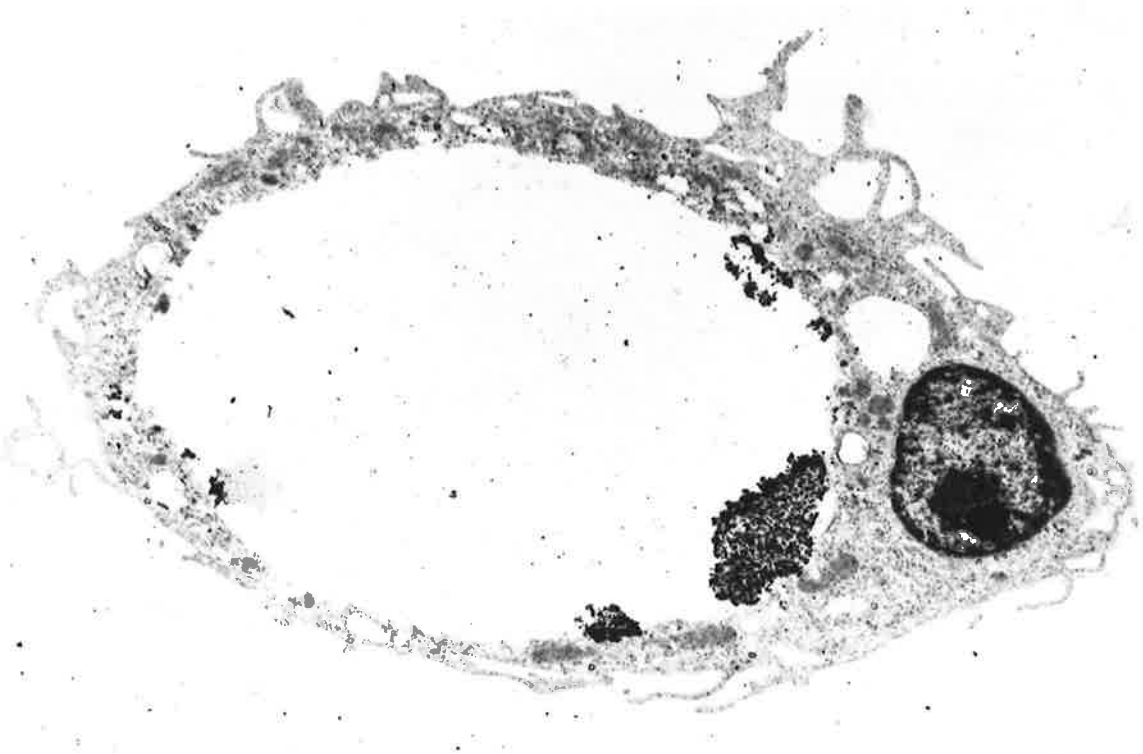


Fig.11.17 Electron micrograph of a macrophage, 2 days after the addition of large HAC. The nucleus is confined to a small cytoplasmic extension. The remainder of the cytoplasm surrounds a phagosome containing remnants of HAC. x6,400

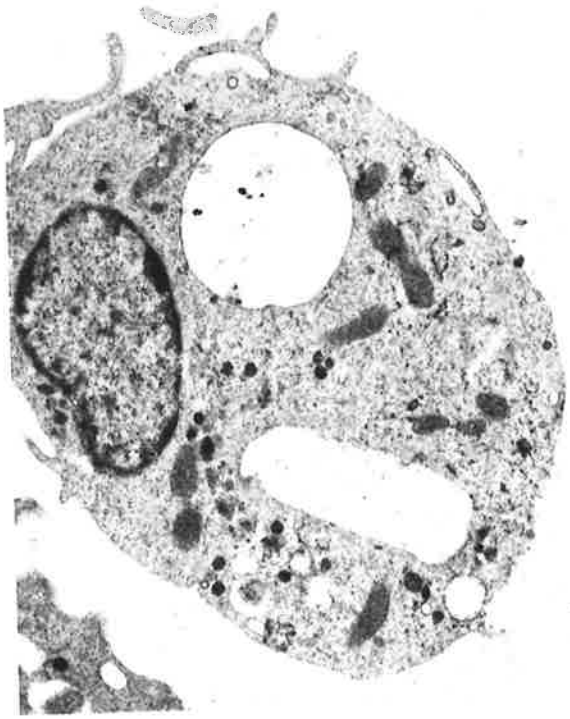


Fig.11.18 Electron micrograph of a macrophage, 60 minutes after the addition of MSU. Membrane bound circular and oblong vacuoles in the cytoplasm are present, presumably the sites of uptake of MSU. x10.000

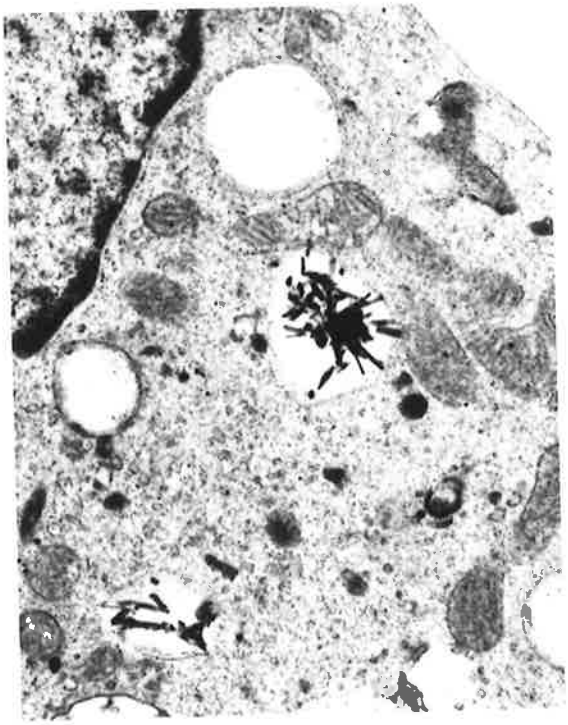


Fig.11.19 Electron micrograph of portion of a macrophage, 60 minutes after the addition of CPPD. Two crystals containing phagosomes are evident. x16,700

had been dissolved by the solutions used for processing the samples. The cells were intact.

11b.5 CPPD Crystals

After 60 minutes, small aggregates of CPPD were found in macrophage phagosomes (fig.11.19). The cells were intact.

11c Scanning Electron Microscopy

11c.1 Control Cell Culture

The SEM appearance correlated with that observed by TEM. Most cells had a flattened appearance and their cell processes interigitated (fig.11.20). A few rounded cells were perched upon the underlying macrophages.

11c.2 Small HAC Aggregates

After 24 hours, the density of cells on the filters had decreased, revealing the surface morphology of the filters. The cells were rounded (fig.11.21) and had retained cell membrane attachments to the filter, reminiscent of tent guy ropes. Cell processes and crystalline material on the cell surface were also seen (fig.11.22).

11c.3 MSU Crystals

After 24 hours, it appeared that the MSU crystals had been completely endocytosed. The elongated crystal was covered by a folded cell membrane (fig.11.23). Adjacent cels were similar in appearance to control macrophages.

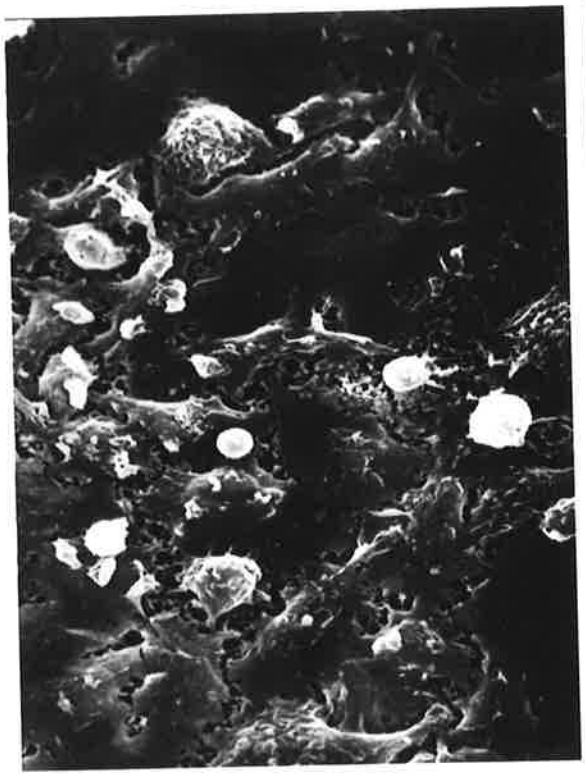


Fig.11.20 Scanning electron micrograph of control macrophages grown on millipore filter. Most of the cells are flattened and their cell processes are contacting. x1,000

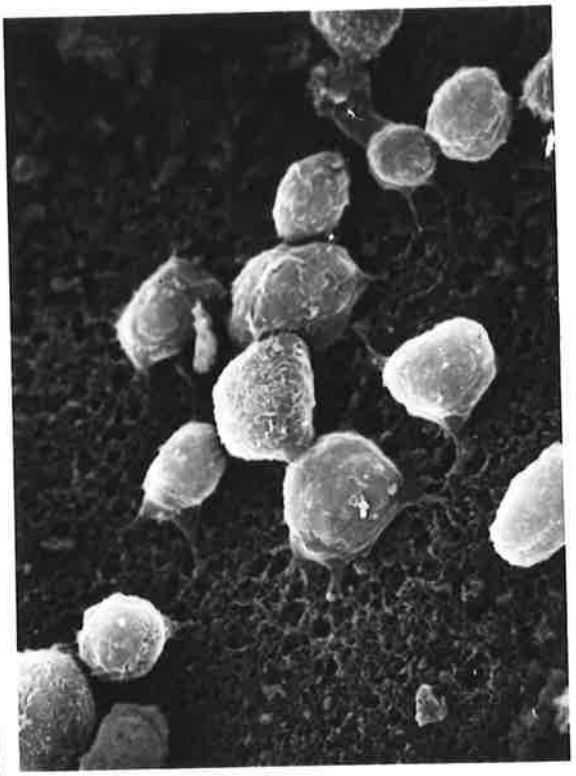


Fig.11.21 Scanning electron micrograph of macrophages, 24 hours after the addition of small HAC. The cells are rounded but are still attached to the millipore filter by cell processes. x1,000

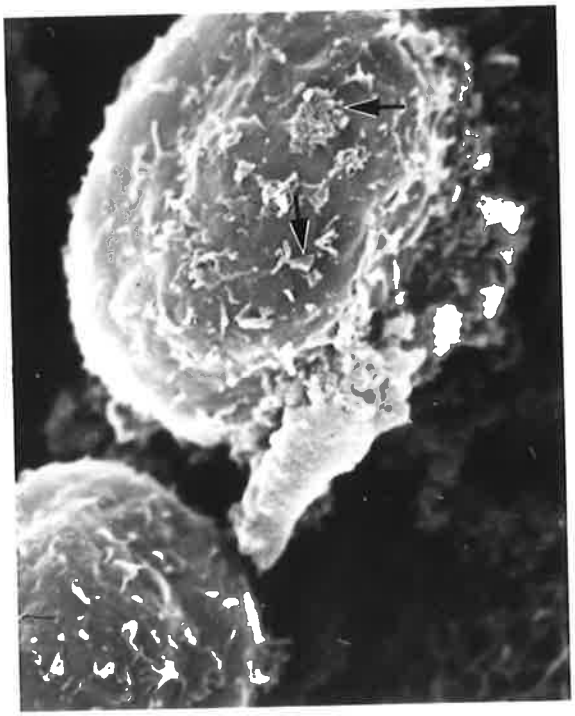


Fig.11.22 Higher magnification of fig.11.21 showing folded cell processes and crystalline material (arrows) on the cell surface. x4,000

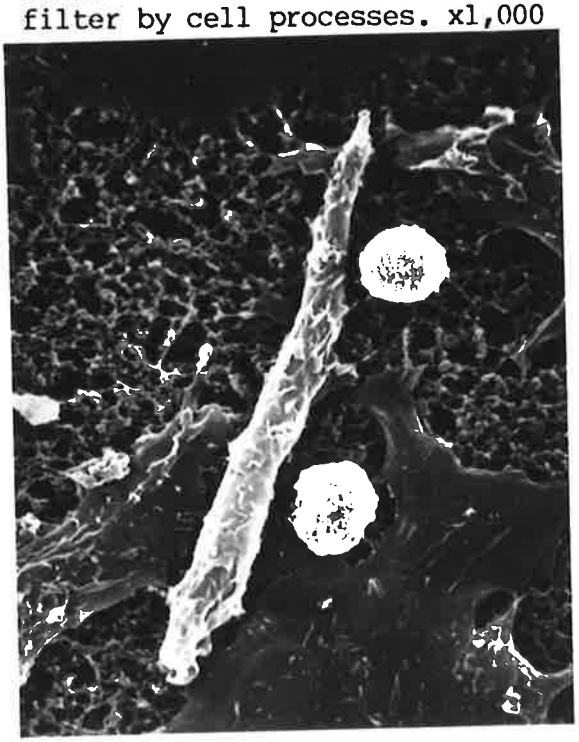


Fig.11.23 Scanning electron micrograph of macrophages, 24 hours after addition of MSU. A macrophage has endocytosed a MSU crystal. Surrounding cells have retained the morphology of control macrophages. x2,000

EXPERIMENT 12

Aim

To study the *in vitro* interaction of bovine synovial cells with HAC and other microcrystals.

Method

A forelimb of a freshly slaughtered calf was obtained from the South Australian Meat Corporation and transported to the laboratory on ice. The skin overlying the metacarpophalangeal joint was incised and removed. The subcutaneous tissue was swabbed with 70% ethanol and the following procedure was carried out using a sterile technique. The method was adapted from that used by Fraser and McCall (1965) to obtain human synovial cells for tissue culture.

An 18 gauge needle was inserted into the flexed ankle joint. The joint was rinsed twice with 20ml calcium and magnesium free Dulbecco's balanced salt solution at 37°C, followed by 20ml of 0.25% trypsin in PBS, also at 37°C. The trypsin was withdrawn after 15 minutes and the synovial pouches were gently massaged. The trypsin solution was re-injected into the joint cavity and withdrawn into the syringe where it was inspected for opalescence in strong light. The number of cells were estimated with a haemacytometer and if the yield was low, the trypsin was re-introduced into the joint and the same procedure was repeated.

The cell suspension was centrifuged at 1,000 rpm for 10min. and resuspended in tissue culture medium (supplemented RPMI-1640). The cell suspension was transferred to Corning tissue culture flasks and

incubated at 37°C. The culture medium was replaced every second day with fresh medium. The cells became confluent after 7-10 days and were then subcultured.

After decanting the medium, the culture was gently rinsed with Dulbecco's balanced salt solution for 5 minutes at 37°C. After discarding this solution, 1ml of trypsin solution (made by combining 0.1ml trypsin, 0.9ml Versine (CSL) and 9ml Dulbecco's balanced salt solution) was added to the culture flask which was incubated at 37°C for two minutes. Phase contrast light microscopy was used to check that the cells had detached from the plastic surface of the culture flask. Fresh culture medium was added and aliquots of the cell suspension (usually containing 4-10x10^6 cells/ml) transferred into new tissue culture flasks. The cells were used in microcrystal interaction experiments were in their third passage.

Cells were detached from tissue culture flasks by trypsin treatment (as above) and suspended in fresh culture medium. They were added to the 16mm wells of Costar tissue culture plates (half of the wells used had sterile millipore filters placed prior to addition of the cells). Approximately 1x10^6 cells were added per well and the plates were incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide and air until the cells were almost confluent (1-2 days later).

Microcrystals were added to the synovial cell cultures at this stage and a similar experimental design as for Experiment 11 was used. The cell culture medium was changed every second day. Small and large HAC aggregates and MSU crystals were added so that each well contained 120µg of microcrystalline material. Wells without filters were used for examination and photography of the cells and crystals by phase

contrast light microscopy.

Small and large HAC aggregates were added to cells grown on millipore filters which were subsequently processed for TEM using the same method as for macrophage cultures (Experiment 11).

Results

12a Light Microscopy

12a.1 Control Cell Culture

At the time of addition of crystals, the synovial cells were well spread on the floor of the culture wells. Long cytoplasmic processes interconnected with those of neighbouring cells (fig.12.1a). The cytoplasm was relatively profuse in relation to nuclear size and spread irregularly with numerous thin polar processes. After 7 days, considerable cell division had occurred and the cells were piled up on each other and formed a reticular pattern.

12a.2 Small HAC Aggregates

Immediately after addition, the HAC were randomly scattered throughout the culture. Uptake of crystals by the synovial cells was seen at 4 hours after their addition and by 24 hours, the HAC was localized within the cells (fig.12.1b). The cells were intact and were packed together in a reticular pattern. At 7 days (fig.12.1c) the cells were densely packed. The HAC was localized in intracellular vacuoles. All cells were intact.

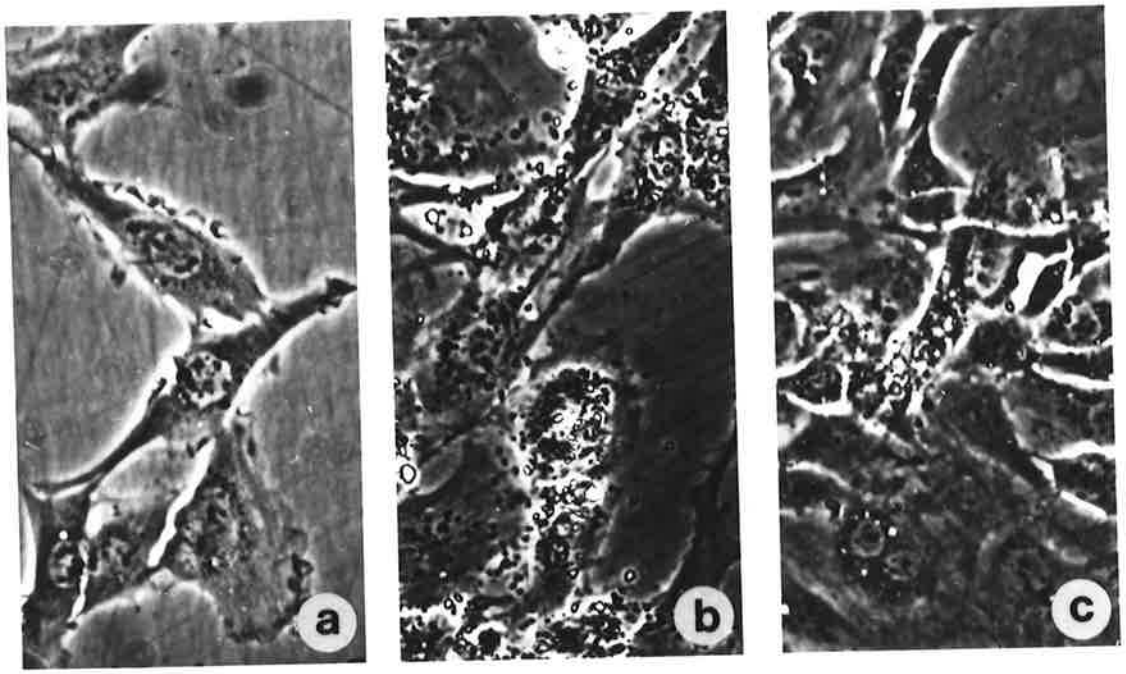


Fig.12.1 Light microscopy of synovial cell cultures
a. control culture showing cytoplasmic spreading
b. 24 hours after small HAC addition. The HAC is localised within and around the cells.
c. 7 days after addition of small HAC. The crystals are present in cell vacuoles. Note large numbers of cells.
All phase contrast x410

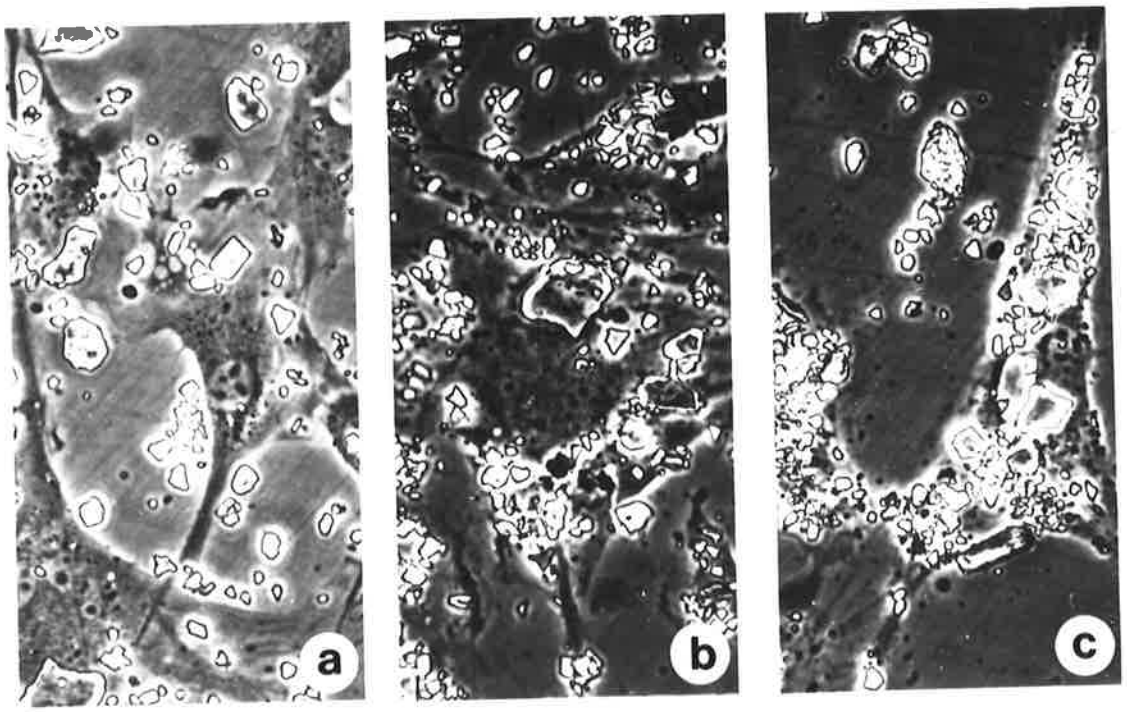


Fig.12.2
Light microscopy of synovial cells after the addition of large HAC.
a. several minutes after addition
b. after 2 hours showing localization of the HAC around the cells
c. after 24 hours. Uptake of the HAC has occurred.
All phase contrast x410

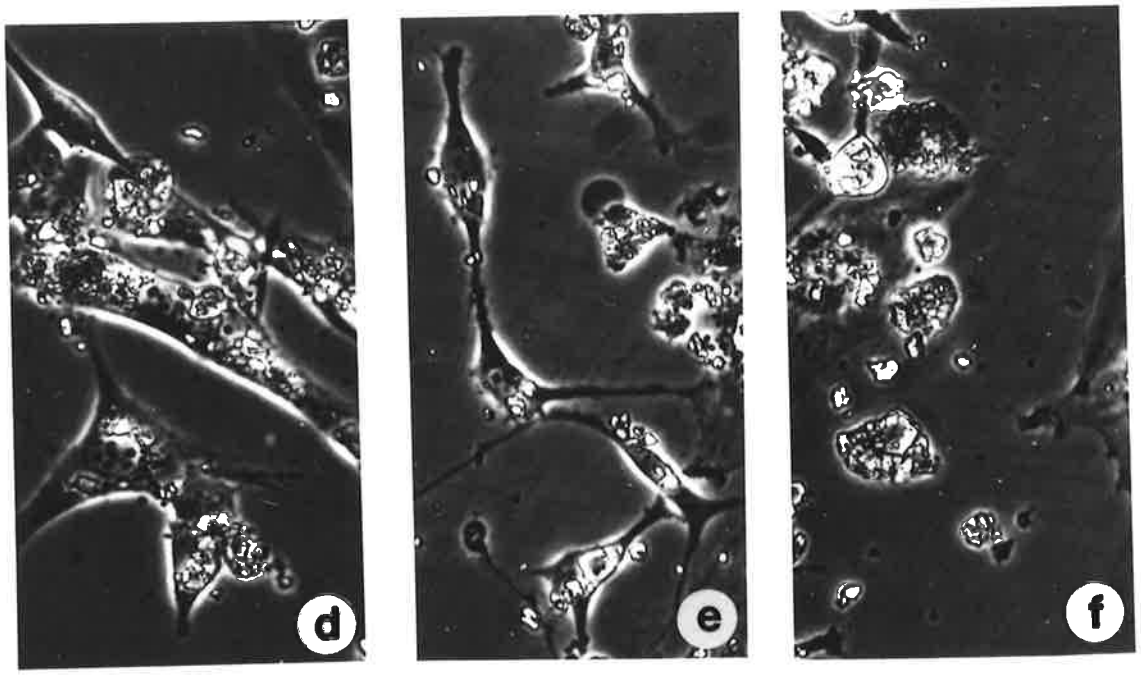


Fig.12.2 (cont.)
d. after 4 days, uptake of large amounts of HAC and decreased numbers of cells
e. after 7 days with spindle-shaped cells showing signs of degenerative changes
f. after 7 days with HAC near cell remnants.
All phase contrast x410

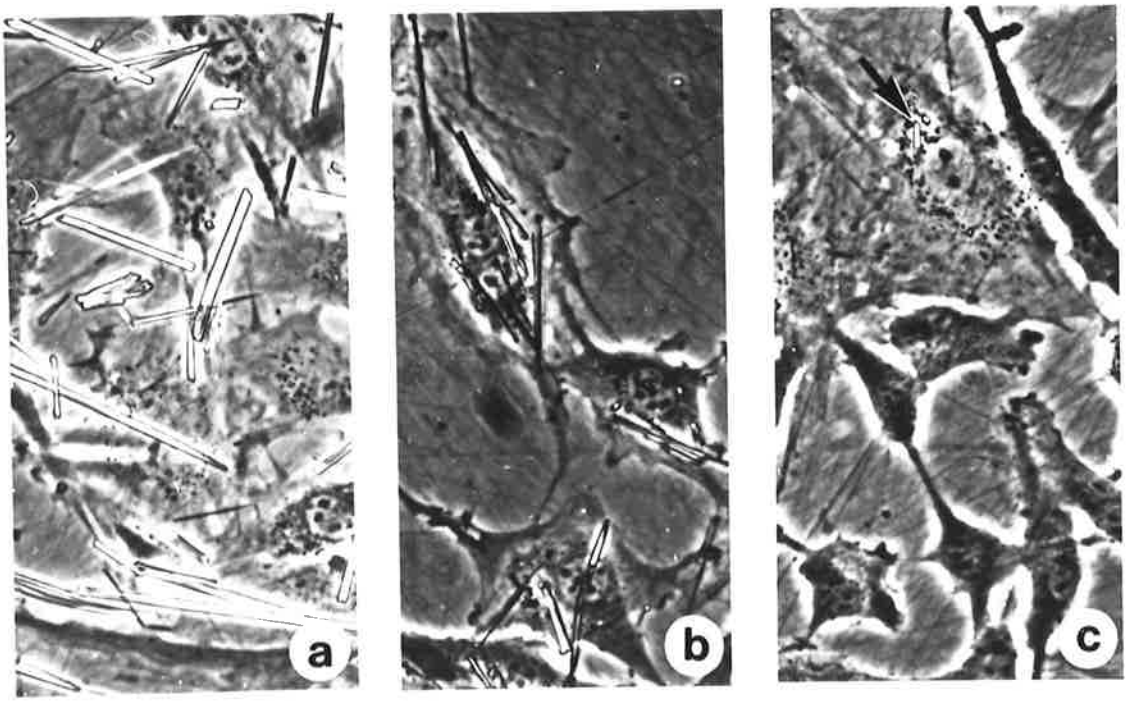


Fig.12.3 Light microscopy of synovial cells after the addition of MSU.
a. several minutes after addition of MSU
b. after 2 hours endocytosis is evident
c. after 24 hours, few MSU crystals (arrow) are present.
All phase contrast x410

12a.3 Large HAC Aggregates

Immediately after addition, the HAC were randomly scattered throughout the culture (fig.12.2a). The localization of HAC around the cells was seen at two hours after their addition (fig.12.2b) and by 24 hours, this effect was strikingly evident (fig.12.6). Some of the HAC had been endocytosed by the cells and some of it appeared to be attached to the cell processes.

At 4 days (fig.12.2d), the cells had enocytosed large amounts of HAC. The numbers of cells in the culture was significantly less than in the control wells.

At 7 days (figs.12.2e,f), spindle shaped cells showing signs of degenerative change were present. Large amounts of HAC aggregates were associated with cell remnants.

12a.4 MSU Crystals

Immediately after addition, the MSU were randomly scattered throughout the culture (fig.12.3a). After one hour, some of the crystals had become orientated along the cell processes and by two hours, endocytosis of some of the particles was evident (fig.12.3b). After 24 hours, only a few small MSU particles located intracellularly could be identified in the cultures (fig.12.3c).

12b Transmission Electron Microscopy

12b.1 Control Cell Culture

Fig.12.4 Electron micrograph of portion of a control bovine synovial cell during the third passage of the cell culture. The flattened cell is well spread on the millipore filter and contains lipid droplets (L). x3,270

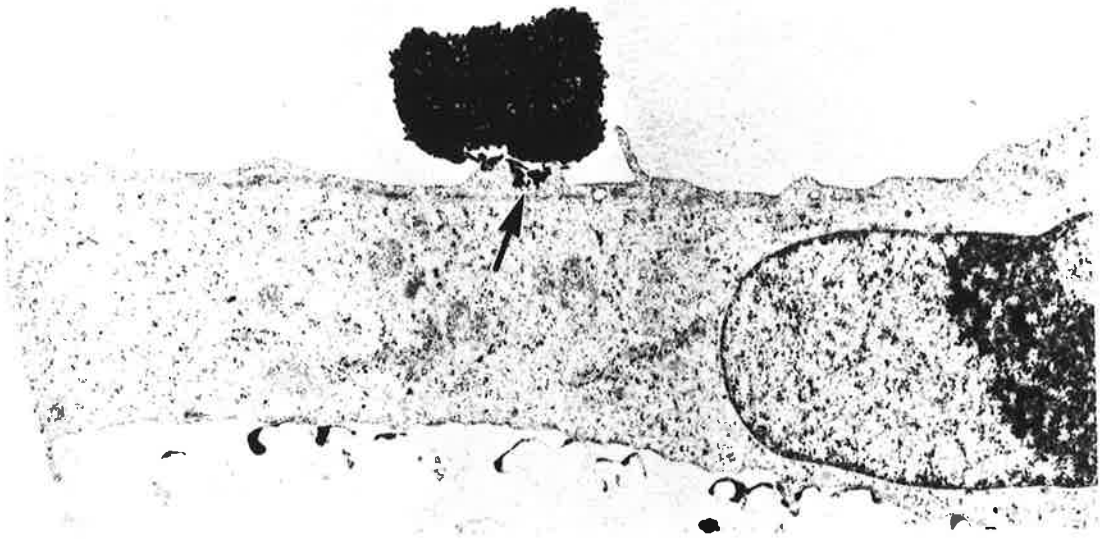


Fig.12.5 Electron micrograph of portion of a synovial cell, 10 minutes after the addition of large HAC particles. Endocytosis of some individual crystals has begun (arrow). x12,600

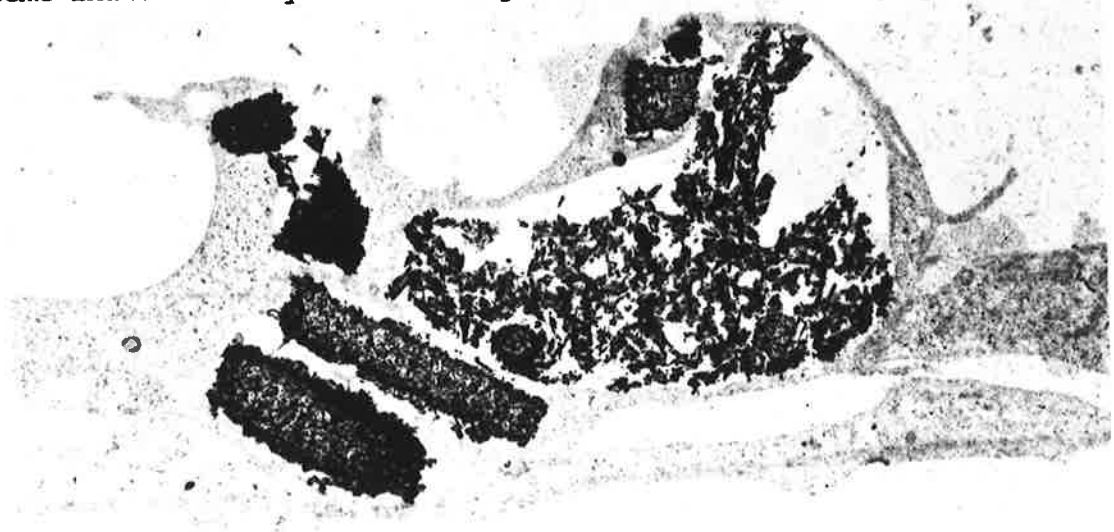


Fig.12.6 Electron micrograph of portion of a synovial cell, 60 minutes after the addition of large HAC particles. The crystal aggregates are contained within membrane bound phagosomes. Disaggregation of some of the material has occurred. x8,500

Fig.12.7 Electron micrograph of portion of a synovial cell, 7 days after the addition of small HAC particles. Many HAC-containing phagosomes are scattered throughout the cytoplasm. x5,300

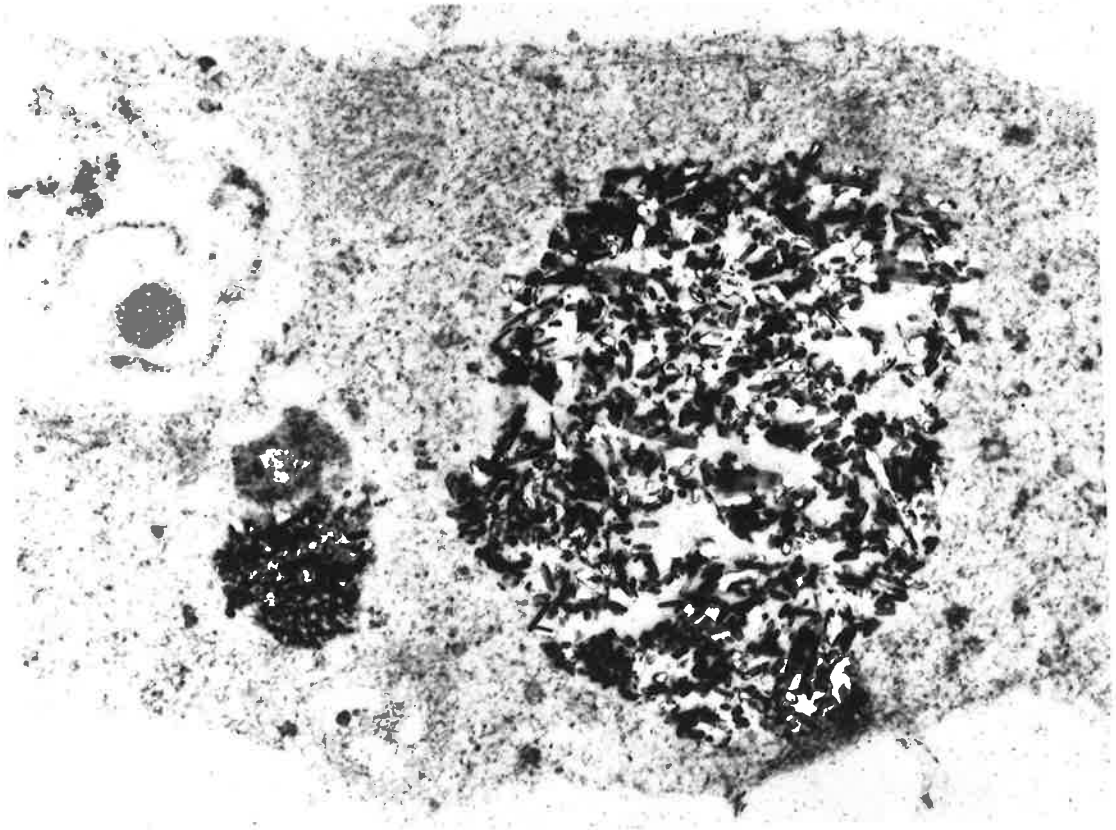


Fig.12.8 Electron micrograph of portion of a synovial cell, 7 days after the addition of small HAC particles. Disaggregation of the HAC into individual crystals is evident. x38,600

Synovial cells grown on millipore filters did not exhibit the same degree of confluency as cells grown on plastic. The cells had elongated cytoplasmic processes which tapered off at the cells' extremities (fig.12.4). The nuclei were ovoid and were generally positioned off-centre. Some cell processes extended a short distance into the millipore filter.

12b.2 Small and Large HAC Aggregates

Ten minutes after addition, the process of endocytosis of large HAC aggregates had commenced (fig.12.5). Small cell processes had almost closed around individual crystals that had separated from the larger crystal aggregates.

After 60 minutes, the cells had endocytosed large amounts of large HAC aggregates contained within membrane bound phagosomes (fig.12.6). Disaggregation of some of the material was evident and the cells were otherwise intact.

After 7 days, synovial cells had taken up considerable quantities of small HAC aggregates (fig.12.7) without any apparent detrimental effect on the cells. In some specimens, disaggregation of the individual crystals was seen within the phagosomes (fig.12.8).

Chapter Four

Discussion

CHAPTER FOUR
DISCUSSION

1. Definition of the Sponge Implantation Model

Subcutaneous implants of polyurethane sponges in young adult male rats elicited responses similar to those reported previously for a variety of sponge implant materials (Dasler et al, 1960; Viljanto and Kulonen, 1962 and Boyle and Mangan, 1980).

While the dry weight gain of left and right sided sponges was similar in all rat strains and at all time periods studied, sponge dry weight gain showed a progressive increase from 5 to 14 days that was not uniform for all rat strains tested. The strain differences observed may have been an effect of variations in the ages of the animals across the strains, since young Dark Agouti rats generally weighed less than Porton and Lewis rats of the same age. Weight was used as an approximate measure of animal age. However, the influence of rat age on the reaction to subcutaneous implants was not investigated in this study and the literature reviewed did not provide any relevant information. In subsequent implantations, single rat strains were used and the animals' weights were within a small range.

The method of measuring the dry weight increase of sponges based on using a portion of the sponge (approximately half) gave similar results to those obtained by measuring the dry weight gain of whole sponges. Consequently, the technique enabled the acquisition of an increased amount of information from each sponge in the form of weight gain, ingrowth of granulation tissue, and histology and also reduced the number of animals required for each series of implantations.

Sponge dry weight gain was considered to be an indirect measure of the inflammatory activity occuring around and within the sponges.

For the first three hours following implantation, cotton pellet implants are permeated with a transudate (a non-inflammatory fluid that has a low protein content) which accounts for approximately 50% of the wet weight measured after 6 days (Swingle and Shideman, 1972). Although the implants absorb a large amount of fluid in this time, its contribution to their dry weight is minimal because of its low content of proteinaceous material. The implantation of cotton pellets by Penn and Ashford (1963) however, initiated an initial period of exudation as determined by leakage of intravenously injected Pontamine Sky Blue dye. Exudation was seen as soon as 20 minutes after implantation and was followed by a period of impermeability which persisted for up to 2.5 hours after implantation. Differences in the preparation of the cotton pellets prior to implantation may account for the disparate findings of these two groups of workers.

A "delayed-prolonged" type exudative phase occurs between 3 and 72 hours after implantation (Swingle and Shideman,1972) and it is during the first 24 hours that cotton pellet dry weight increases dramatically.

The dry weights of individual polyvinyl sponges implanted for up to 56 days in guinea pigs varied considerably from the mean (Bole and Robinson,1962). The water content of the sponges, which was calculated by subtracting dry weight from wet weight, decreased from an initial high of 96.7% at four hours after implantation to 87% after 14 days.

The dry weight gain of sponges impregnated with heat killed TBC was attributed to the protein content of the fluid exudate when implanted for 5 days (Clarke et al,1975). Five days after implantation of sponges in the present study, it is also likely that the protein content of the exudate contributed the major amount to sponge dry weight gain. However, it is not clear why sponges implanted in Lewis rats should have gained 45mg more weight than sponges implanted in other rat strains at this time.

Seven days after implantation, granulation tissue occupied approximately 25% of the sponges and probably accounted for the significant increase in sponge dry weight gain that was measured between 5 and seven days. The contribution of macromolecular material to the dry weights of 4-6 day cotton pellet implants has been demonstrated by Swingle and Shideman, 1972).

Although granulation tissue ingrowth continued in an approximately linear fashion when measured at 10 and 14 days, the dry weight gain of the sponges did not reflect this trend. This finding may be explained by the different densities and water contents of proteinaceous exudates and granulation tissue.

It is apparent therefore, that the interpretation of the data pertaining to sponge dry weight gain in terms of inflammatory activity is fraught with difficulties. One of the main sources of error in these experiments was the variable amount of fluid that could be displaced from the sponges when they were bisected immediately after their removal from the animals. Although care was taken to apply minimal pressure to the scalpel and a new blade used for each sponge, it was not possible to standardize the forces applied during bisection.

Granulation tissue ingrowth into the sponges followed a linear pattern after 7 days for Porton and Lewis rats, but in DA rats the amount of ingrowth had declined between 10 and 14 days after implantation. At 14 days, Dark Agouti rats had 21% less ingrowth into the sponges than the other strains, a difference that could have a genetic basis. The varying responses to injection of adjuvant in different rat strains has been shown to be genetically determined (Zidek and Perlik, 1971). Of the three rat strains used in the present study, DA rats develop the most severe adjuvant disease yet were least reactive to plain sponge implants. This disparity in strain response to sponge implants was exploited in subsequent experiments in which DA rats were used to test the effect of a variety of microcrystals on granulation tissue ingrowth. The extent of tissue ingrowth in sponges impregnated with microcrystals was significantly greater than the ingrowth into plain sponges implanted for 14 days in DA rats.

A similar amount of tissue ingrowth was measured in left and right sided sponges at all time periods and for each rat strain. This finding confirmed that the sites chosen for implantation reacted uniformly to the sponge implants. It was considered appropriate therefore to use the left side as the control implantation site and the right side as the test implantation site in subsequent experiments.

The method used for measuring the extent of granulation tissue ingrowth has not been previously reported. Despite the need to trace the outer sponge perimeter and the limit of granulation tissue penetration twice: first when outlining the projected image of the stained histological section; and, second, when retracing with the digitiser stylus; the error involved was only 0.12%. The limit of tissue ingrowth was readily discernible on the projected images of the slides. This method measured the entire inside perimeter of tissue and was therefore unaffected by local variation in ingrowth that would not have been detected by other techniques such as ocular micrometry at preselected sites (Bole and Heath, 1967; Clarke et al, 1975). The main limitation of using area digitisation as described was that it only gave an indication of the extent of tissue ingrowth into the mid section of the sponges. Serial sections through sponges were not measured for tissue ingrowth. However, as care was taken to bisect the sponges as accurately as possible, the sections measured for ingrowth represented coincident zones in all sponges. In addition, these sections were thought to be the most useful in the assessment of ingrowth because they were through parts of sponge that had the least ingrowth. Cross sections near the edge of the sponges invariably would be fully occupied by tissue after 14 days since tissue could penetrate directly from five sides rather than four and the depth of tissue penetration required to fill these sections would be less.

The histological reaction to polyurethane sponge implants also paralleled those previously reported (Bole and Robinson, 1962; Boyle and Mangan, 1980) and correlated with the normal events in wound healing (Glynn, 1978). Granulation tissue was first noticeable 5 days after implantation and its appearance was similar to granulation tissue at various sites.

The sponges gradually underwent fatty infiltration. Occasional fat cells were seen 10 days after implantation and by 14 days they were prominent in some areas in the periphery of the sponges. At 20 weeks, the infiltration was extensive, involving approximately one third of a sponge cross-section. An interesting feature of the pattern of fatty infiltration was that it only involved the subcutaneous aspect (superficial when in position in vivo) rather than the deep part of the sponge in contact with the muscles of the back. Other workers reported fatty infiltration and a high lipid content of polyvinyl sponge implants in rats. (Moore and Brown, 1952; Dasler et al, 1960 and Edwards et al, 1975) However Bole and Robinson (1962) found no evidence of fatty infiltration of similar implants even after 144 days in guinea pigs.

There was a tissue reaction to the sponge matrix commencing at 5 days as discerned by light microscopy. It occurred at first contact of granulation tissue with sponge. This involved condensation of a fibrillar substance and the appearance of flattened cells around the sponge matrix. By 10 days, the thickness of the cell layer had increased, and by 14 days multinucleated giant cells (MNGC) were prominent around the sponge matrix. The formation of MNGC around polyurethane sponges at this time had been previously reported (Paulini et al, 1974; Gabbiani et al, 1976). Seven weeks after implantation of sponges, all sponge

matrices had been surrounded by aggregates of MNGC and there was evidence of the breakdown of matrx material. By 20 weeks, it was apparent from light microscopy and TEM that advanced breakdown of the sponge was occurring with subsequent phagocytosis by the MNGC Pieces of sponge (or sponge breakdown products) could be observed within the cytoplasm of these cells. The extensive infiltrate of lymphocytes in the granulation tissue may have been elicited by the sponge breakdown products or by mediators released from the MNGC during phagocytosis.

The large artefactual spaces that invariably were seen between the tissue and the sponge matrix up to 49 days after implantation indicated that this junction involved only weak attachment (if any) of cells to the polyurethane. Similar artefacts were found by Moore and Brown (1952); Boucek and Noble (1955) and Bole and Heath (1967). However, with increasing time of implantation, as observed at 7 and 20 weeks, artefactual separation of the tissue from the sponge was no longer prominent. This latter observation could have been a result of:

a) development of a strong attachment of the MNGC to the sponge as part of the engulfing and degradative process;

b) development of well defined collagen bundles within the granulation tissue which rendered the sponge less liable to undergo separation artefact during processing for histology;

c) a combination of a) and b).

Further definitive evidence of cellular reactivity to the sponge matrix was evident using TEM. After 14 days, close apposition of a MNGC to the sponge was evident with a multitude of mitochondria and intractyoplasmic vesicles suggestive of intense cellular activity. At 20 weeks there was positive evidence of endocytosis of large pieces of sponge. Degradation and packaging of breakdown products into large

membrane-bound intracytoplasmic vesicles was also a predominant feature. This clearly indicated that a cellular reaction to the polyurethane sponge was mounted as soon as granulation tissue came into contact with it, and that the reaction was localized around the sponge and increased in intensity with the duration of implantation. Active breakdown of the polyurethane therefore was confirmed.

Polyvinyl sponges also underwent degradative changes after long periods of implantation (4-7 months) in experiments undertaken by Bole and Robinson (1962), while Parnham (1980) saw granuloma models in general as the host attempt at isolating a foreign body. The findings of this study are in strong agreement with this latter concept.

The sponge-granuloma model defined above provided a unique matrix within which a variety of inflammatory reactions were studied. The granulation tissue growing in the sponge interstices was of a uniform nature throughout the sponges and contained a minimal inflammatory cell infiltrate for up to 7 weeks after implantation. After that time, cell mediated sponge breakdown and breakdown products induced a moderate lymphocytic infiltration in the granulation tissue.

In conclusion, the findings confirmed that subcutaneously implanted polyurethane sponges offer a satisfactory model for studying granulation tissue and superimposed inflammatory changes induced by impregnating the sponges with a variety of materials prior to implantation. Polyurethane sponge implants may be studied for at least 7 weeks in the rat without undergoing breakdown.

The site of sponge implantation had a significant influence on both the dry weight gain of the sponges and the extent of tissue in-

growth after 14 days. Posterior implantation sites reacted less vigorously and less uniformly to the implants.

The different reactivities of the anterior and posterior sites may have been due to the different patterns of forces applied to the sponges by muscular action. Alternatively, anterior sites may have been endowed with a richer blood supply which supported the ingrowth of a greater amount of tissue. Dasler et al (1960) had also noted variations in responses at different implant sites in rats and found that comparable sites were limited to a small area of the mid-back. No explanation of this variability was given. Bole and Robinson (1962) also found that sponge dry weight gain varied considerably at different implantation sites in guinea pigs.

As a consequence, the number of sponges implanted per rat in subsequent experiments was limited to one left- and one right-sided implant at the anterior implantation site.

2 Reaction to HAC Impregnated Sponges

The synthetic hydroxyapatite chosen for investigation in this series of experiments was selected using the following criteria:

a) its ready availability;

b) different batches had similar characteristics;

c) its use in previous studies in dog stifle joints (Schumacher et al, 1977), in PMN suspensions (Maurer and Schumacher, 1979) and in synovial cell culture (Cheung et al, 1981a).

The crystal aggregates as supplied by the manufacturer varied greatly in size and shape. The aggregates consisted of closely packed,

randomly orientated rod-shaped crystals when examined by TEM.

The technique used to impregnate the sponges with HAC was developed to ensure an even distribution of the crystal aggregates on all surfaces of the sponge. By suspending the HAC in 90% alcohol and turning the sponges frequently during drying, the sponges dried quickly as the alcohol evaporated and gravitational settling of the particles to the bottom of the sponges was avoided. The SEM appearance of part of a sponge impregnated with 20mg HAC confirmed the even distribution of the HAC. An interesting observation was that the sponge fibres were concave and consequently were efficient at retaining the impregnated material.

Reweighing the dried impregnated sponges ensured that they held the required amount of HAC. Sponges weighing less than the target weight were discarded and those weighing more were gently tapped to shake out excess HAC.

Sponges impregnated with from 1 to 50mg HAC and implanted for 14 days in Porton rats had no effect on rat weight gain. This parameter was used as a measure of the general health of the animals since they were undergoing a rapid growth phase. Even 50mg of HAC per sponge did not influence the rate or amount of weight gained when compared with the weight gain of control rats implanted with plain sponges. The HAC therefore did not influence the systemic status of the animals as measured by their weight gain.

The dry weight gain of plain (control) sponges was similar in all experimental groups. Sponges impregnated with 5, 10, 20 and 30mg HAC gained significantly less weight than control sponges while sponges

containing 50mg HAC gained significantly more weight. This response is difficult to explain but may reflect a more intense inflammatory response and proteinaceous exudate directed towards the 50mg HAC sponges. When comparing the weight gain with extent of granulation tissue ingrowth, it was evident that sponges containing 50mg of HAC had the least ingrowth, although there was a large variation within these sponges ($\pm$ 22%S.D. compared with $\pm$ 6%S.D. for controls). Less tissue ingrowth would suggest that there was more sponge volume that could be occupied by a relatively heavier fluid exudate.

Sponges containing 30mg HAC were also significantly less infiltrated by granulation tissue than control sponges. The variation in ingrowth in these sponges was $\pm$ 14% S.D. which was also considerably larger than the controls. However, the smaller degree of ingrowth was associated with decreased rather than increased weight gain as had been measured for 50mg HAC sponges.

Only sponges containing 20mg HAC had a significantly greater amount of tissue ingrowth than plain sponges, although the difference was only 7%.

The most egregious feature of the histological response was the tendency for the HAC to form large clumps which appeared to be randomly distributed throughout the sections. Clumps of HAC were also found in the central zone of the sponges which was free of granulation tissue. Clumping was probably the result of the wound and inflammatory fluid entering the sponge in the first few days after implantation and dislodging the HAC from the sponge surface. HAC has a marked tendency to form clumps, as has been observed by Schumacher (1977) and Cheung et al (1981a).

Quantities of HAC in sponges of 20mg and over elicited an intense macrophage and MNGC reaction that was confined to the immediate vicinity of the HAC clumps. The granulation tissue between the HAC clumps had a moderate lymphocytic infiltrate. These cells appeared to be more numerous than in granulation tissue that had grown into plain sponges. Perivascular aggregates of lymphocytes and plasma cells were found in human joint capsules affected by heterotopic calcification (Doyle, 1982). These cells were not directly associated with the calcified material and the present finding corroborates Doyle's report.

An acute inflammatory response to as much as 50mg HAC per sponge was not observed 14 days after implantation. Previous studies have found HAC to elicit an acute inflammatory response for a few days after its administration in rat foot pads (Denko and Whitehouse,1976), in rat pleural cavities (Glatt et al,1979) and at human subcutaneous injection sites (Dieppe et al,1982). None of these investigations had examined the longer term effects of HAC.

In subsequent experiments, a standardized amount of 20mg of particulate material was used for sponge impregnation. This quantity was chosen because it evenly covered all surfaces of the sponge matrix elicited an acute and chronic inflammatory reaction and caused stimulation of granulation tissue ingrowth.

3 Modification of Shape and Size of HAC Aggregates

The tissue response to physical modifications of the HAC aggregates was evaluated for alterations in inflammagenic potential. Smaller crystals of HAC and CPPD provoked a more intense acute inflammatory

reaction (Dunn et al, 1978). In general, it is considered that smaller crystals are more inflammagenic than larger ones, although definitive evidence is lacking.

Both sonication and grinding of the HAC resulted in the reduction in size of the crystal aggregates and the production of rounded particles. Smashing the HAC produced irregularly shaped particles of varying sizes. Of all the techniques used, only sedimentation produced very small crystal aggregates the majority being less than 0.04μ in diameter. These were in the mid-range of size of synovial fluid HAC particles detected by Dieppe et al (1976).

The successful separation of the smallest crystal aggregates present in the HAC supplied by the manufacturer by overnight settling in PBS in the presence of 5% rat serum demonstrated the avidity of HAC for protein and the abolition of the marked clumping tendency of HAC. The aggregation of milled cobalt-chrome particles was inhibited by the addition of 20% foetal calf serum to the milling fluid (Garrett et al, 1983). This effect was attributed to protein adsorption by the particles which altered the surface charge characteristics so that particle repulsion superceded particle attraction. A similar phenomenon is considered to have occurred with the HAC in the present study.

Sponges impregnated with sedimented HAC gained significantly more dry weight (an extra 17%) than plain control sponges or the other impregnated sponges. According to the preceding discussion regarding the significance of sponge dry weight gain, this result indicated that smaller crystal aggregates had elicited a more intense inflammatory response. The histological appearance however, did not support this view.

The extent of granulation tissue ingrowth was greatest into sponges impregnated with unmodified HAC (11% more than controls). Sponges containing ground HAC had 11% less ingrowth than controls and the other preparations responded similarly to the controls. Unmodified HAC aggregates had a slight inductive effect on granulation tissue ingrowth of the same order as that observed using 20mg of unmodified HAC per sponge.

The smashed HAC attracted the most persistent and intense PMN reaction of all the HAC preparations tested. This reaction was only observed in the central tissue-free zone of the sponges. The reaction to clumps of HAC in granulation tissue was similar for all preparations and consisted of an intense macrophage and MNGC reaction with endocytosis of crystal aggregates. Granulation tissue between the HAC was moderately infiltrated by lymphocytes in the sonicated and sedimented HAC preparations. These cells were less prominent with the other HAC preparations.

A noteworthy feature of the ground, smashed and sedimented HAC impregnated sponges was the formation of very large clumps of HAC up to 1mm in diameter. Clumping was less prominent for unmodified HAC and sonicated HAC. The reason for this pattern of clumping is not clear. The protein rich exudates in which the sponges are soaked soon after implantation should have resulted in protein adsorption by the HAC which had prevented aggregation of the particles *in vitro*. The behaviour of HAC *in vivo* therefore did not parallel their *in vitro* characteristics. Cellular and humoral products released as part of the acute inflammatory reaction occurring in the first few days may have caused removal of adsorbed protein from the HAC and alteration of the crystal

surface. The aggregates would then be more liable to undergo clumping. The degree of clumping could have been an indication of the intensity of the inflammatory reaction induced by a particular modification of the HAC. If this premise was valid, then the ground, smashed and sedimented HAC were associated with the most intense inflammatory responses.

Sponges impregnated with sedimented HAC and implanted for 8 weeks demonstrated the persistence of large clumps of HAC and a macrophage and MNGC reaction. In addition, the HAC had been encapsulated by well defined collagen bundles. Breakdown of the sponge itself was also seen and corresponded with observations discussed earlier. Longer term implantation may be of value to observe the fate of the HAC but an alternative implantation model would be required since tissue reaction to sponge breakdown products is superimposed on the pathological process being studied.

TEM of the impregnated sponges provided further valuable insight into the cellular reaction to HAC. TEM of unmodified HAC containing sponges vividly demonstrated the endocytosis of large amounts of crystal aggregates by macrophages and MNGC, thus confirming the information obtained by light microscopy. The cells were capable of taking up large pieces of HAC (relative to the cell size) without any apparent affect on cell viability, as assessed by their intact cell membranes and lack of internal degenerative changes. The endocytosed HAC was <u>non toxic</u> to the cells. Although the crystal aggregates did not appear to be encompassed by a membrane it is possible that the intense electron density of the HAC had masked one from view. The cells were actively engaged in the breakdown of pieces of HAC by disaggregation into its component microcrystals. Some of these were separately packaged into

intracytoplasmic vacuoles. The intracytoplasmic fate of the HAC for implantation periods longer than 14 days has not been studied.

Ground HAC aggregates had also been avidly endocytosed by macrophages and MNGC. Endocytosis of large amounts of HAC was compatible with cell viability. The ground HAC was membrane bound and a similar pattern of disaggregation as for unmodified HAC was observed, presumably as a result of cell activity. It is unclear why the ground HAC was less electron dense than the unmodified HAC. TEM of ground HAC before implantation had not been carried out as a control measure.

The significance of these findings in relation to apatite deposition disease lies in the capacity of the phagocytic cells to take up massive amounts of HAC without detrimental effect. Subsequently, the intracellular clumps of HAC are slowly disaggregated and broken down. The release of chemical mediators such as prostaglandins, collagenase, neutral proteases as a result of phagocytosis could influence the metabolism of adjacent cells. This experiment has provided morphological evidence to support the theories of Dieppe (1977); Dieppe and Doherty (1982) and McCarty (1983) who have implicated crystal phagocytosis indirectly in the mediation of apatite deposition disease by the release of inflammatory mediators or proteolytic enzymes. McCarty (1979) had suggested that rupture of phagolysosomal membranes may result from hydrogen bond mediated membranolysis by the HAC but there was no evidence of this having occurred in the present study.

The size or shape of the HAC crystal aggregates did not appear to have a significant influence on the tissue or inflammatory response to impregnated sponges. The clumping of the HAC that followed sponge im-

plantation had probably masked any individual differences.

4 Reaction of Dark Agouti Rats to the Implantation of Sponges Impregnated with HAC

Dark Agouti rats responded in a different manner than the Porton rats used for implantation of HAC containing sponges described above. Dry weight gain of HAC sponges was one and a half times that of plain sponges after 7 days, but by 14 days the values were the same for control and test implants. It is conceivable that the large weight gain recorded for HAC sponges after 7 days resulted from the exudative component of the inflammatory reaction stimulated by the HAC. There appeared to be a considerable proteinaceous exudate in the granulation tissue free central part of the sponges and a PMN infiltrate around the HAC. In control sponges there was only a light proteinaceous exudate in this sponge zone and some of the material appeared to be used as a scaffold for the granulation tissue ingrowth. The cellular infiltrate was minimal. The material was most likely the protein component of the inflammatory exudate which was precipitated by the formal-saline fixative. The histological picture therefore provided corroborative evidence for the large increase in dry weight of HAC sponges being the result of increased inflammatory activity.

The most interesting result of this experiment was the significant differences in ingrowth of granulation tissue in plain and HAC sponges at both 7 and 14 days after implantation. The HAC had a stimulatory effect on granulation tissue ingrowth. This effect was more pronounced in DA rats than in Porton rats using the same amount of unmodified HAC in each sponge. After 14 days, HAC sponges in DA rats had 25% more granulation tissue ingrowth than plain sponges and the increase found

in Porton rats was only 11%.

Despite the large difference in the amount of tissue occupying plain and HAC sponges, sponge dry weight gain at 14 days was the same for both. The lack of correlation between the quantity of tissue in a sponge and its dry weight gain has been clearly shown in this experiment.

Histology of the plain and HAC sponges also demonstrated the accelerating effect of HAC on granulation tissue ingrowth. After 7 days, granulation tissue had just started to infiltrate the plain sponges whereas HAC sponges had more extensive ingrowth of better organized tissue. There were more intense acute (PMN) and chronic (macrophage) responses occurring in the HAC sponges at this time. After 14 days, ingrowth into HAC sponges was far in advance of that into plain sponges. In addition, the granulation tissue showed better organization with prominent deposition of collagen bundles and slightly more infiltration of lymphocytes than in plain sponges. The macrophage and MNGC response to the HAC was similar to that of the Porton rats using the same implants.

As in an earlier experiment, tissue ingrowth after 14 days into plain sponges in DA rats was significantly less than in Porton and Lewis rats. However, DA rats responded more vigorously than Porton rats to the HAC sponges which had an extra 25% of granulation tissue than plain sponges in the same time period. DA rats seemed to mount a more active inflammatory reaction in response to the HAC than Porton rats. Subsequent experiments used DA rats to study other aspects of microcrystals and inflammation.

5 Drug Effects on HAC-Induced Inflammation

The drug dosing schedules and route of drug administration were selected from schedules used in large scale studies in rats with adjuvant arthritis (Garrett, personal communication).

Prednisolone was included as a positive control because of its well documented antiinflammatory actions (Popper and Watnick, 1974), including its use in sponge implantation studies (Clarke et al, 1975).

D-penicillamine (DPA) (B,B-dimethylcysteine) is a breakdown product of penicillin used in the treatment of inflammatory diseases associated with enhanced connective tissue growth, including progressive systemic sclerosis and rheumatoid arthritis. It is also used in the treatment of heavy metal poisoning because of its ability to chelate metals. The pharmacological effects of DPA have been summarized by Vapaatalo et al (1979). They include an effect on elastin matrix formation, some immunosuppressive activity and inhibition of lymphocyte transformation. This agent also affects the intra- and intermolecular crosslinking of collagen (Nimni, 1968; Jayson, 1979).

The copper/D-penicillamine complex (copper/DPA) is an experimental antiinflammatory compound that uses the antiinflammatory properties of copper (reviewed by Whitehouse and Walker, 1978) while reducing its toxic effects by binding it to the DPA molecule. The complex molecular formula, determined by Birker and Freeman (1977) is:

$$\left[Cu^{II}_{6}Cu^{I}_{8}\,\text{D-penicillamine}_{12}\,Cl\right]^{5-}$$

Its molecular weight is about 2,200 (Lengfelder and Elstner, 1978).

The physiology and pharmacology of copper complexes and their potential role as antirheumatic agents has been the subject of an extensive review by Sorensen (1978). The biologic activity of copper complexes are thought to be due to:

a) induction of lysyl oxidase mimetic activity.
b) induction of superoxide dismutase mimetic activity.
c) modulation of prostaglandin synthesis.
d) modulation of histamine activity.
e) stabilization of lysosomal enzymes.

Sodium aurothiomalate (SATM) has been used in the treatment of rheumatoid arthritis for over 50 years. There is little understanding of its antiarthritic properties despite wide-ranging investigations. A recent review of the *in vivo* and *in vitro* effects of gold compounds is informative (Liebfarth, 1981). The drug has a number of systemic actions including a suppressive effect on the proliferation of bone marrow and on the differentiation of monocytes to macrophages at sites of inflammation, according to Vernon-Roberts (1979).

Adjuvant injection in rats results in a large range of systemic side effects (Whitehouse et al, 1974) including immune stimulation, altered liver function and arthritic changes in the joints. Adjuvant arthritis in rats mimicks some features of rheumatoid arthritis and is widely used to test the efficacy of antirheumatic and antiinflammatory agents (Billingham and Davies, 1979).

Rat weight gain was used as a measure of the effect of the drugs on the well-being of the animals. Whereas saline treated rats gained 5g

over the 14 day experimental period, all drugs administered resulted in loss of weight by the animals. After 14 days DPA, copper/DPA and SATM caused a similar amount of weight loss (6-11g). The adjuvant treated group lost 23g, an amount consistent with the systemic effects of adjuvant disease and the disabling effects of severe adjuvant induced arthritis apparent at the end of the experiment.

Prednisolone treatment caused the loss of 44g of weight mainly due to the catabolism of protein and fat deposits and other anabolic properties of steroids. The animals appeared healthy and retained sleek, shiny coats throughout steroid treatment. Prednisolone treatment caused a significant decrease in the weight gain of both plain and HAC sponges when compared to sponges in saline treated rats. This effect was due to the suppression of the fluid and cellular phases of the inflammatory response.

Copper/DPA treatment resulted in decreased sponge weight gain in both plain and HAC sponges compared with saline treatment. This effect was less than half that found with prednisolone treatment. Decreased sponge weight gain may have resulted from the antiinflammatory activity of the compound as was evidenced by the lesser degree of collagenization of the granulation tissue in the sponges and a less intense macrophage and MNGC reaction to the HAC. Both a polymeric cuprous complex with DPA containing 26.7% copper, and a cupric disulphide complex containing 15.4% copper administered by subcutaneous injection to rats caused decreased dry weight gain of implanted cotton pellets (Sorensen, 1978). DPA alone was ineffective in reducing the dry weight gain of the pellets. In the present study, the copper/DPA complex also had much more antiinflammatory activity than the DPA alone.

In both SATM and adjuvant treated rats, HAC sponges gained significantly more weight than plain sponges, whereas the weight gain of control and test sponges in the other treatment groups was similar. The intensity of the inflammatory reaction to the HAC and the extensive fibrosis observed in the granulation tissue arguably explains the pattern of sponge weight gain measured. Vernon-Roberts et al (1973) found that SATM treatment reduced the dry weight of cotton pellets implanted for 4 days. The effect was more pronounced with increasing drug dosage (1-10mg given on alternate days) but the reduction in dry weight was less than that produced by prednisolone. The cellular phase of the inflammatory response was suppressed by gold treatment.

The extent of ingrowth of granulation tissue into HAC sponges was significantly greater than into plain sponges for all treatments other than the prednisolone treated group. Sponges in prednisolone treated rats had approximately 10% of their area occupied by granulation tissue, confirming the powerful antiinflammatory effects of this drug at the dosage used.

Tissue ingrowth into plain sponges in DPA treated rats was slightly less than in saline treated rats. The interference induced by DPA on the rate of collagen synthesis in tissue culture (Jayson, 1979) and its inhibitory effects on the cross-linking of newly formed collagen may have accounted for the slight modification of the rate of tissue ingrowth into the sponges.

Plain sponges in SATM and adjuvant treated rats had been infiltrated by significantly more granulation tissue than in saline treated animals. SATM and adjuvant appeared to have accelerated the rate of infiltration of granulation tissue, even in the absence of an irritant

in the sponges.

The differences in the histological responses in the six treatment groups were interesting and informative.

Prednisolone treatment failed to prevent the formation of a fibrous capsule around plain and HAC sponges. In the latter case the capsule was relatively dense and there was a macrophage and MNGC reaction to HAC within the capsular tissue. Complete suppression of an inflammatory response had not occurred. A marked reduction in the cellular infiltration into sponges implanted for 5 days was also measured as a result of prednisolone treatment in acute and chronic inflammatory models (Woodland et al, 1977).

DPA treatment did not appear to have an influence on the histological appearance of either plain or HAC sponges. This histological finding correlated with the biochemical data obtained by Paulini et al (1977a,b) who studied the collagen content of tissue growing into polyurethane sponges. Early and late doses of DPA were administered for periods of 3 weeks but there was no effect on the <u>total</u> collagen content of the sponges. However, there was a significant increase in the neutral salt-soluble collagen fraction associated with both dosing schedules. The DNA content of sponges was also raised and was related to an increased number of fibroblasts, although no histology was presented.

Copper/DPA treatment resulted in the formation of a less structured and less collagenized granulation tissue with a decreased cellular reaction to the HAC. Copper/DPA thus exerted an antiinflammatory effect in addition to modifying the connective tissue response. The

copper/DPA complex used in the present study has a high superoxide dismutating activity *in vitro* (Lengfelder and Elstner, 1978). It would therefore be effective in destroying oxygen derived free radicals that are thought to be associated with the inflammatory process (Whitehouse, 1978). The reduced intensity of the macrophage and MNGC response around the HAC may be linked to this effect of the drug.

The application of an ethanolic copper salicylate complex to the skin of rats suppressed the inflammatory response to HAC in rat foot pad oedema tests (Walker et al, 1980). The toxic properties of copper (II) mentioned by Denko and Whitehouse (1976) had been abolished. Walker et al concluded that clinical trials of copper complexes should be undertaken in OA associated with HAC, which they called the "silent inflammagen".

The reduction in the intensity of the chronic inflammatory response to HAC impregnated sponges as a result of copper/DPA treatment is of relevance to the future treatment of diagnosed cases of apatite deposition disease. Commonly used antiinflammatory agents may bring about remission by copper complex formation *in vivo* (Sorensen,1976). Further evaluation of these compounds is warranted to define their mode of action, efficacy and to determine any side effects associated with their administration.

SATM treatment had a pronounced stimulatory influence on the collagenization of the granulation tissue and the macrophage and MNGC response to the HAC in the test sponges. A similar degree of intensity of the cellular and fibrotic reaction to the HAC deposits had not been observed in previous implants. There is growing evidence that gold may have both stimulatory and suppressive actions, although much of the

information has been derived from in vitro studies. The stimulatory response observed in this model may have involved the following known actions of gold:

a) By stabilizing collagen (Deyl et al, 1970) thereby rendering it more resistant to breakdown during tissue turnover and accounting for the prominent fibrosis in the HAC sponges.

b) Enhancement of T-cell function (observed by Walz and Griswold, 1978) which can increase the level of macrophage activation via lymphocyte products (eg macrophage activating factor). The intense concentration of macrophages around the HAC provided evidence for this effect of gold. Alternatively, the HAC itself or its protein coating may have been chemotactically attractive for macrophages and may have encouraged the formation of MNGC observed around HAC in sponges in saline treated rats.

c) Enhanced cellular response to the HAC could have been stimulated by other known side-effects of gold therapy (reviewed by Vernon-Roberts, 1979) such as suppression of a wide range of lysosomal enzyme activity, inhibition of enzyme release and extended depression of phagocytc activity of macrophages. The phagocytic function of macrophages arriving at the site of HAC deposition may have been impaired by SATM and the cells were consequently less able to deal with the HAC.

This experiment has provided evidence for an enhanced collagenous and macrophage and MNGC respose attributable to gold therapy in sponges impregnated with HAC. As a corollary, since HAC has recently been found in patients suffering from RA (Reginato et al, 1982b), gold therapy given to treat the RA may also act in a stimulatory fashion.

Adjuvant treatment also stimulated inflammatory activity and fibrosis, this effect being most noticeable in HAC sponges. The presence of an increased infiltrate of lymphocytes near the HAC aggregates, compared with HAC sponges in saline treated rats suggests an immunostimulatory action of the adjuvant. The number of fibroblasts in granulation tissue growing into polyurethane sponges implanted for 6 weeks was increased by adjuvant treatment, although the collagen content was the same as the controls (Paulini et al, 1976). Nakamura and Shimizu (1979) also found that the granulation tissue that formed around felt pellets implanted in adjuvant treated rats was qualitatively different from that in intact rats. The felt pellets had been implanted subcutaneously for periods up to 245 days. Implants in adjuvant treated animals gained 30-40% more weight than implants in control rats. It was speculated that the production of excess tissue was related to the numerous systemic and immunological side effects of adjuvant treatment. Parnham (1980) produced corroborative evidence for lymphocyte mediated enhancement of the cellular reaction to sponges implanted in adjuvant treated rats and suggested that it was lymphocyte-mediated.

It would be interesting to extend the present impregnated sponge model in adjuvant treated rats to study the effects of the same series of drugs on enhanced granulation tissue formation. Another obvious and potentially useful extension of the present study would be the evaluation of some non-steroidal antiinflammatory agents, including those currently in clinical use for the treatment of osteoarthritis.

6 The Inflammatory Nature of a Variety of Microcrystals in DA Rats.

This experiment was carried out to assess the inflammatory nature

of some calcium phosphates and other microcyrstalline substances using the impregnated sponge model. It was also designed to give information regarding the comparative nature of the inflammatory response to the microcrystals, most of which are associated with crystal deposition diseases.

Small and large HAC aggregates were included for further assessment of the relationship of aggregate size to inflammatory potential.

CPPD crystals which form the intraarticular deposit in pseudogout (reviewed by Resnick and Resnick, 1983) were included in this study as well documented inflammagens. The CPPD preparation impregnated into the sponges had been used in acute rat foot pad oedema tests by Denko and Whitehouse (1976).

Brushite is another calcium phosphate that has been found within the menisci of human knees (Faure et al, 1977; McCarty, 1983). Its occurrence is rare and little is known about its role (if any) in joint disease. However, it served as a convenient positive control for the other forms of calcium phosphate known to be associated with arthropathy.

MSU crystals, (the inflammagen in gout) were another positive control against which the inflammatory nature of HAC could be compared.

Calcium carbonate was chosen as a microcrystalline material that is not associated with human disease.

Sponge dry weight gain after 3 days was essentially similar in all treatment groups with the exception of MSU sponges which gained consid-

erably less weight. This finding is paradoxical since histology revealed an intense acute inflammatory response with an abundant fibrinous exudate within the sponges. The response to MSU was the most florid of the microcrystals implanted. As discussed earlier, the dry weight gain of sponges in the first few days after implantation is attributable to the protein content of the inflammatory exudate. However, the intensity of the acute inflammatory response observed towards MSU crystals did not correlate with the small increase in sponge weight measured.

Immersion of the sponges in liquid nitrogen to prevent excessive and uncontrollable loss of fluid during bisection may have resulted in altered fluid content. The varying intensity of the inflammatory reaction in the sponges may have affected the rate of penetration of the liquid nitrogen and therefore the rate of displacement of inflammatory exudate. The dry weight gain measured would not have been representative of the actual weight. An alternative explanation is that the abundance of fibrin resulted in contraction of the sponge, thereby reducing the sponge volume and limiting the content of proteinaceous exudate.

Little difference was found in the dry weight gain of small and large HAC and CPPD sponges after 10 days but, after 14 days the weights of sponges impregnated with these microcrystals were significantly higher than the controls. The mild chronic inflammatory cell response observed in these sponges is considered as an unlikely explanation for this finding. The considerably increased amount of tissue ingrowth compared with control (plain) sponges did not account for this disparity either, since it was common to brushite, MSU and calcium carbonate sponges which recorded similar dry weight increases as control sponges.

The results obtained from the measurement of the extent of granul-

ation tissue ingrowth is of more interest. After 10 days, small and large HAC had a similar stimulatory effect on tissue ingrowth. CPPD sponges were slightly less infiltrated than plain sponges. By 14 days, the pattern had changed considerably. Ingrowth (above that into the respective control sponges) into small HAC sponges was 3.5 times more extensive than into large HAC sponges, indicating a basic difference in the tissue response to the size of the crystal aggregates in DA rats. Despite the extensive clumping of both small and large HAC aggregates observed histologically, the small HAC nevertheless exerted a greater stimulatory effect on granulation tissue ingrowth than large HAC. Previous experiments in Porton rats showed that unmodified HAC (containing a large range of aggregate sizes) caused a slight stimulation of granulation tissue ingrowth whereas ingrowth into small HAC sponges was similar to that into plain sponges. Differences in the response of each rat strain probably accounted for the disparate findings of the two experiments. DA rats have been found to react quite differently than Porton rats to plain and HAC sponge implants in terms of ingrowth dynamics.

It was interesting to observe that all sponges impregnated with the microcrystals had significantly more granulation tissue ingrowth than plain sponges after 14 days. MSU had stimulated the most ingrowth and calcium carbonate was the second most effective. The stimulation of tissue ingrowth into sponges in DA rats therefore was not specifically related to the presence of HAC in sponges. Rather, the presence of an irritant material eg calcium carbonate, in the sponges induced alteration of the growth rate of granulation tissue. The mechanisms whereby this phenomenon occurs are obscure but they may be related to the release of stimulatory factors from cells during phagocytosis. Alteration of connective tissue metabolism and the consequent tissue

destruction seen in human joints affected by crystal deposition disease may be mediated by similar mechanisms.

Histology of the sponges provided confirmatory evidence for the disparate degree of intensity and type of inflammatory response to the different microcrystals at the time periods studied. At 3 days after implantation, MSU elicited the most intense acute inflammatory response which was characterized by a dense fibrinous exudate. The inflammagenic properties of MSU have been reviewed by Garcia Leme (1978). Fibrin was not a prominent component of the response to the other materials tested, as determined histochemically using the MSB stain.

Calcium carbonate elicited an intense and persistent acute inflammatory response that was confined to the vicinity of the particles.

The acute inflammatory response to both small and large HAC aggregates was consistent with those previously reported in human and animal models of acute inflammation (Denko and Whitehouse, 1976; Glatt et al, 1979; Dieppe et al, 1982). The acute inflammatory response to HAC declined after three days. The brushite particles showed a less pronounced clumping tendency than the HAC and this may have accounted for the dispersal of the PMN infiltrate in the sponges. Many PMN were necrotic as judged by the large amount of nuclear dust around the HAC. CPPD particles were also less subject to clumping and were widely distributed throughout the sponges, but the intensity of the PMN response was less than to the HAC.

At 10 days after implantation there was little evidence of persistence of an acute inflammatory response to small HAC, large HAC or CPPD. Macrophages and MNGC were becoming more prominent around the

clumps of the calcium phosphates. Brushite, calcium carbonate and MSU were not tested at this time period because of a shortage of animals.

At 14 days after implantation an acute inflammatory response persisted in sponges impregnated with calcium carbonate and MSU particles. Considerable fibroblastic activity was also a feature of the granulation tissue in these sponges. In calcium carbonate impregnated sponges, macrophages that had endocytosed von Kossa positive material were focally distributed in the granulation tissue. No accumulation of macrophages were observed in MSU sponges. However, the prominent fibrosis in these sponges was similar to that in HAC sponges implanted in gold and adjuvant treated rats. None of the calcium phosphates tested elicted an acute inflammatory response after 14 days. Both small and large HAC clumps were surrounded by macrophages and MNGC and it was evident that large amounts of material had been endocytosed by the MNGC. CPPD and brushite particles elicited a mild macrophage response with little evidence of giant cell formation.

Histologically, there did not appear to be any marked difference in the acute and chronic inflammagenic properties of small or large HAC aggregates. The predisposition of HAC to form clumps probably negated any increased inflammagenic properties of smaller crystals. The comparative inflammatory propensities of the microcrystals implanted were rated in the following descending order of intensity:

Acute response at 3 days	1. MSU
	2. Calcium carbonate
	3. Small, large HAC
	4. CPPD
	5. Brushite

Chronic response
1. Small, large HAC
2. CPPD
3. Brushite
4. Calcium carbonate
5. MSU

The acceleration of granulation tissue ingrowth into sponges implanted in DA rats was common to a wide range of particulate materials, including HAC. However the HAC evoked the most intense macrophage and MNGC reaction.

7 Induction of HAC Deposition in vivo by Lead Acetate Injection into Sponges Containing Granulation Tissue

The results clearly demonstrated that calcergic reactions can be induced in vascular granulation tissue within the matrix of subcutaneously implanted polyurethane sponges. Moreover, the observation that calcific deposits were present in the centres of sponges unoccupied by cells or fibres as early as five hours after the injection of calcergen into the sponges indicated that, in the initial stages at least, the calcergic reaction is essentially chemical in nature.

The detection of calcification in calcergic lesions by histochemical techniques has been discussed previously (McClure, 1979; 1980). A positive von Kossa reaction was indicative of the presence of phosphate which was usually, but not always, associated with calcium ions. In the present study, confirmation of the presence of calcium occurring at the site of VK positive material was achieved using the alizarin red S stain on serial histological sections.

The presence of calcification in zones free of cells and fibres, and the finding of intensely electron dense areas within aggregates of

needle-shaped crystals two days after lead acetate injection, supports the postulate of Gabbiani (1964) that an insoluble salt of a calcergen represented the first nucleus that elicited the accumulation of calcium phosphate. Thus the electron dense areas were found to contain lead and phosphate, whereas the surrounding material contained calcium and phosphate as determined by Xray dispersive analysis. These findings are similar to the lead triphosphate structures, described by Gabbiani et al (1970), around which thin rod-shaped crystals appeared five hours after lead acetate injection. Later studies showed that lead pyrophosphate is the first lead containing mineral deposited at sites of calcergy induced by PbAc (Kato and Ogura, 1978).

The Ca:P ratios of the calcergic deposits in the present investigation were slightly less than that of synthetic HAC controls. The ratios also varied marginally for different calcific deposits in the same TEM section. Calcified lesions in simple calcergy have layers exhibiting different Ca:P ratios (Shah et al, 1982). However, the Ca:P ratios were consistent with those of HAC rather than other calcium phosphates and the morphological appearance of the material was consistent with that of HAC in bone (Cameron, 1972).

While the studies of Gabbiani et al (1970) involved mature connective tissues, the present experiments clearly showed that the early reaction may take place in a cell- and fibre-free environment. However, hydroxyapatite crystals thereafter rapidly formed in close association with collagen fibres in the granulation tissue, and nucleation by these fibres may play a role in the promotion of the calcergic reaction.

The absence of mast cells from the granulation tissue that had grown into the sponges was a consistent finding in sponges injected

with saline or lead acetate. That the absence of mast cells from sponges was not artefactual was supported by the observation that mast cells still could be demonstrated histochemically around the sponge perimeter in the zone of dense connective tissue formed at the junction of sponge and subcutaneous tissue. No mast cells were found in the granulation tissue inside polyvinyl sponge implants in guinea pigs, even at 144 days after implantation (Bole and Robinson, 1962). However, mast cells were present in the loose tissue at the periphery of the sponges.

The role of mast cells as mediators of local calcergy was proposed initially by Selye (1962), and Selye et al (1964) later claimed that mast cells had a causal role in calcergy. The present findings clearly demonstrate that the calcergic reaction may occur without the participation of mast cells or their granules. This is the first report of a lack of involvement of mast cells in calcergy occurring in rodents, although Bridges and McClure (1972) successfully induced simple calcergy in the wattle of the domestic fowl which does not possess conventional mast cells.

Comparison of the dry weight increases of PbAc and saline injected sponges shows significantly earlier and greater increases for the PbAc treated sponges. Presumably this is due largely to the weight of the induced calcific deposits and the inflammatory response associated with calcification. The total dry weight of subcutaneous sites injected wth PbAc showed an identical pattern of change over the same time period (Takimoto, 1973). The increase in weight of these areas was due to increases in their calcium and phosphorus content.

A morphological difference in the leading edge of the granulation

tissue between test and control sponges, with condensation of the innermost border of the tissue in sponges injected with PbAc persisted for two days following injection.

It must be stressed however, that there was no apparent arrest of ingrowth following either injection. The interpretation of this aspect of the present study needs to take into account the fact that, at the time of injection of saline or PbAc, granulation tissue already occupied up to 70% of the sponge and rendered difficult the assessment of rates of further ingrowth. With that qualification, the findings support the concept that the calcific deposits do not exert a fibrogenic response up to 21 days after calcification has been initiated. However, there were qualitative differences in that the number of macrophages and MNGC was greater in the PbAc injected sponges, and these cells were associated closely with the calcific deposits. In the saline injected sponges MNGC also were observed but these were fewer in number and related to the surfaces of the sponge matrix.

An acute inflammatory reaction was not a prominent feature in these studies. A few acute inflammatory cells appeared early in the calcergic reaction but disappeared once this was established after two days. Thereafter, the calcific deposits were associated with macrophages and MNGC. Similar findings have been reported by Doyle et al (1979a) following the induction of calcergy in rats by the subcutaneous injection of potassium permanganate. Thus, while Dieppe et al (1976) induced acute inflammation in the pleural cavities of rats by the injection of HAC, it would appear that HAC formed within granulation and connective tissue <u>in vivo</u> provoke a transient acute inflammatory reaction. This latter conlusion is in keeping with the histopathological findings in calcifying tendinitis, in which condition the deposits of

hydroxyapatite are surrounded by macrophages and MNGC without the presence of PMN (Thompson et al, 1968; Uhthoff, 1975; Uhthoff, Sarkar an Maynard, 1976).

The hydroxyapatite deposited within the granulation tissue of sponges injected with PbAc elicited a less intense macrophage and MNGC reaction than that which was observed at the junction between sponge and surrounding connective tissue, or at the sites of subcutaneous injection of PbAc. This observation may reflect a basic difference in the reactivities of granulation tissue and mature connective tissues in dealing with biologically deposited particulate materials. The disparity may be related to the different degree of collagen maturity of the two tissues. In contrast, the subcutaneous injection of PbAc in mice resulted in the deposition of a calcific material that was accompanied by a minimal cellular reaction when studied for eight days (McClure and Gardner, 1976). However, the concentration of PbAc injected was 10 times less than that used in the present study.

Treatment of the animals with the antiinflammatroy drugs indomethacin and dexamethasone (Doyle et al, 1979a) and prednisolone and SATM (McClure, 1982) failed to inhibit the formation of calcified plaques in rats and mice. However, prednisolone reduced the intensity of the cellular response to the calcific material. McClure concluded that the agents tested:

> "do not influence the development of a local calcergy and might therefore be unlikely to influence the development of a crystal arthropathy."

The success of inhibitors of calcification in preventing the cal-

cergic response (EHDP) or delaying the rate of resorption of the calcergic deposits (Cl_2MDP) led Doyle et al (1979) to suggest a role for these drugs in the treatment of apatite deposition disease.

These studies of calcergy have shown that early calcification can occur independently of connective tissue components; that calcification of granulation tissue can take place in the absence of mast cells; and that calcification may not have any effect on the rate of ingrowth of pre-existing granulation tissue.

8 Induction of HAC Deposition in vivo by the Implantation of Sponges Soaked in Lead Acetate

The calcergic reaction that occurred within and around PbAc soaked sponges differed considerably from that observed when the sponges had been injected with PbAc in vivo . In general the deposits consisted predominantly of lead, although there was morphological and EDAX evidence of HAC deposition. The long term implantations also gave interesting information regarding breakdown of the sponge matrix.

Sponges soaked in lead acetate had gained significantly more weight than plain sponges at all time intervals. This difference was attributed to the deposition of calcergic material in PbAc sponges.

Dry weight gain of plain sponges increased steadily up to Day 14. The slight decrease in sponge weight measured between Days 14 and 35 coincided with maturation of the granulation tissue as the degree of collagenization increased. In fact, the shrinkage of sponges that occurred during the same period provided confirmatory evidence of collagen maturation and was similar to later events in wound healing, such as

the cicatrization of scars (Glynn, 1978). The same effect was even more evident between 49 and 140 days after sponge implantation when sponge dry weight decreased by 42% and total sectional area by 51%. The large reduction in dry weight reflected the extensive shrinkage of the sponges as well as the fatty infiltration that had affected approximately one third of the area of the histological sections examined. The same pattern of change in sponge dry weight gain occurred in polyvinyl sponge implants in guinea pigs at similar time periods (Bole and Robinson, 1962). Although degenerative changes related to breakdown of the sponge matrix were evident after 14 days, the sponge implantation model can be usefully applied to the study of the dynamics of the repair process and the contraction of wounds.

Myofibroblasts, cells intermediate between fibroblasts and smooth muscle cells, are probably responsible for the shrinkage of granulation tissue, owing to their contractile apparatus (Gabbiani et al,1972). The granulation tissue that grew into polyvinyl sponges implanted for up to nine months in rats was studied with ultrastructural, biochemical and immunological techniques by Gabbiani et al, (1976). Myofibroblasts were the most frequent type of fibroblast in the granulation tissue near the central part of the sponges after 21 days. The sponges gradually contracted with increasing duration of implantation and the proportion of normal fibroblasts and well-formed collagen fibres increased significantly. Evidence was presented that linked myofibroblasts with the synthesis of Type III collagen (usually associated with embryonic dermal tissues) in sponge granulation tissue.

In sponges soaked in PbAc, dry weight gain peaked after 7 days and then slowly decreased until Day 49. Although granulation tissue ingrowth was rapid between 7 and 14 days, no direct effect on dry weight

gain was measured. Between 49 and 140 days after implantation, the decrease in dry weight of PbAc sponges was half that measured in plain sponges. Persistence of the calcific deposits, less extensive fatty infiltration and decreased degree of degradation of the sponge matrix than in plain sponges all contributed to this phenomenon. PbAc soaked sponges did not shrink as much as plain sponges between 49 and 140 days (23% decreased area for PbAc soaked sponges vs. 51% for plain sponges), and it was evident that the calcergic material deposited within the sponges and in their capsular tissues had inhibited the extensive contraction seen in plain sponges.

Granulation tissue ingrowth into plain sponges followed a similar pattern as that reported in a previous experiment for Lewis rats. After 14 days, the rate of ingrowth of granulation tissue had slowed, and the sponges were completely filled by tissue after 28 days. However, in PbAc soaked sponges the amount and rate of tissue ingrowth was significantly less than in plain sponges at 5 and 7 days after implantation. It is probable that the high levels of lead on the sponge surface were inhibitory to cellular proliferation, and the calcergic deposits containing lead salts were toxic to the macrophages that had undertaken endocytosis. Gabbiani et al (1970) also noticed necrosis of cells that had taken up lead triphosphate in early calcergic lesions.

After 7 days, the rate of tissue ingrowth in PbAc soaked sponges increased markedly so that the extent of ingrowth equalled the ingrowth into plain sponges after 14 days. Removal of inhibition or stimulation of granulation tissue proliferation may have been encouraged by the appearance of HAC as outgrowths on the dense rounded deposits of lead, thus masking the inhibitory effects of lead on tissues and cells.

This type of calcergic reaction represents an extreme "end" of the calcergic response as it has been induced by very high local concentrations of the calcergen. No other reports in the literature of similarly induced calcergic reactions have been found. The basis of calcergic reactions per se may involve localization of the noxious substance (the calcergen) to prevent its wider distribution in the animal's system. An alternative view is that calcification is induced by the removal of the inhibitory effects of pyrophosphate on the calcification process after its chemical combination with lead (Takimoto, 1973). Localization is accomplished by the rapid influx of phosphate ions which combine with the soluble calcergen to form a precipitate of lead pyrophosphate. Although little is known about the physicochemical interaction of lead ions with calcified tissue, the formation of lead hydroxyapatite has been described (Verbeeck, 1981). It is likely that lead hydroxyapatite is the mineral deposited with calcium hydroxyapatite in bones and teeth in the presence of high blood lead levels. Lead pyrophosphate is the mineral found at early calcergic sites since the intensity of the characteristic Xray diffraction peak diminishes with time, indicating slow dissolution of the material (Kato and Ogura, 1978). The coating of the initial precipitate with calcium phosphate salts could be seen as the next stage in the rapid detoxification of the calcergen. As has been apparent in granulation tissue injected with PbAc, lead pyrophosphate favours the deposition of calcium phosphates which take on the morphological and EDAX characteristics of HAC after a few days (Takimoto, 1973). The reaction to sponges soaked in PbAc support this concept of the nature and function of the calcergic reaction.

Indeed, the histological and TEM appearance of sponges at 5 hours after implantation graphically reinforced the hypothesis that calcergy

is essentially a chemical reaction. At 5 hours, the sponges had been barely permeated by "wound fluid" which contained few inflammatory cells. Yet white deposits of calcergic material were visible macroscopically on the tissues that had come into contact with the sponge as well as in the central part of the sponge. The sponge implantation model thus has provided a unique method of determining to what extent fluids such as transudates and exudates and/or cells initiate and promote calcergic reactions. It can be stated confidently that the calcergic response in sponges soaked in PbAc almost certainly was a chemical reaction between calcergen and component(s) of the fluid transudate or exudate that permeated the sponges soon after their implantation. Intact cells were not associated with the reaction in its very early stages, although the soluble products of acute inflammatory cells or cells in the surrounding tissues can not be entirely excluded from involvement in the calcergic phenomenon.

Cells became a more prominent part of the reaction to the calcergic deposits one day after implantation when focal aggregates of PMN were found. At five days, the material had elicited an extensive acute inflammatory response and an intense macrophage response was seen near the calcergic deposits at the sponge periphery. In sponges injected with PbAc, PMN were not a prominent part of the inflammatory reaction at any time period after injection. The PMN infiltrate seen in sponges soaked in PbAc were probably chemotactically attracted to the deposits of lead pyrophosphate and were attempting to render the material less toxic. Subsequent coating of the lead compound with HAC removed the chemotactic stimulus for PMN and encouraged a macrophage and MNGC response. The acute inflammatory reaction did not play a role in the pathogenesis of PbAc induced calcergy in the mouse, according to McClure (1982) who found that prednisolone did not influence the simple calcer-

gic response.

As granulation tissue filled the sponges, the macrophage and MNGC response to the calcific deposits within the sponges was not as intense as that seen in sponges injected with lead acetate. MNGC were rare even at 21 days after implantation. Between 28 and 42 days, a considerable amount of material deposited in the capsular tissues had been removed, presumably as a result of the action of macrophages and MNGC. The slowly decreasing dry weight gain of the sponges over this time period supports the concept of removal of calcergic material.

The histological appearance of sponges soaked in PbAc after 140 days is worthy of comment because of its striking difference to plain sponges at the same time. Well collagenized granulation tissue with few chronic inflammatory cells occupied the PbAc sponges and only a small degree of sponge breakdown was observed. In contrast, plain sponges had been extensively infiltrated by fat, breakdown of the sponge matrix was conspicuous and much of the granulation tissue was densely infiltrated by lymphocytes and macrophages. This was most likely to be an immunological reaction in response to sponge breakdown products or indirectly to macrophage or MNGC by-products that were released during sponge degradation.

The calcergic deposits in sponges clearly had inhibitory effects in delaying sponge breakdown, fatty infiltration and contraction of sponges implanted for 140 days. The meagre cellular (macrophage) reaction towards the calcergic material is unlikely to have regulated these extensive inhibitory effects. The calcergic deposits may have physically strengthened the sponges thereby making them less susceptible to shrinkage. The degree of fatty infiltration may have been

related to the process of sponge degradation and the apparent replacement of granulation tissue by the lymphocytic infiltrate in plain sponges. Thus the intact granulation tissue in PbAc sponges may have been more resistant to fatty infiltration than tissue infiltrated by lymphocytes. The matrices of sponges soaked in PbAc may have been less suject to breakdown by cellular action because of persistence of lead in the calcergic deposits, affecting the phagocytic ability of macrophages and MNGC.

9 Intraarticular Injection of Microcrystals in Rats

Despite the considerable effort expended in processing embedding and cutting the undecalcified sections of rat stifle joints, the artefactual tearing of the soft tissues that occurred in most blocks precluded determination of the fate of the injected material. The disturbance in soft tissue morphology probably occurred during the lengthy processing and impregnation technique rather than during sectioning of the blocks since sections cut with different knives and at varying thicknesses were also badly disrupted.

A few intact sections did show the presence of HAC in the synovium. The synovial hyperplasia that was consequent on HAC uptake correlated well with the inductive effect of HAC on granulation tissue infiltrating HAC impregnated sponges. It also correlates with the finding that HAC was mitogenic to cultures of canine synovial cells (Cheung and McCarty, 1982).

The MNGC response of synovium 14 days after HAC injection was consistent with the MNGC seen around HAC deposits in sponge granulation tissue. Doyle (1982) in his extensive study of the frequency of occur-

rence and response to calcific deposits in osteoarthritic joints in humans, found that the most prominent cellular reaction was the formation of MNGC in the synovium. Patchy lining cell hyperplasia was present in all synovial specimens. As Sokoloff (1979) has pointed out:

> "Villous hypertrophy and fibrosis of the synovium are the rule rather than the exception in clinically obtrusive osteoarthritis."

In addition, fibrosis of the synovial surface and a progressive increase in the amount of fibrous tissue separating the synovial capillaries from the joint space may occur in OA.

When the findings of the present study are considered together with the well-documented pathological changes observed in advanced OA, it is tempting to implicate the intraarticular deposits of HAC as important factors in the induction of synovial thickening. This could occur via uptake of HAC by macrophages, Type A synovial cells and MNGC and the subsequent release of collagenase, proteases, prostaglandins and factors regulating fibrogenesis. Uptake of crystals by synovial cells is seen as a major factor in the initiation of chronic synovitis and the development of synovial thickening (Dieppe and Calvert, 1983a). Synovium was described as being "peculiarly susceptible to inflammation".

The implications of HAC in the pathogenesis of apatite deposition disease is further considered below.

Effective mechanisms exist for the rapid clearing of artificially introduced crystals from human and animal joints (McCarty et al, 1979a, b; Halverson et al, 1981). Blood-borne PMN and macrophages pour into

the synovial fluid in response to the crystals and endocytose some of the material. The remaining particles or particles that had been re-released into the synovial fluid from necrotic phagocytic cells may be taken up by the synovial macrophage-like cells. It is interesting to compare the sparse numbers of PMN seen in synovial fluid smears at 24 hours after injection of HAC into rat stifle joints with other studies. Schumacher et al (1977) reported that 90% of the mean leucocyte count of 33950/ml in synovial fluid smears were PMN at 4 hours after injection of 15mg of HAC in dog stifle joints. In saline injected joints, PMN comprised 80% of the leucocyte count of 11537/ml. Before injection, the synovial fluid aspirates contained 6-18% PMN out of a mean leucocyte count of 1250/ml. No studies beyond this time period were carried out by Schumacher et al (1977). HAC found in human synovial fluid samples are phagocytosed by macrophages rather than by PMN (Schumacher et al, 1983).

Injection of HAC into the rat pleural space resulted in an exudate containing 90% PMN for the first 12 hours of the reaction (Glatt et al, 1979). After this time, mononuclear cells predominated. In the present study, macrophages have been shown to be able to phagocytose large amounts of HAC both in vitro and in vivo without apparent damaging effects on the cells. Macrophages may in fact be more efficient than PMN at removal of HAC because of this property, since PMN have been shown to degenerate as early as 2 hours after the addition of HAC in vitro (Maurer and Schumacher, 1979).

The toxic nature of HAC to PMN suspensions in vitro was confirmed by Dieppe et al (1983). HAC was more toxic than MSU and caused the release of 2.5 times as much β-glucuronidase into the culture medium.

PMN are short-lived cells that appear in large numbers at the site of a noxious stimulus such as injured tissue or in response to bacterial invasion. Despite their protective role in attempting to neutralise the offending stimulus, the enzymic content of the cells is released following cell death and has the potential to cause tissue damage. PMN structure and function has been reviewed by Hirsch (1974). The toxic nature of HAC to PMN *in vitro* and the lack of association of HAC in synovial fluids with PMN suggest that PMN are not efficient at removing HAC *in vivo* .

Macrophages are long-lived cells that are both tissue bound eg Type A synovial cells and present in inflammatory exudates eg in synovial fluid (Vernon-Roberts, 1972a). The cells can synthesise a large array of enzymes and chemical mediators controlling the function of other cells, such as fibroblasts. Extensive reviews of macrophage source, structure and function have been published (Vernon-Roberts, 1972; Adams et al, 1981; van Furth, 1981).

In the present study, macrophages have been shown to take up large amounts of HAC *in vitro* and *in vivo* without detrimental effects to themselves. In both MSU and CPPD induced synovitis, PMN predominate in the synovial fluid, although phagocytosis of the crystals by macrophages and synovial cells is frequent (McCarty, 1979).

The preferential uptake of HAC by macrophages and MNGC observed in this study parallels the cellular response to *M. tuberculosis* . The organisms stimulate phagocytosis by macrophages (Vernon-Roberts, 1972b) and their dissemination in the host is effected by the cells. In tuberculosis, PMN are the first cells to arrive in response to the tubercle bacilli. They are actively phagocytic but are unable to damage the

organisms. However, the PMN are successful in localizing the spread of infection by preventing the spread of bacilli through the tissues. Within 24 hours, the PMN are replaced by macrophages which endocytose free bacilli and PMN that contain bacilli. Some macrophages are transformed into epithelioid cells which gradually break the bacilli down. The organisms can multiply within the macrophages.

Intraarticular brushite particles induced an intense acute exudative response with prominent PMN as seen in synovial fluid smears at 24 hours after injection. The reaction was similar to that induced by the implantation of sponges impregnated with brushite.

There was no evidence of pathological changes in joint tissues other than the synovium in the sections suitable for examination.

The few rounded deposits of calcergic material found in synovial tissues of PbAc injected joints 28 days after injection were also associated with synovial hyperplasia. No other pathological changes were observed in the joint tissues. Biological deposition of HAC in rabbit knee synovium has been induced by calciphylaxis (Reginato et al, 1982). At 45 days after challenge, synovial proliferation and MNGC formation was evident in relation to masses of calcified tissue in the synovium. The deposited material had not caused any osteoarthritic changes.

To use the present model to study mechanisms involved in human crystal-related arthropathy, the following important modifications of the current experimental design would be necessary:

a) a longitudinal regime of joint injections with daily injections of crystals or calcergen for at least 6 months.

b) a refined histological technique for the processing of undecalcified sections of the joints to enable study of the fate of and reaction to the crystals and calcergic material.

10 Macrophage Interactions with Microcrystals in vitro

The method of obtaining stimulated mouse peritoneal macrophages has been described and discussed by Adams (1979) who emphasized that the cells were not the equivalent of mature resident peritoneal cells. Although they were mononuclear phagocytes recently derived from peripheral blood, many enzymatic and functional activities of mononuclear phagocytes are found only in these young cells (Meltzer, 1981). The cell yield is dependent on the nature of the irritant injected and the time between injection and harvesting the cells. The optimum cell yield was determined by harvesting the cells at various times after injection of protease-peptone. For C57 mice the highest yield of cells was obtained 24h after injection.

Meltzer (1981) pointed out that macrophages are very responsive to environmental changes and that tissue culture conditions are artificial. The cells attempt to endocytose the plastic culture plates on which they are grown. Meltzer concluded:

> "The longer these cells are in culture, the less they resemble their in vivo counterparts. Working with these responsive cells is in effect a race, a race against time and continuing culture-induced morphologic, functional and biochemical changes."

Kavet and Brain (1980) described how macrophages attach to plastic surfaces via cytoplasmic projections and the formation of intracellular arrays of microtubules and by microfilaments directly subjacent to the

plasma membrane's point of contact. The literature reviewed supported the view that macrophage monolayers can be cultured for weeks. Most populations of macrophages were heterogenous with respect to their phagocytic ability (Kavet and Brain, 1977), indicating that the cells were a mixture of resident macrophages and recently recruited monocytes. Subpopulations of both these cell types exist, as expressed by varying functional capabilities.

The mouse was chosen as the source of macrophages rather than the rat because of the consistently larger number of cells obtained. Teitelbaum et al (1979) have also used stimulated mouse macrophages for experiments investigating the interaction of macrophages with rat calvaria and ground rat bone. These workers noted that mouse macrophages were more efficient at breaking down the mineralized bone added to the culture than rat macrophages. Chambers (1980, 1981) also used mouse macrophages in a similar culture system and found that the cells caused contact-dependent resorbtion of bone powder, an effect that was inhibited by the addition of EHDP to the medium.

Growing cells on millipore filters is widely used in ultrastructural studies of cell monolayers where the filters support the cells during the embedding process (McCombs et al, 1968). The filter pore size was selected on the basis of a study by Wennberg et al (1979) who found that monolayers do not form readily when the pore size was greater than 0.45μ. In the present study, culturing the macrophages on millipore filters proved to be a most effective medium for the subsequent preparation of the cells for TEM and SEM examination. The filters were easily lifted out of the culture wells, could be readily cut into pieces for TEM and SEM and were not affected by the processing solutions.

The avidity and speed with which the macrophages endocytosed the materials added to the cultures corresponded with previous reports. Quartz particles (1-3μ, 100μg/ml of culture medium) were endocytosed within 2-4 minutes of their addition to cultures of rat alveolar macrophages (Miller et al, 1978). It was not known whether this phenomenon was due to the size and shape of the quartz or to its physicochemical properties.

The light microscopic appearance of the control cells examined 24h after seeding into the wells was characteristic of macrophages in culture as described by Adams (1979). With the passage of time, the numbers of cells decreased. This phenomenon reflected the heterogeneity of the macrophage population with varying degrees of attachment capability and ability to survive in vitro . The proliferative activity after 9 days indicated that the initial culture contained cells (probably not macrophages), that were capable of replication.

The light microscopic TEM and SEM appearance of cell cultures to which small HAC aggregates had been added showed the rapid uptake of large quantities of crystals. Although the number of cells decreased with time, no free crystals were observed around the remaining cells, indicating that crystals released from necrotic cells had been endocytosed by the remaining cells. In general, it appeared that only the cells that had endocytosed very large amounts of crystals were damaged. The majority of cells were intact.

The observation that the cells were capable of taking up large amounts of HAC corresponds to the finding that murine macrophages were able to interiorize up to 50% of their plasma membrane during phagocyt-

osis before reaching capacity (Werb and Cohn, 1972). The activity of the plasma membrane marker enzyme 5'nucleotidase, decreased for six hours after endocytosis. The decrease was in proportion to the amount of latex particles ingested. During this time, new plasma membrane was being synthesised and the macrophages were subsequently able to ingest more particles.

Uptake of crystals caused rounding of cells and loss of cell processes which gave the cell surfaces a smooth appearance when examined by SEM. The addition of milled cobalt-chrome alloy particles to cultures of murine macrophages also caused rounding of the cells (Rae, 1975; Garrett et al, 1983). The cell processes were most likely lost as a result of their interiorization during phagocytosis of the particles.

Dissolution of the HAC by cellular action was apparent since the amount of particulate material decreased with increasing time of incubation. Macrophages are capable of resorbing devitalized radiolabeled bone particles and rapidily releasing free calcium into the medium (Teitelbaum et al, 1979). In the present study, the macrophages formed clusters around clumps of HAC remaining from two days after addition of the crystals until 14 days. The clustering of cells may have been representative of *in vitro* giant cell formation, paralleling the *in vivo* response to HAC. Spontaneous fusion of macrophages *in vitro* has been demonstrated by Sutton and Weiss (1966) and Chambers (1977).

Stimulated macrophages are more apt to form clusters than unstimulated cells (Stewart et al, 1975) and Adams (1976) suggested that the macrophages might secrete substances inducing aggregation. Mature cells tend to aggregate more readily than immature macrophages (Golde

et al, 1972).

The loss of HAC from the tissue culture wells could have occurred during the replacement of the culture medium, although only extracellular HAC or HAC attached to non-adherent cells would have been removed in this way.

The cellular response to the large plate-like HAC aggregates involved their mobilization and concentration and the formation of prominent clusters of macrophages around the HAC. A similar effect had been observed in vivo where clumping of HAC in impregnated sponges had occurred with a subsequent macrophage and MNGC response. However, the clumping observed in vivo was thought to have been a physico-chemical phenomenon rather than a result of cellular activity. Large HAC added to wells containing culture medium only, carpeted the floor of the wells and did not form clumps. In vitro , therefore, the concentration of the HAC aggregates was a result of cellular action.

The prominent cytoplasmic ballooning observed by light microscopy and TEM was a consequence of the endocytosis of considerably quantities of large HAC aggregates. The cells remained intact despite the uptake of large amounts of HAC.

The ability of the macrophages to endocytose the elongated MSU crystals was remarkable. SEM showed that a single cell was capable of ingesting a crystal many times its size by enveloping it in cytoplasmic extensions. Similarly, crocodilite fibres (20μ or longer) were phagocytosed by macrophages by the development of large flattened pseudopodia to which the fibre was attached by small cytoplasmic tendrils. The crocodilite fibres were ingested end first and endocytosed as soon

as 10 minutes after addition of the fibres (Miller et al, 1978). Very long fibres (50μ) were concurrently attached to and engulfed by several cells.

The MSU were no longer visible by light microscopy five days after addition due to their solubility in aqueous solutions in addition to cell-mediated dissolution.

The calcium carbonate particles were present in clumps from the time of their addition to the cell culture, clumping was also a feature when the particles were added to wells in the absence of cells. Further concentration of the aggregates occurred as a result of cellular activity.

The calcium carbonate, compounds derived from it as a result of cellular activity, or products of its dissolution in aqueous solution induced intense proliferation of cells after 14 days. Since all wells had been plated from the same pool of cells harvested from the animals, it is unlikely that the increased proliferative activity observed with calcium carbonate compared to the proliferation of cells in control wells was related to a greater number of replicating cells. Rather, it was related to a stimulatory property of the material added and it would be of interest to follow up this finding.

11 Synovial Cell Interactions with Microcrystals in vitro

The morphological appearance and behaviour of the cells obtained from a bovine metacarpophalangeal joint was indentical to that described for normal human synoviocytes in tissue culture (Fraser and McCall, 1965; Smith, 1971; Marsh et al, 1978). Cytoplasmic spreading is con-

sequent on cell attachment to the floor of the culture well and confluent cultures form a characteristic reticular pattern and dense multilayers of cells. This feature can be used to distinguish synovial cultures from fibroblastic cell strains, suggesting that the cells obtained in the present study were of synovial origin. Since the morphology of synovial cell cultures alters with repeated passage of the cells (Marsh et al, 1978), the cells used for all crystal-interaction experiments were in their third passage.

Synovial cells are concerned with the regulation of the composition of synovial fluid and maintenance of the ordered structure of synovial membrane. The cells are classified as Type A and Type B on the basis of ultrastructural and cytochemical differences (Mankin and Radin 1979). Type A cells are macrophage-like with numerous cell processes and prominent Golgi complexes. B cells resemble fibroblasts and have abundant granular cytoplasmic reticulum. The cells form a discontinuous layer, lacking a basement membrane, and cell processes project from the cells towards the joint space.

It is likely that both types of cells were collected by the present harvesting technique. However, cells released from rabbit knee joint synovium by trypsinization showed extensive morphological and functional alterations (Glen-Bott, 1972). The alterations brought about by growing the cells in an artificial environment were so striking that Glen-Bott stated:

> "It can be concluded that significant alterations in the form and structure of these cells occur as a result of their altered environment. Cells, whose shape in the intact animal is determined by their position on the synovial surface, all become actively phagocytic when suspended in a fluid medium; they take on a round shape after 4 hours in culture and

later some selected cells spread out over a smooth surface."

Cells grown from synovial explants exhibited features of both types of synoviocytes in the one cell (Castor and Muirden,1964). Consequently, it is unlikely that the cells used in this study had the same characteristics that their progenitors possessed in vitro . Type A synoviocytes, had they retained their in vivo macrophage-like characteristics would have been lost during the three passages of the culture which occurred over a period of 21 days after obtaining the cells.

The phagocytic ability of synovial cells in culture is of considerable interest. Previous studies have shown that the endocytosis of indigestible latex particles by rabbit synovial "fibroblasts" (Werb and Reynolds,1974) and of HAC by human rheumatoid and canine synovial cells (Cheung et al, 1981a) caused the release of collagenase and neutral proteases.

The uptake of small HAC, as observed by light microsopy, was delayed by approximately two hours compared with large HAC aggregates. Cell proliferation continued at the same rate as in control cell cultures, indicating that the endocytosed material did not effect cell viability.

Cells to which large HAC had been added seemed to concentrate the particles in the vicinity of their cytoplasm as soon as two hours after addition of the material. Large amounts of HAC had been endocytosed by four hours. As a consequence, these cells did not undergo the same rate of cell division observed in control or small HAC cultures and showed signs of degradative changes with release of HAC into the medium after

seven days. This response could have been due to:

a) large HAC aggregates being toxic to the cells,

b) the phagocytic process utilizing cell metabolism in such a way as to preclude cell division,

c) the presence of large intracytoplasmic particles interfering with the mechanics of cell division.

Since inhibition of replication and toxicity had not been observed in small HAC cultures, it is concluded that the large HAC physically prevented cell division.

The sparsity of cells growing on the millipore filters suggested that this surface was not as conducive to cell proliferation as was the plastic surface of the culture wells, since all other conditions were identical.

TEM of control synovial cells confirmed that the cells were well spread and were attached to the millipore filters by small cytoplasmic extensions. Lipid containing vesicles observed in the cells' cytoplasm had been previously reported in synovial cells grown on polyester sheets (Glen-Bott, 1972). No degenerative changes were evident.

The synovial cells were actively phagocytic and the uptake of HAC occurred within a similar time scale as was seen in the macrophage cultures. The synovial cells were capable of ingesting considerable quantities of large HAC aggregates as seen at 60 minutes after addition of the crystals. The degree of cellular disruption and degenerative changes observed at this time suggests that the cells had overindulged and correlates with the light microscopic findings of degenerative changes in the cultures after 4 days.

The uptake of small HAC aggregates, observed by TEM, 7 days after their addition was not associated with degenerative changes. The particles contained within membrane bound phagosomes were disaggregating, suggesting enzymic or acid activity by the cell against the crystals.

Although both small and large HAC aggregates were taken up by the synovial cells, small HAC aggregates caused less cellular disruption. If this in vitro finding is relevant to the in vivo situation, then it would appear that larger crystals or crystal aggregates may be more disruptive to the cells once phagocytosed than small crystals and could therefore initiate more cellular damage and have more inflammatory potential.

12 Relevance of the Present Findings to the Pathogenesis of Apatite Deposition Disease

The pathogenesis of apatite deposition disease, as far as it has been elucidated to this time, has been described in some detail by Dieppe and Calvert (1983b). The mere presence of HAC in articular cartilage and synovial fluids in some patients with advanced OA does not necessarily imply a cause and effect relationship. Indeed, HAC may be found in fluid aspirates from asymptomatic, apparently healthy joints. Dieppe and Calvert suggested five possible interrelationships of HAC and OA:

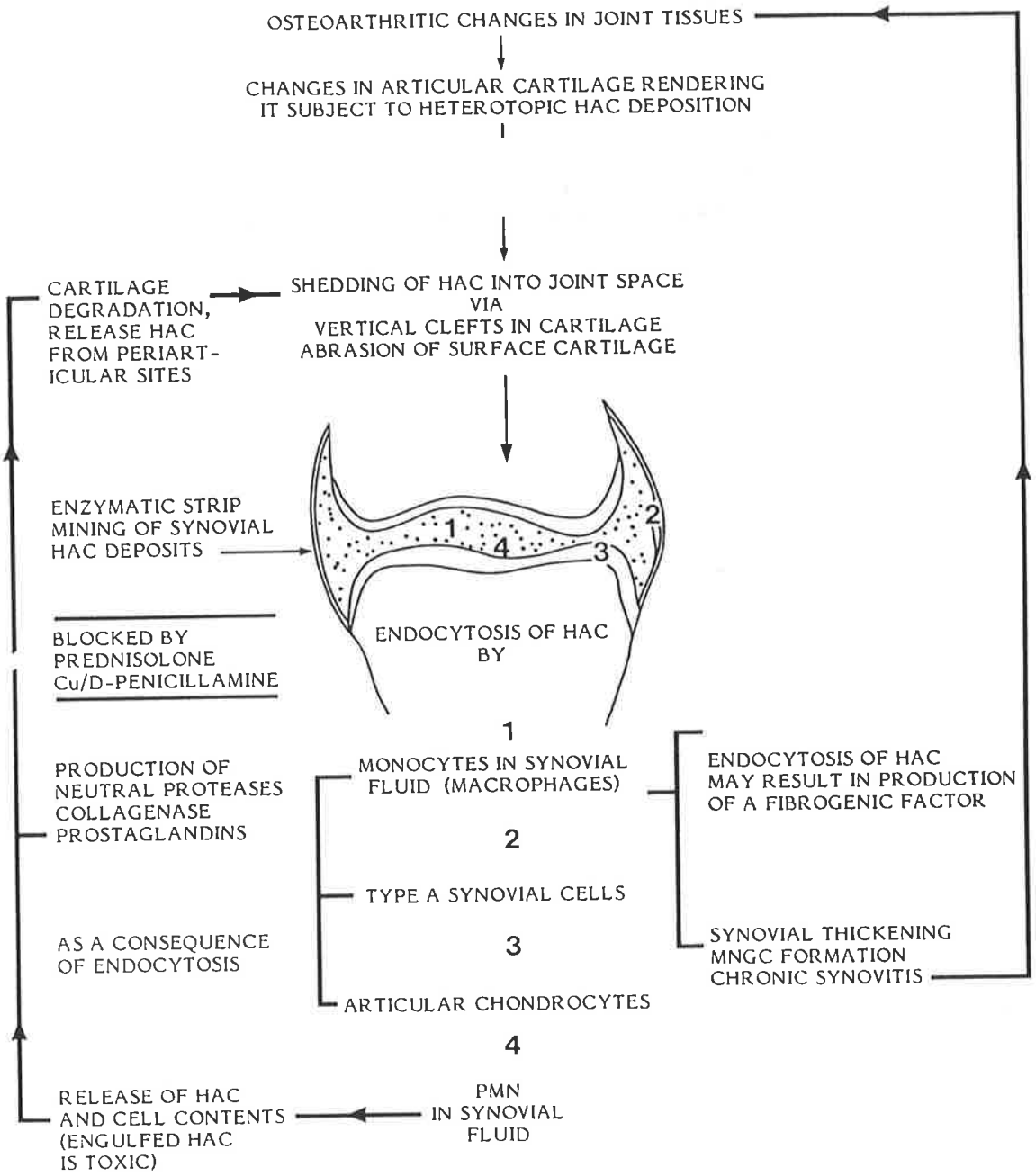
a) OA causes crystal deposition
b) Crystal deposition is a marker of osteoarthritic change
c) Crystal deposition causes OA
d) Crystal deposition is independent of OA but occurs at the same time
e) Crystal deposition and OA are interrelated

With the exception of possibility (c), none of these scenarios explains the aetiology of OA, and in the case of (c), does not explain the source of the HAC in the absence of preceding osteoarthritic change. The following flow chart summarizes the events that may follow HAC deposition in joints as a result of OA. The chart has been compiled using data obtained in the present investigations and from the literature. A more detailed discussion of these events follows below.

There is strong evidence that OA induces metabolic and morphological alterations in articular cartilage metabolism (Ali, 1977; 1980). The chondrocytes become more active, the levels of alkaline phosphatase increase, and free calcium is released from proteoglycans, predisposing to the formation of matrix vesicles and the deposition of small clumps of HAC. The HAC is usually deposited in the mid and lower zones of the cartilage, near the bone-cartilage junction. The presence of HAC in synovium is thought to be a result of uptake of material from the synovial fluid (Doyle et al, 1979b), rather than a de novo deposition there. HAC deposition in articular cartilage is unlikely to be a calcergic phenomenon because of the lack of blood supply to cartilage, making the access of calcergens difficult.

The HAC deposited in the articular cartilage may be shed into the joint space, directly through vertical clefting and fibrillation that develop in osteoarthritic cartilage (Ali, 1980) or indirectly via abrasion of cartilage during joint movement (Dieppe, 1977). Dieppe and Calvert (1983b) also referred to recent findings of the presence of small HAC embedded in the superficial parts of fibrillated articular cartilage which would make the cartilage subject to increased wear and result in shedding of crystals into the joint space.

FLOW CHART OF INVOLVEMENT OF HAC IN OSTEOARTHRITIS



Another possible source of HAC in the joint space could be a result of a calcergic phenomenon. As has been demonstrated in the present study, deposition of HAC mediated by a calcergen can occur in the absence of intact cells and independently of collagen fibres. The presence of an increasing burden of contaminants in the environment, including lead (Goyer and Rhyne, 1973) may initiate calcergic reactions in the joint spaces. Calcification of human dental plaque in vivo by an environmental calcergen has been suggested by Hirsch et al (1983).

The different morphological forms of HAC in joint fluid include individual crystals and particles varying in size, shape and crystallinity (Dieppe and Calvert, 1983b). The latter could be deposited as a result of a calcergic reaction. However, the calciphylactic reaction induced in rabbit knees by Reginato et al (1982) occurred within the synovial tissues; no crystals were found in the synovial fluid at any time period after initiation of the reaction. Similarly, rat knees injected with lead acetate in the present study showed deposition of calcific material in the synovium rather than in the synovial fluid. It is possible, however, that a calcergic reaction had occurred in the joint space and that the synovium had endocytosed the deposits, as had been demonstrated with the injection of HAC into the joints. Further elucidation of the nature and effect of the calcergic phenomenon in joints is indicated.

Once shed into the joint space, the HAC would quickly acquire a protein coating derived from the synovial fluid. In health, the protein content is almost all albumin, but with active synovitis, immunoglobins gain access to the joint space (Simkin, 1979). Binding of IgG to the HAC surface is rapid and may enhance phagocytosis. However, rapid phagocytosis can occur in the absence of IgG, as has been demon-

strated with murine macrophages in vitro . Owing to the affinity of the HAC surface for proteins, particularly IgG (Glueckauf and Patterson, 1974), it is highly likely that uncoated crystals do not exist in vivo but the significance of the protein coating in relation to the rate and efficiency of phagocytosis is unknown.

Dieppe et al (1983) have demonstrated the toxic nature of HAC to PMN suspensions in vitro . Perturbation of the PMN plasma membrane causes the cell to undergo an oxidative burst which is characterized by the extramitochondrial uptake of oxygen, the release of the superoxide anion, hydrogen peroxide and the hydroxyl radical (Sbarra and Karnovsky, 1959; Babior, 1978). Phagocytosis of crystals is a stimulus for the production of oxygen derived free radicals by PMN. The radicals are released into the phagosome and into the medium. In this way, they represent a threat to the viability of adjacent PMN and undoubtedly, some degree of back diffusion into the cytosol of the stimulated PMN would occur, leading to cell death. It may well be that the particularly toxic nature of HAC to PMN is related to its ability to switch on the oxidative burst.

The lysosomal enzymes released as a consequence of cell death may disrupt the synovial cells and cause the release of endocytosed HAC.

Phagocytosis of HAC by macrophages (Schumacher et al, 1983) in the synovial fluid and by Type A synovial cells (Garancis et al, 1981) appears to be the predominant cellular response. Preferential handling of HAC by macrophages and MNGC rather than by PMN has been found in the present investigation. Adherent human blood mononuclear cells stimulated by PHA and pokeweed mitogen, produce one or more factors (called Catabolin by Dingle, 1981) that induce the degradation of matrix pro-

teoglycan and collagen in cartilage explants (Jasin and Dingle, 1981). Macrophage products stimulate collagenase and neutral protease release from articular chondrocytes (Desmukh-Phadke et al, 1980) although Ridge et al (1980) showed that the enzymes were released in latent form.

Particles such as latex that have been ingested by macrophages can also trigger the production of neutral proteases, collagenases and elastases by the cells (Werb and Gordon, 1975a,b). HAC may act as the stimulatory factor once endocytosed by macrophages and cause the release of a similar battery of enzymes capable of breaking down joint tissues. The production of these enzymes is related to endocytosis rather than to the substance endocytosed.

The uptake of HAC in vitro by human rheumatoid synovial cell strains and rabbit articular chondrocytes results in the release of collagenase, protease and large amounts of prostaglandin E_2 (Cheung et al, 1981a, 1983; Hasselbacher, 1982). These findings led McCarty et al (1981) to propose enzymatic strip mining of calcific deposits remaining in synovium or in the periarticular tissues and release of more HAC into the joint space. The proteases can digest cartilage proteoglycan, collagen and elastin. The rabbit chondrocytes were stimulated to release an amount of collagenase that was out of proportion to the increased synthesis of extracellular protein, suggesting a specific response to the phagocytosis of HAC.

The large amounts of prostaglandin elaborated by synovial cells and chondrocytes in response to HAC may be involved in bone resorbtion and further destructive joint changes.

An important finding of the present study was the stimulatory

nature of the HAC in accelerating granulation tissue ingrowth into HAC impregnated sponges. Synovial hyperplasia was also found wherever synovial cells had taken up HAC or where calcergic deposits had formed in rat knee joints. HAC has been shown to be mitogenic for synovial cells in vitro (Cheung and McCarty, 1982; McCarty, 1983), partly explaining the synovial response observed. The apparent inductive effect of HAC, the expression of which was largely genetically determined in rats, can be implicated in the synovial thickening that is observed around synovial and soft tissue deposits of crystals (Moskowitz et al, 1971; Dieppe and Calvert, 1983a). Fibrosis is often found at the site of chronic inflammation.

A mechanism whereby crystals may indirectly exert a fibrogenic reaction has been outlined by Allison (1978). Diffusion chambers made of millipore filters (pore size = 0.8μ) cemented to both sides of a plastic ring were filled with a mixture of mouse peritoneal macrophages and 25ug of quartz particles. The chambers were implanted in the peritoneal cavity of mice for one month. The mixture of macrophages and quartz particles were associated with fibrosis of the peritoneum in the vicinity of the chamber. No such effect was seen with implants filled with saline, macrophages only or quartz particles only. Allison concluded that the macrophages secreted a factor that stimulated collagen synthesis by fibroblasts and/or increased the multiplication of fibroblasts. The fibrosis of the lung that occurs following inhalation of silica (quartz) or asbestos particles may be induced by the fibrogenic macrophage product.

The same (but as yet undefined) factor produced by macrophages in response to endocytosis of HAC may well be exerting the fibrogenic stimulus in vivo . Further investigation is warranted into this fas-

cinating area of cell interactions.

Finally, this investigation has shown the efficacy of a copper coordination compound (copper/D-penicillamine) in reducing the intensity of the macrophage and MNGC reaction to HAC in impregnated sponges as well as modifying the fibrogenic influence of the HAC. Similar drugs may be of use in the treatment of diagnosed cases of arthropathy associated with HAC.

CONCLUSIONS

Consideration of the literature reviewed and the findings of the present series of investigations have enabled the following conclusions to be drawn regarding the inflammatory potential of HAC and its role in apatite deposition disease.

1. PMN are probably not involved in apatite deposition disease.

a) Minimal PMN response to HAC impregnated sponges was observed after 14 days

b) Few PMN were seen in synovial fluid smears even as early as four hours after injection of HAC

c) The literature showed that HAC is toxic to PMN, even more so than is MSU.

2. Macrophages, Type A synovial cells, MNGC and possibly articular chondrocytes have important roles in the removal of HAC from synovial fluid and in the pathogenesis of OA.

a) Macrophages and MNGC have the abilitiy to endocytose large amounts of HAC in vivo and in vitro without any apparent toxic effects. TEM suggests that the HAC is slowly degraded.

b) The literature suggests that macrophages predominate in synovial fluids in which HAC are found.

c) HAC has been shown to be fibrogenic, possibly via macrophage-mediated influences on fibroblasts. The synovial thickening seen in OA may be related to this property of HAC together with the chronic inflammatory response elicited by HAC.

d) The literature shows that endocytosis of HAC by the cells mentioned above stimulates the production of collagenases, neutral protease and protaglandins which can be destructive to joint tissues, especially cartilage.

e) Following endocytosis, large crystals or crystal aggregates may be more damaging or disruptive to synovial cells than smaller crystals.

3. Drugs can affect the chronic inflammatory response to HAC.

a) Prednisolone can totally suppress the response.

b) Copper/D-penicillamine can reduce the macrophage and MNGC response.

c) Sodium aurothiomalate and adjuvant treatment can accentuate the response to HAC.

4. Biologically induced deposition of HAC using the phenomenon of calcergy elicited a similar cellular response as synthetic HAC.

a) Macrophages and MNGC predominated

b) HAC deposition was not mediated by cells, cell products or collagen

c) HAC deposition was seen as a chemical reaction between the calcergen and elements of serum.

* * *

DIRECTIONS FOR FUTURE STUDY

The results of this series of investigations have provided widely based morphological and semi-quantitative evidence for the involvement of HAC in chronic inflammation and in fibrogenesis. It would be of interest to follow up this study to examine the following issues:

a) *In vitro* and *in vivo* studies, such as those used by Allison (1978) for silica particles, of factor(s) produced by macrophages, synovial cells and possibly articular chondrocytes in reponse to the endocytosis of HAC that can stimulate collagen production.

b) *In vitro* studies of the interaction of peripheral blood monocytes, macrophages, synovial cells and articular chondrocytes with HAC involving the measurement of enzyme release and determination of the rate of breakdown of radiolabeled HAC. The mitogenicity of HAC to these cells should also be more extensively investigated.

c) Biochemical studies of sponges impregnated with HAC in which the amount and types of collagen are evaluated in addition to investigations of proteoglycan synthesis and degradation in granulation tissue in the sponges. The use of radiolabeled matrix component precursors would be of value in this regard.

d) Long term studies of repeated intraarticular injections of HAC and calcergens employing modified histological techniques as well as TEM. Calcergic deposition of HAC in the synovial membrane could be used to test the enzymatic strip mining hypothesis of Halverson et al (1982).

e) Studies of the effects of HAC in the air pouch model of Edwards et al (1981), which provides a cavity lined by cells similar to Type A and B synoviocytes, would be of interest. This model would provide a convenient method of studying the interaction of exogenous and endogenous HAC with synoviocytes and allow easy sampling of cells and fluid exudate during the experimental period.

f) Drug studies using the HAC impregnated sponge model and a range of drugs including non-steroidal antiinflammatory agents, diphosphonates and copper coordination componds. The effects of these agents on the uptake and subsequent production of enzymes and fibrogenic factor(s) by macrophages, synovial cells and articular chondrocytes in vitro could provide valuable corroborative information.

* * *

Chapter Five

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CHAPTER FIVE

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Chapter Six

Appendix

STATISTICS - EXPERIMENTS 1-9

Experiment	N	Mean	Variance	Skewness	Std.Error	Coeff. Variation	Kurtosis
1 Sponge wgt	96	122.1458	1256.7154	-0.2949	3.6181	29.02	2.3519
Ingrowth	96	38.3865	586.4766	0.3981	2.4717	63.09	2.1192
2 Sponge wgt	16	137.4375	313.1958	0.0501	4.4243	12.88	2.1926
Ingrowth	16	74.8875	127.1172	-0.2777	2.8187	15.06	1.9816
3 Rat wgts	56	66.6607	259.0646	0.1464	2.1508	24.15	1.5758
Sponge wgt	48	124.8958	939.2868	0.2381	4.4236	24.54	3.1221
Ingrowth	48	74.2708	146.9338	-1.4356	1.7495	16.32	5.7722
4 Sponge wgt	24	147.2083	280.433	0.7	3.4183	11.38	3.6963
Ingrowth	24	59.2875	107.0742	-0.084	2.1122	17.45	2.5604
5 Sponge wgt	16	203.4375	3129.5958	1.2708	13.9857	27.5	3.2673
Ingrowth	16	50.1813	465.1376	0.3905	5.3918	42.98	1.7977
6 Rat wgt	72	-13.0	186.3944	-0.9381	1.609	-105.02	3.4075
Sponge wgt	48	147.8125	839.5598	-0.3462	4.1822	19.6	2.8492
Ingrowth	48	61.9542	744.8617	-0.7399	3.9393	44.05	2.5884
7L Sponge wgt	60	102.5167	710.2201	0.1441	3.4405	26.0	1.9504
R	60	111.5833	1281.6370	0.2631	4.6218	32.08	2.1555
L Ingrowth	36	61.5056	125.6634	-0.5083	1.8683	18.23	2.1980
R	36	77.5528	262.8608	-0.6277	2.7022	20.91	2.2172
8 Sponge wgt	82	165.1707	2769.0569	1.3660	5.8111	31.86	5.0652
Ingrowth	74	78.7811	237.1500	-0.3417	1.7902	19.55	2.6554
9 Sponge wgt	80	158.05	1775.3646	-0.1886	4.7108	26.66	2.7914
Ingrowth	72	74.5736	1335.2273	-1.1332	4.3064	49.00	2.5487
F p.75 Sponge wgt	24	136.7917	441.1286	-0.2402	4.2872	15.35	2.3116

TABLE F: Sponge dry weight gain (mg) estimated by three methods

	Left Implantation Site			Right Implantation Site		
	Portion A + B	Portion A	Portion B	Portion A + B	Portion A	Portion B
Mean	140.25	142.25	135.75	137.00	132.25	133.25
S.D.	17.36	24.86	20.25	21.11	29.83	24.78

Single Factor Analysis of Variance

	SS	DF	MS	F
Method of weight gain estimation	304.208	5	60.842	0.111
Error	9841.749	18	546.764	

Table 1a: Dry weight increase (mg) of left (L) and right (R) side plain polyurethane sponges implanted for 5, 7, 10, and 14 days in three rat strains

RAT STRAIN	PORTON				LEWIS				DARK AGOUTI			
Implantation Site	L		R		L		R		L		R	
	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
Time (Days)												
5	58.00	7.07	66.25	5.67	112.75	11.02	105.25	16.07	62.25	8.05	67.00	4.96
7	101.75	14.54	94.25	11.95	128.50	18.84	133.75	5.31	125.75	6.60	142.00	7.43
10	103.25	14.90	123.25	15.30	139.00	18.12	132.00	13.49	121.00	21.77	130.50	14.52
14	135.75	8.80	136.00	7.87	153.25	11.67	145.00	9.09	158.00	17.53	157.75	24.04

	Analysis of variance	S.S.	DF	M.S.	F	P
Porton	Implantation Site	220.500	1	220.500	1.708	
	Time	23037.625	3	7699.208	59.481	<.001
	Site/Time	828.250	3	276.083	2.138	
	Error	3098.500	24	129.104		
Lewis	Implantation Site	153.125	1	153.125	.821	
	Time	6661.125	3	2220.375	11.900	<.001
	Site/Time	248.625	3	82.875	.444	
	Error	4477.999	24	186.583		
Dark Agouti	Implantation Site	51.005	1	51.005	1.043	
	Time	17827.818	3	5942.606	121.484	<.001
	Site/Time	252.313	3	84.104	1.719	
	Error	1174.005	24	48.917		

Table 1b: Extent of granulation tissue ingrowth (%) into left and right side plain polyurethane sponges implanted for 5, 7, 10 and 14 days in three rat strains.

RAT STRAIN	PORTON				LEWIS				DARK AGOUTI			
Implantation Site	L		R		L		R		L		R	
	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
5	18.85	5.31	10.25	2.50	9.25	1.38	8.82	0.83	3.95	1.62	4.95	1.94
7	20.39	6.73	19.95	3.46	29.35	3.53	30.35	1.98	27.05	5.55	26.07	3.66
10	52.29	8.97	40.12	11.30	41.54	7.04	42.37	8.00	47.82	2.63	53.42	5.67
14	76.60	7.90	77.72	5.35	77.45	12.43	78.25	7.21	55.80	7.07	58.60	10.09

	Analysis of variance	S.S.	DF	M.S.	F	P
Porton	Implantation Site	51.005	1	51.005	1.043	
	Time	17827.818	3	5942.606	121.484	< .001
	Site/Time	252.313	3	84.104	1.719	
	Error	1174.005	24	48.917		
Lewis	Implantation Site	2.420	1	2.42	0.057	
	Time	19982.003	3	6660.666	157.03	< .001
	Site/Time	2.583	3	0.861	0.02	
	Error	1017.995	24	42.416		
Dark Agouti	Implantation Site	35.49	1	35.49	1.174	
	Time	13929.093	3	4643.031	153.621	< .001
	Site/Time	46.811	3	15.604	0.516	
	Error	725.372	24	30.224		

Table 2a: Dry weight increase (mg) of left and right sided, anterior and posterior sponges implanted for 14 days in Porton rats.

	Left Anterior	Right Anterior	Left Posterior	Right Posterior
Mean	153.75	146.25	127.00	122.75
S.D.	14.29	9.6	11.4	15.75

Analysis of variance

	SS	DF	MS	F	P
Implantation Site	2673.687	3	891.229	5.283	.01
Error	2024.25	12	168.687		

Table 2b: Extent of granulation tissue ingrowth (%) in left and right anterior and posterior sponges

	Left Anterior	Right Anterior	Left Posterior	Right Posterior
Mean	83.15	83.00	70.77	62.62
S.D.	5.92	7.89	10.18	5.71

Analysis of variance

	SS	DF	MS	F	P
Implantation Site	1205.452	3	401.817	6.875	.006
Error	701.305	12	58.442		

Table 3a: Rat weight gain at 7 and 14 days after implantation of plain sponges and sponges impregnated with different amounts of HAC.

Group	1	2	3	4	5	6	7
7 DAYS							
Mean	52.25	57.00	49.25	51.00	50.50	51.50	53.25
SD	5.18	4.83	3.86	8.12	4.20	7.14	3.59
14 DAYS							
Mean	77.25	81.75	74.50	83.00	86.50	80.50	84.50
SD	8.61	7.22	5.80	12.30	5.44	4.04	5.44

Analysis of variance

	SS	DF	MS	F	P
Time	11803.018	1	11803.018	275.442	<.001
Exptal Groups	342.000	6	57.000	1.330	.264
Time/Exptal Groups	222.357	6	37.059	.865	
Error	1799.750	42	42.851		

Table 3b: Dry weight increase (mg) of left (plain) and right sided (HAC) sponges

Group	1	2	3	4	5	6
Left Side						
Mean	129.75	135.75	147.25	131.50	130.50	142.00
SD	10.24	20.25	16.07	11.38	10.59	8.86
Right Side						
Mean	134.50	91.00	94.25	92.25	88.25	181.75
SD	9.88	16.75	20.12	18.92	11.32	24.04

Analysis of variance

	SS	DF	MS	F	P
Left Dry Weight Gain	1008.375	5	201.675	1.106	.392
Error	3282.249	18	182.347		
Right Dry Weight Gain	28259.333	5	5651.867	18.35	<.001
Error	5544.000	18	308.000		

Table 3c: Extent of granulation tissue ingrowth (%) in left (plain) and right (HAC) sponges

Group	1	2	3	4	5	6
Left Side						
Mean	77.65	83.15	70.12	75.95	80.72	76.85
SD	9.56	5.92	10.46	4.64	5.71	6.10
Right Side						
Mean	69.67	76.52	72.50	88.30	65.00	54.79
SD	4.52	6.68	5.92	5.51	15.43	21.73

Analysis of variance

	SS	DF	MS	F	P
Left					
Ingrowth	398.043	5	79.609	1.458	.251
Error	983.015	18	54.612		
Right					
Ingrowth	2528.618	5	505.724	3.608	.019
Error	2523.235	18	140.180		

Table 4a: Dry weight increase (mg) of plain sponges and sponges containing modified HAC crystal aggregates

Treatment	Control	Unmodified	Sonicated	Ground	Smashed	Sedimented
Mean	143.25	139.25	143.75	141.75	142.75	172.50
SD	14.72	10.62	12.28	16.49	11.84	14.70

Analysis of Variance

	SS	DF	MS	F	P
Treatment	3121.208	5	624.242	3.376	0.025
Error	3328.749	18	184.931		

Table 4b: Extent of granulation tissue ingrowth (%) into plain sponges and sponges containing modified HAC crystal aggregates

Treatment	Control	Unmodified	Sonicated	Ground	Smashed	Sedimented
Mean	60.67	71.40	57.67	49.22	57.05	59.70
SD	2.98	8.31	8.63	10.79	10.48	9.91

Analysis of Variance

	SS	DF	MS	F	P
Treatment	1030.674	5	206.135	2.591	0.061
Error	1432.032	18	79.557		

Table 5a: Dry weight increase (mg) of plain and HAC impregnated sponges in Dark Agouti rats at 7 and 14 days after implantation

	Plain Sponges	HAC Sponges
7 Days		
Mean	177.25	278.15
SD	14.56	57.80
14 Days		
Mean	180.00	177.75
SD	42.94	12.68

Analysis of variance

	SS	DF	MS	F	P
Implantation Site(L/R)	9850.563	1	9850.563	7.088	.019
Time	9653.063	1	9653.063	6.946	.020
Sides Time	10764.062	1	10764.062	7.746	.015
Error	16676.249	12	1389.687		

Table 5b: Extent of granulation tissue ingrowth (%) into plain (L) and HAC (R) impregnated sponges in Dark Agouti rats at 7 and 14 days after implantation

	Plain Sponges	HAC Sponges
7 Days		
Mean	27.05	37.42
SD	5.55	4.44
14 Days		
Mean	55.80	80.45
SD	7.07	5.56

Analysis of Variance

	SS	DF	MS	F	P
Implantation Site(L/R)	1226.751	1	1226.751	37.279	<.001
Time	5151.651	1	5151.651	156.550	<.001
Sides Time	203.776	1	203.776	6.192	.027
Error	394.887	12	32.907		

Table 6a: Weight changes (g) of drug treated Dark Agouti rats

Group		1	2	3	4	5	6
4 days	Mean	0.25	-21.50	-6.00	-15.75	-9.50	-8.25
	SD	5.73	2.38	2.94	2.75	7.18	2.06
8 days	Mean	1.50	-39.25	-11.75	-16.75	-4.00	-8.00
	SD	1.91	2.06	1.49	2.87	1.63	3.26
14 days	Mean	5.75	-44.25	-11.00	-11.50	-6.00	-23.25
	SD	7.13	2.62	6.21	1.73	5.35	9.77

Analysis of variance

	SS	DF	MS	F	P
Weight change	9366.736	5	1873.347	92.069	<.001
Time	293.444	2	146.722	7.211	.002
Weight change/Time	1729.056	10	172.966	8.498	<.001
Error	1098.750	54	20.347		

Table 6b: Dry weight increase (mg) of plain(L) and HAC(R) impregnated sponges in drug treated Dark Agouti rats

Group		1	2	3	4	5	6
Plain sponges	Mean	155.50	98.25	149.50	132.25	147.25	162.50
	SD	9.03	22.63	32.64	6.55	16.97	12.23
HAC sponges	Mean	162.25	109.50	163.25	134.00	173.25	186.25
	SD	15.56	12.89	12.86	10.42	25.25	9.14

Analysis of variance

	SS	DF	MS	F	P
L/R	2310.187	1	2310.187	7.821	.008
Treatment	25617.437	5	5123.487	17.346	<.001
L/R vs Treatment	898.437	5	179.688	.608	
Error	10633.249	36	295.368		

Table 6c: Extent of granulation tissue ingrowth (%) into plain (L) and HAC impregnated sponges (R) in drug treated Dark Agouti rats

Group		1	2	3	4	5	6
Plain sponges	Mean	55.80	8.97	46.67	58.10	62.92	63.80
	SD	6.57	6.44	4.71	4.47	5.23	2.48
HAC sponges	Mean	85.75	14.37	82.75	88.50	90.92	84.90
	SD	2.66	2.39	2.15	10.83	7.63	8.42

Analysis of variance

	SS	DF	MS	F	P
L/R	7592.785	1	7592.785	214.405	<.001
Treatment	24959.364	5	4991.873	140.961	<.001
L/R Treatment	1169.091	5	233.818	6.603	<.001
Error	1274.877	36	35.413		

Table 7a: Dry weight increase (mg) of plain(L) sponges and sponges impregnated with a variety of microcrystals (R) implanted in Dark Agouti rats

1. Plain Sponges

Group		1	2	3	4	5	6
3 days	Mean	70.25	74.25	73.25	73.75	74.25	73.50
	SD	3.40	6.13	3.77	4.99	6.02	3.87
10 days	Mean	122.50	115.25	135.50	-	-	-
	SD	20.07	7.76	22.39			
14 days	Mean	119.25	109.25	126.00	127.00	121.75	122.00
	SD	7.80	8.13	17.37	13.88	10.07	9.27

Analysis of variance (Groups 1-3 at Days 3,10,14)

	SS	DF	MS	F	P
Time	19214.389	2	9607.195	59.324	<.001
Groups	884.056	2	442.028	2.730	.081
Time vs Groups	560.944	4	140.236	.866	
Error	4372.499	27	161.944		

Analysis of variance (Groups 1-6 at Days 3 and 14)

	SS	DF	MS	F	P
Time	27265.334	1	27265.334	334.767	<.001
Groups	419.667	5	83.933	1.061	.398
Time vs Groups	439.917	5	87.983	1.113	.371
Error	2846.999	36	79.083		

2. Impregnated Sponges

Group		1	2	3	4	5	6
3 days	Mean	73.75	75.75	82.00	72.50	64.00	86.25
	SD	23.30	8.05	8.28	9.53	6.68	5.85
10 days	Mean	123.00	124.75	112.00	-	-	-
	SD	17.45	11.17	13.19			
14 days	Mean	174.50	155.75	159.00	122.75	122.75	125.00
	SD	12.66	13.57	18.81	8.84	16.58	12.27

Analysis of variance (Groups 1-3 at Days 3,10,14)

	SS	DF	MS	F	P
Time	44290.389	2	22145.195	100.728	<.001
Groups	252.722	2	126.361	.575	
Time vs Groups	1080.778	4	270.194	1.229	.321
Error	5935.999	27	219.852		

Analysis of variance (Groups 1-6 at Days 3 and 14)

	SS	DF	MS	F	P
Time	54810.084	1	54810.084	321.022	<.001
Groups	6320.000	5	1264.000	7.403	<.001
Time vs Groups	5105.417	5	1021.083	5.980	<.001
Error	6146.499	36	170.736		

Table 7b: Extent of granulation tissue ingrowth % into plain (L) sponges and sponges impregnated with a variety of microcrystals (R) implanted in Dark Agouti rats

1. Plain Sponges

Group		1	2	3	4	5	6
10 days	Mean	45.50	46.97	52.60	-	-	-
	SD	8.01	3.16	5.99			
14 days	Mean	64.55	66.17	67.65	71.62	67.50	70.97
	SD	8.00	4.78	6.70	2.24	8.57	4.56

Analysis of variance (Groups 1,2,3 at Days 10 and 14)

	SS	DF	MS	F	P
Time	1893.927	1	1893.927	46.962	<.001
Groups	109.373	2	54.687	1.356	.282
Time vs Groups	22.163	2	11.082	.275	
Error	725.915	18	40.329		

2. Impregnated Sponges

Group		1	2	3	4	5	6
10 days	Mean	67.02	61.42	48.57	-	-	-
	SD	8.72	5.79	7.40			
14 days	Mean	88.12	72.37	84.22	88.92	95.75	91.55
	SD	4.10	8.86	6.63	2.98	3.26	3.10

Analysis of variance (Groups 1,2,3 at Days 10 and 14)

	SS	DF	MS	F	P
Time	3055.527	1	3055.527	60.318	<.001
Groups	637.563	2	318.782	6.293	.001
Time vs Groups	616.543	2	308.272	6.085	.001
Error	911.825	18	50.657		

Analysis of variance (Plain vs Impregnated sponges at Day 14)

	SS	DF	MS	F	P
Plain/Impregnated	4216.875	1	4216.875	126.509	<.001
Groups	882.418	5	176.484	5.295	.001
Plain/Impregnated vs Groups	562.393	5	112.479	3.374	.013
Error	1199.972	36	33.333		

Table 8a: Dry weight increase (mg) of sponges injected with saline and lead acetate

Time after injection	Dry weight increase (mg)			
	Saline injected		PbAc injected	
	Mean	SD	Mean	SD
10 mins	135.8	8.8	155.5	33.7
1 day	139.5	30.9	169.3	25.7
2 days	119.5	4.7	158.5	13.7
3 days	114.8	10.7	193.8	16.3
4 days	116.0	9.9	165.0	14.1
5 days	125.8	9.7	182.0	16.8
6 days	115.0	4.8	206.0	18.3
7 days	125.0	13.6	187.8	9.4
14 days	173.3	55.4	306.7	55.2
21 days	205.3	26.5	281.8	19.2

Analysis of Variance

	SS	DF	MS	F	P
Saline vs PbAc	71456.49	1	71456.49	132.913	<0.001
Time	96997.762	9	10777.529	20.047	<0.001
Saline/PbAc vs Time	17574.293	9	1952.699	3.632	<0.001
Error	29031.331	54	537.617		

Table 8b : Extent of ingrowth of granulation tissue into sponges injected with saline and lead acetate (PbAc)

Time after injection	Per cent granulation tissue ingrowth			
	Saline injected		PbAc injected	
	Mean	SD	Mean	SD
10 mins	65.3	7.2	71.2	18.0
1 day	82.8	3.6	72.9	9.5
2 days	82.7	10.7	64.2	8.6
3 days	70.6	10.8	71.6	9.5
4 days	81.8	5.0	75.1	7.5
5 days	85.9	10.8	79.9	7.6
6 days	87.9	14.4	87.7	11.4
7 days	90.7	7.1	88.3	15.2
14 days	100.0	0	99.1	1.6
21 days	100.0	0	100.0	0

Analysis of Variance

	SS	DF	MS	F	P
Saline vs PbAc	244.189	1	244.189	2.689	0.103
Time	7719.740	9	857.749	9.445	<0.001
Saline (PBAc vs Time	718.810	9	79.868	.878	
Error	4722.465	52	90.817		

Table 9a: Dry weight increase (mg) of plain and lead acetate (PbAc) soaked sponges

Time (days) after implantation	Dry weight increase (mg)			
	Plain sponges		PbAc soaked sponges	
	Mean	SD	Mean	SD
1	82.50	9.84	176.75	37.85
5	104.00	27.78	202.50	9.53
7	128.50	18.84	208.75	44.58
14	153.25	11.67	190.75	15.75
21	147.75	19.05	207.25	11.05
28	140.25	9.74	186.00	25.49
35	131.50	12.04	194.50	14.38
42	143.75	30.04	184.50	13.52
49	150.75	11.17	192.50	21.76
140	87.75	20.36	147.50	12.39

Analysis of variance

	SS	DF	MS	F	P
Plain vs PbAc	77128.200	1	77128.200	172.241	<.001
Time	27371.051	9	3041.228	6.792	<.001
Plain/PbAc vs Time	8887.048	9	987.450	2.205	.033
Error	26867.498	60	447.792		

Table 9b: Extent of ingrowth (%) of granulation tissue into plain and lead acetate (PbAc) soaked sponges

Time (days) after implantation	Granulation Tissue Ingrowth (%)			
	Plain sponges		PbAc soaked sponges	
	Mean	SD	Mean	SD
5	9.25	1.38	3.37	1.78
7	29.35	3.53	8.70	6.57
14	77.45	12.43	73.70	9.80
21	83.77	10.08	91.12	7.81
28	98.00	2.36	95.65	2.33
35	97.62	1.49	99.32	1.34
42	100.00	0	100.00	0
49	100.00	0	100.00	0
140	100.00	0	100.00	0

Analysis of variance

	SS	DF	MS	F	P
Plain vs PbAc	123.507	1	123.507	4.543	.035
Time	87204.599	8	10900.575	400.959	<.001
Plain/PbAc vs Time	951.365	8	118.921	4.374	<.001
Error	1468.057	54	27.186		

Table 9c: Total area (arbitrary units) of histological sections of plain and lead acetate (PbAc) soaked sponges

Time (days) after implantation	Plain sponges		PbAc soaked sponges	
	Mean	SD	Mean	SD
5	3669.75	228.05	3997.75	297.20
7	3775.00	67.71	3994.50	25.95
14	3889.00	282.34	3968.25	255.61
21	3623.75	40.99	3710.25	131.12
28	3448.00	277.62	3611.50	202.70
35	3412.25	268.24	3889.66	93.35
42	3449.50	176.78	3896.75	173.38
49	3208.75	244.38	3499.50	174.91
140	1586.75	271.79	2691.25	263.45

Analysis of variance

	SS	DF	MS	F	P
Plain vs PbAc	2229529.170	1	2229529.170	49.213	<.001
Time	18814724.200	8	2351840.520	51.912	<.001
Plain/PbAc vs Time	1557685.470	8	194710.684	4.298	<.001
Error	2401115.500	53	45304.066		