ANTI-INFLAMMATORY PROTEINS

AND

OLIGOAMINES

A thesis submitted by

JOHN BIRD, B.Sc.

for the degree of

Doctor of Philosophy

at

The University of Aston in Birmingham

Department of Pharmacy,
University of Aston in Birmingham
Gosta Green,
Birmingham. B4 7ET

July, 1981

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. David A. Lewis, for allowing me the opportunity to undertake this study and for his constant advice and helpful criticism.

I also wish to thank Mr. Ralph Best for the many helpful discussions we shared concerning this work.

Finally, I am indebted to my parents, whose constant support and encouragement made this task very much easier.

The University of Aston in Birmingham June, 1981

Anti-inflammatory Proteins and Oligoamines

A thesis submitted by JOHN BIRD, B.Sc. for the degree of Doctor of Philosophy.

SUMMARY

1. The proteinase inhibitor Trasylol was shown to be unable to reduce the formation of granulation tissue and the development of inflammation in rats with adjuvant induced arthritis. In contrast Trasylol clearly inhibited the proteinases found in human rheumatic synovial fluid samples which are responsible for tissue damage.

2. It was confirmed that alpha-2-macroglobulin and proteinase levels are related to the total protein content of human rheumatic synovial fluid. In addition, a relationship between proteinase and alpha-1-antitrypsin was established.

3. Bacterial endotoxin was shown to be anti-inflammatory when given intraperitoneally and not irritant. When incubated with macrophages the endotoxin was lytic. It also contained small amounts of oligoamines.

4. Putrescine was proven to be a potent anti-inflammatory agent as was spermidine, while spermine and cadaverine had little or no activity. These activities were not related to the irritancy of the compounds and none of the oligoamines had any effect on the development of a local anaphylactic reaction in the mouse ear. Both putrescine and cadaverine were capable of stabilizing the membranes of macrophages *in vitro* while spermine and spermidine were lytic towards them. In addition putrescine inhibited the development of adjuvant induced arthritis in the rat. Drugs which increased the levels of putrescine were also anti-inflammatory.

5. Human rheumatic synovial fluid was found to contain two or more substances which were anti-inflammatory, but irritant. Fractions of the synovial fluid also stabilized the membranes of macrophages.

6. Fractions of liver extracts from DMNO treated rats were anti-inflammatory and this was related to their irritancy. It was shown that the high molecular weight fraction degraded both *in vitro* and *in vivo* producing dialysable molecules which were anti-inflammatory. Putrescine and cadaverine were greatly elevated by DMNO treatment and may explain these results.

7. Inflammatory exudate produced by the implantation of polyester sponge stabilized the membranes of macrophages as did the two fractions produced by gel filtration. Sponge exudate was shown to possess inherent proteolytic activity which caused the breakdown of the exudate both *in vitro* and *in vivo* releasing dialysable molecules which had an anti-inflammatory effect. Sponge exudate both contains and is capable of releasing oligoamines which have been shown to be directly anti-inflammatory and may, at least in part, account for the activity exhibited by the exudate.

Key Words: Anti-inflammatory, oligoamines, Inflammatory exudates.

CONTENTS

3. Results

TABLES AND FIGURES

ABBREVIATIONS

The following abbreviations have been used in the text:-

ODC, Ornithine decarboxylase

DMNO, Dimethylnitrosamine

Cyclic AMP, Adenosine 3':5' - cyclic Monophosphate

1. INTRODUCTION

Inflammation is the process by which living tissue responds to injury. In many instances it is trivial, such as with an insect sting, but sometimes it can be a serious and even life threatening condition. In man the most prevalent inflammatory conditions are known collectively as the rheumatic diseases. This classification covers a wide variety of syndromes and includes diseases associated with high mortality such as Systemic Lupus Erythematosus (SLE) and diseases such as rheumatoid arthritis and osteoarthrosis which are severely crippling, but are not usually life threatening.

Both of these crippling diseases are prevalent,but rheumatoid arthritis can be more serious since severe forms of the disease can quickly lead to loss of mobility. Osteoarthrosis is not a well defined disease, but may be considered to be largely a "wear and tear" disease that represents a gradual loss of function of joints as part of the aging process. Due to the serious nature of rheumatoid arthritis it has received great attention from research workers.

The researcher into rheumatoid arthritis must accept the discouraging statement that the syndrome is of largely unknown cause and aetiology and, in addition, there is no known cure.

It is for these reasons that many researchers assume that a study of inflammation will help our understanding of rheumatoid arthritis, and much of modern drug therapy is based on this belief. It is certain that inflammation plays a large part in the disease and the work described in this study has been concerned with controlling inflammation as a means to treat rheumatoid arthritis.

1.1 The Nature of Inflammatory Stimuli

The stimulus giving rise to inflammation may be one of

several factors, but trauma of one type or another is a common feature.

The ability of a drug or chemical substance to provoke an inflammatory response is termed irritancy. In this case the tissue damage may be restricted to the site of administration and is not usually severe, although some irritants, for example: croton oil or corrosive poisons, are very potent and may cause severe and prolonged inflammation.

Inflammation may also develop due to infection, the tissue damage being caused by toxins or the organisms themselves. The response may be a consequence of activity of the immune system to the infectious organism (Hicks, 1975). Several organisms have been implicated in the search for an infectious agent in rheumatoid arthritis. Marmion (1976) concluded that there was no evidence that diptheroids, mycoplasmas or other prokaryotes were the missing exogenous antigens in rheumatoid arthritis, but there were some preliminary hints that a defective rubella, or other, unidentified virus may be involved.

In addditon, inflammation,both mild and severe, may be the consequence of an allergic malfunction of the immune system as typified by rheumatoid and other "autoimmune"diseases.

1.2 The Inflammatory Process

Inflammation involves change in the connective tissues and vascular supply of damaged tissue. The process may be considered as a protective measure, designed to minimise or eliminate the cause of damage and subsequently to remove the damaged tissue and lead to its repair. The acute inflammation following a stimulus involves two components, fluid and cellular,both arising from a vascular source.

The first changes are a brief arteriolar constriction followed by prolonged dilation of arterioles, capillaries and venules after the stimulus is received (Spector and Willoughby, 1968).

This process is accompanied by an increased permeability of the venules and capillaries (Cotran, 1967) with a consequent loss of fluid into the surrounding tissue.

The increase in permeability of blood vessels to plasma protein following injury is now known to have a biphasic nature. The initial phase, which has not been shown for all experimental inflammations, has a transient character and primarily involves the venules, whereas the delayed or prolonged phase of increased permeability involves the capillaries as well (Cotran, 1967). However, in certain inflammations the venules appear to be solely responsible for the delayed phase (Hurley and Spector, 1965).

In general, the initial phase of this response can be inhibited by antagonists of known endogenous permeability factors (Logan and Wilhelm, 1966). Histamine is a likely candidate as a mediator of this increased vascular permeability and considerable evidence is available to support this view. For example, histamine has been found in the exudate after intrapleural injection of turpentine for up to thirty minutes after initiation of the pleurisy (Spector and Willoughby, 1957). Venule labelling with colloidal carbon can be inhibited by anti-histamine treatment during this initial phase (Hurley and Spector, 1965) and, also, tissue depletion of histamine has been shown to delay the onset of inflammation produced by injection of bacteria (Sheldon and Bauer, 1960). Thus histamine appears to be the major mediator in this phase of increased vascular permeability although it plays no part in the prolonged phase of the response (Spector and Willoughby, 1968). In rats and mice 5-hydroxytryptamine is thought to have a role in addition to histamine, but this fact seems peculiar to these species.

In the prolonged phase many mediators may be involved in the response, but in the case of the carrageenan induced oedema in

the rat (Di Rosa and Willoughby, 1971; Di Rosa, Giroud and Willoughby, 1971) kininlike substances are thought to maintain the oedema during its plateau phase and prostaglandinlike substances promote the second accelerating phase of swelling, both these events occurring after the initiation of the response by histamine and 5-hydroxytryptamine. The involvement of kininlike substances is suggested by work which shows an inhibition of the oedema by antiproteinases (Von Arman, Begany, Miller and Pless, 1965; Di Rosa and Sorrentino, 1968) . Briseid, Arntzen and Dyrud (1971) have correlated changes in the plasma kinin system with inhibition of the oedema, other work has also supported this involvement of kinins (Di Rosa, Giroud, and Willoughby, 1971; Vinegar, Macklin, Truax, and Selph, 1971). Prostaglandin-like substances have been implicated in the second phase of the oedematous response (Di Rosa and Willoughby, 1971) and the isolation of E-type prostaglandins from oedema fluid has been claimed although artifacts may be present in this case (Willis, 1969). Chang, Murota and Tsurufugi (1976) concluded that E-type prostaglandins, at best, have a minor role as mediators of vascular permeability. However, it is now known that many of the non-steroidal anti-inflammatory drugs inhibit prostaglandin synthesis which is thought to be their mode of action and, indeed, the ability of these compounds to inhibit prostaglandin synthesis seems to parallel their therapeutic efficacy (Vane, 1971; Paulus and Whitehouse, 1973). Recently, leukotriene B, a product of the lipoxygenase pathway of arachidonic acid metabolism, has been found to increase vascular permeability in the presence of a vasodilator such as PGE_2. (Bray, Cunningham, Ford-Hutchinson and Smith,1980).

These changes in the vascular channels together with the altered permeability cause an increase in viscosity of the blood which is responsible for pushing the leukocytes against the vascular

endothelium to which they adhere and eventually migrate into the extravascular tissues.

The cellular component of an acute inflammatory exudate is now thought to be of haematogenous origin, as opposed to local proliferation of histiocytes and reticulo-endothelial cells (Spector, Walters, and Willoughby, 1965). For most experimentally induced inflammations, it has been found that the polymorphonuclear leucocytes are the first cells to enter the injured area, but, with time, the mononuclear cells (monocytes, lymphocytes, and plasma cells) predominate (Martin and Southwell, 1965; Hurley, Ryan and Friedman, 1966; Ryan, 1967). These results conflicted with the earlier work of Spector, Walters and Willoughby (1965) and Spector and Coote (1965) who found mononuclear cells emigrating concurrently with the polymorphonuclear leucocytes, though in far smaller numbers.

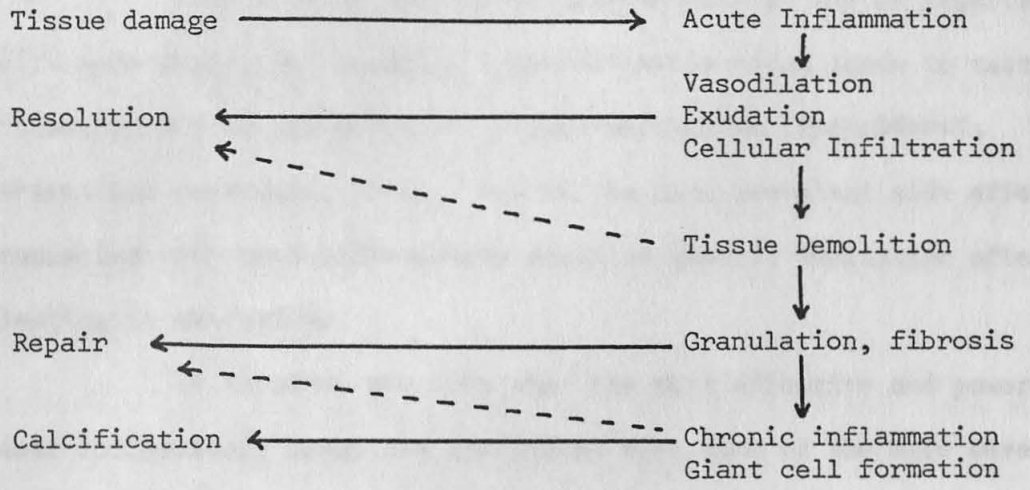
The emigration of cells from small blood vessels is now well documented, largely from electron microscope studies, and reveals that, after injury, leucocytes adhere to the vascular wall and emigrate across the endothelium by extending pseudopodia between the endothelial cells. The emigration of leucocytes appears to be divorced from the leakage of protein and indeed emigration can take place with little or no permeability increases.

The various types of inflammatory cells proceed to remove the foreign material and to demolish the severely damaged tissue prior to repair. Polymorphonuclear (PMN) leucocytes play a key role in this phase of the process. During the process of phagocytosis these cells release a number of biological molecules (Weissmann, Smolen and Hoffstein, 1978). For example derivatives of molecular oxygen ($O_2^{-\cdot}$, $OH^{\cdot}$ and H_2O_2) which have a bactericidal action, inflammatory mediators such as prostaglandins and thromboxanes are also released. The PMN leukocyte also secretes lysosomal proteinases

which aid tissue demolition.

The process of repair may now take place with the development of granulation tissue. The tissue is reformed by fibroblast cells which migrate from surrounding connective tissue and deposit collagen fibres around the lesion. If at this point the inflammatory stimulus still persists, macrophages often undergo a process of fusion, giving very large multinucleate "giant cells" which attempt to surround the stimulus. Ultimately persistent inflammatory sites may become calcified.

The development of inflammation is summarised in the following diagram:-



(After Hicks, 1975)

1.3 The Use of Drugs in Inflammation

In order to evaluate the importance of endogenous anti-inflammatory substances it is necessary to consider the drugs currently being used to treat inflammation and some of the problems associated with them.

The drugs in current use may be conveniently split up into five groups; antimalarials, gold salts, nonsteroidal anti-inflammatory drugs, immunosuppressives and immunostimulants. None

of these drugs eliminate the cause of the disease (Mathies, 1979) but they can alleviate the symptoms.

In general the five groups of drugs provoke common side effects due to the same pathological processes occurring. These side effects are commonly immunological in nature due to either the immunological effects of the compounds or of the immunogenicity acquired by the compounds upon their formation of complexes with proteins or platelets (Franchimont, Heyren and Hanvwaert, 1978). The side effects may include skin rashes, leukopenia, glomerulonephritis and symptoms comparable with those found in viral diseases (fever, chills etc.).

Side effects associated with metabolism may be important with some drugs, for example, D-penicillamine often leads to taste disorders due to its effect on copper metabolism (Franchimont, Heyren and Hanvwaert, 1978). One of the most prevalent side effects connected with anti-inflammatory drugs is gastric irritation often leading to ulceration.

It is often the case that the most effective and powerful anti-inflammatory drugs are associated with some of the more severe side effects seen.

From this brief review it is clear that all the drugs which are currently in use in the treatment of inflammation suffer from the disadvantage that their use may cause severe side effects.

It is for these reasons that endogenous anti-inflammatory substances have been the subject of intensive study by experimental investigators. An endogenous anti-inflammatory substance would have the benefit that it would not be so liable to produce injurious side effects and also a study of the organisms natural defence mechanism may elucidate the inflammatory process.

1.4 Antiproteinases

Antiproteinases may be considered to be endogenous anti-inflammatory substances since they occur naturally and may play a role in inflammation. The involvement of lysosomal enzymes (elastase, collagenase, cathepsin G) released by polymorphonuclear leukocytes in inflammation is well known (Weissmann, Smolen and Hoffstein, 1978). The overall activity of proteinases is the result of the activity of the proteinase and the activity of any proteinase inhibitor present. Thus antiproteinases may have a role in inflammation and have been extensively studied.

1.4.1 Alpha-1-antitrypsin

Alpha-1-antitrypsin was isolated, characterized and named by Schultz, Heide and Haupt (1962). Of all the proteinase inhibitors alpha-1-antitrypsin has the highest molar concentration in blood (40-100 μ mol/L) and is responsible for up to 90% of the trypsin inhibiting capacity of plasma (Ganrot, P.O., 1972). This inhibitor has been structurally designated as an alpha-1-glycoprotein which is composed of a single polypeptide chain having 12% carbohydrate and containing galactose, mannose, acetylglucosamine and sialic acid and has a molecular weight of between 47,500 and 55,000 daltons (Crawford, 1973; Musiani and Tomasi, 1976). The protein is heat labile and can be irreversibly inactivated at low pH (<5).

Alpha-1-antitrypsin has been found to inhibit the action of a number of serine proteinases beside trypsin, these include: chymotrypsin, plasmin, thrombin (Rimon, Shamash and Shapiro, 1966), kallikrein (Haberman, 1968), elastase (Kaplan, Kuhn and Pierce, 1973), granulocytal elastase (Ohlsson, 1971) and granulocytal collagenase (Harris, Di Bona and Krane, 1969), all of which are associated with inflammation. It should be noted that alpha-1-antitrypsin does not inhibit all collagenases. It does not, for example, inhibit acid

proteinases (Kopitar and Lebez, 1975) and also a neutral collagenase isolated from human gastric mucosa is unaffected (Woolley, Tucker, Green and Evanson, 1976).

Alpha-1-antitrypsin is a so called acute phase reacting (APR) protein which implies that the concentration in plasma increases in infection and inflammatory conditions. Patients suffering from rheumatoid arthritis show a significant rise in alpha-1-antitrypsin levels in both serum and synovial fluid. A large molecular protein with the immunological specificity of alpha-1-antitrypsin, presumably a complex of the inhibitor and a proteinase, was detected in the synovial fluid of subjects with confirmed or suspected rheumatoid arthritis (Brackertz, Hagmann and Kueppers, 1975).

The inhibitor can exist in a large number of genetic variants of which the so called MM type is the form found in normal individuals. A slowly migrating component, called ZZ is seen on electrophoresis in cases of genetic deficiency. Cox and Huber (1976) claimed an association between heterozygosity for the Z allele of alpha-1-antitrypsin and classical rheumatoid arthritis. This finding has been disputed by several workers (Sjöblom and Wollheim, 1977; Brackertz and Kueppers, 1977; Buisseret, Pembrey and Lessof, 1977) who claim the influence of alpha-1-antitrypsin polymorphism on the incidence and course of rheumatoid arthritis is modest at most. Lewis and Capstick (1977) claimed to have found an inverse relationship between alpha-1-antitrypsin and neutral proteinase levels in human rheumatoid synovial fluid. A similar relationship was demonstrated in the sera from adjuvant arthritic rats, the alpha-1-antitrypsin levels falling as the disease progressed (Parrot and Lewis, 1977). The levels of plasma alpha-1-antitrypsin are also elevated during pregnancy (Fagerhol and Laurell, 1970) and viral

hepatitis (Kindmark and Laurell, 1972) both of which are known to produce a remission of rheumatic disease.

1.4.2 Alpha-2-macroglobulin

The second major antiproteinase found in plasma is alpha-2-macroglobulin (2.5 - 5 μmol/l) and it accounts for nearly 10% of the trypsin inhibiting capacity of plasma (Ganrot, P.O. and Scherstén, 1967). The molecular weight of alpha-2-macroglobulin has been estimated to be in the range of 725,000 to 820,000 daltons (Schönenberger, Schmidtberger and Schultz, 1958; Roberts, Riesen and Hall, 1974), but there is no information regarding its quaternary structure. Jones, Creeth and Kekwick (1972) have suggested an eight chain subunit structure, with dimers of these chains forming the 196,000 dalton subunits produced by limited thiol reduction of alpha-2-macroglobulin (Frenoy, Razafimahlaeo and Bourrillon, 1972). Four of these subunits may then form an alpha-2-macroglobulin molecule.

Alpha-2-macroglobulin exhibits a wide spectrum of activity. It can bind and inhibit proteinases from all groups of endopeptidases. These include trypsin, chymotrypsin, plasmin, thrombin, kallikrein, granulocytal elastase, granulocytal collagenase, cathepsin, B_1, cathepsin D, thermolysin and chlostridio peptidase A (Barrett and Starkey , 1973). Cathepsin B_1 and neutral collagenases are known to be capable of attacking the triple helix of collagen (Burleigh, 1973) and together with cathepsin D of degrading proteoglycans (Burleigh, Barrett and Lazarus, 1974). The levels of alpha-2-macroglobulin are also elevated during pregnancy (Schumacher and Schulmberger, 1963) and the protein may be detected in the synovial fluid of rheumatoid arthritic patients although it was in the inactive form in 25% of the patients. It was, however, active in the sera from these subjects.

In the rat, alpha-2-macroglobulin does not occur in the plasma of normal rats, but certain conditions, for example, foetal and neonatal states, pregnancy, neoplastic growth, interference with the kidney and inflammation cause it to appear in the plasma of such rats (Heim, 1968). K. Ganrot (1973) has found that this alpha-2 acute phase globulin binds plasmin and trypsin and can be considered as a human alpha-2-macroglobulin homologue. In the normal rat the function of alpha-2-macroglobulin is performed by alpha-1-macroglobulin (Ganrot, K. 1973).

1.4.3 Other Antiproteinases

A potentially important antiproteinase was discovered by Woolley, Roberts and Evanson (1976) in human plasma. Migrating electrophoretically as a beta-1-globulin, with a molecular weight of about 40,000 daltons, it has been named beta-1-anticollagenase since it specifically inhibits neutral collagenases. Its importance may lie in the fact that it can inhibit collagenases which are normally inhibited by alpha-2-macroglobulin, but its smaller molecular size would enable it to reach inflammatory sites much more easily.

Other proteinases are present in human and animal plasma, but since their concentrations are extremely low they may be of less importance. However, inter-alpha-trypsin and alpha-1-antichymotrypsin have been found to inhibit trypsin, chymotrypsin and chymotrypsin respectively (Heide,Heimburger and Haupt, 1965;Heimburger and Haupt, 1965).

Rat plasma appears to have another antiproteinase in addition to alpha-1-antitrypsin, alpha-1-macroglobulin and alpha-2-acute phase protein. This protein has an approximate molecular weight of 90,000 daltons and inhibits trypsin and chymotrypsin, but its trypsin inhibitory capacity in plasma is less than that of alpha-1-antitrypsin and alpha-1-macroglobulin (Huttunen and Korhonen,

1973).

A subject of recent investigations has been the proteinase inhibitor Trasylol (Bayer). Trasylol appears to influence many functional systems, these include blood clotting, thrombolysis, complement and plasma kinin formation.

Förster (1969), using a variety of inducing agents showed that Trasylol inhibited the formation of rat paw oedema and the arthus reaction. The activity of acid and neutral proteinases were reduced in the serum of these animals. Spilberg and Kirk-Osterland (1970) using sodium urate crystals to induce an acute arthritis in the rabbit found Trasylol to be anti-inflammatory and suggested that Trasylol may act by inhibiting acid and neutral polymorphonuclear leukocyte lysosomal proteinases. The evidence that Trasylol is beneficial in the treatment of human rheumatoid arthritis is contradictory. It has been claimed (Bruckner and Eisen, 1970) that Trasylol compares favourably with hydrocortisone acetate in the treatment of the disease and may be useful where steroids are contra-indicated. In contrast Marcy, Loyan and Dumas (1972) found Trasylol to be relatively ineffective in the treatment of this condition.

1.5 Endogenous Anti-Inflammatory Substances

There are many reports in the literature concerning inflammatory exudates, but from early work (Rindani, 1956) it became clear that anti-inflammatory substances were present in such exudates.

Lyophilized granuloma pouch exudates obtained from intact or adrenalectomised rats were found to contain a substance capable of inhibiting granulation tissue formation (Dispasquale and Girerd, 1961). The activity contained within this exudate was later found not to be due to inherent ACTH or adrenal corticoid activity

(Dispasquale, Girerd, Beach and Steinetz, 1963).

Many workers have used in vitro tests for anti-inflammatory activity and using such techniques Persellin (1972) found that a protein derived from arthritic rat plasma, that migrated electrophoretically as an alpha globulin with a molecular weight of 170,000 daltons stabilized rat liver lysosomes against thermallysis. Human sera has been found to inhibit the lysis of rabbit polymorphonuclear leukocytes by Triton X-100 (Hempel, Fernandez and Persellin, 1970), this ability, which is not due to increased corticosteriod levels, being increased during pregnancy.

Lewis, Capstick and Cosh (1975) demonstrated that human rheumatoid synovial fluid stabilized rat liver lysosomes in vitro. This stabilizing effect was abolished by pre-incubation of the synovial fluid with trypsin. Later work (Lewis, 1977) has shown that two proteins were responsible for this effect and they migrate in the alpha/beta globulin region during electrophoresis.

A high molecular weight protein fraction from the sera of normal rats was isolated by Lewis, Capstick and Best (1976). This fraction was shown to be anti-inflammatory against the carrageenan induced oedema and the adjuvant arthritic animal models.

Antilymphocytic serum was shown to contain two anti-inflammatory components which could be separated by gel filtration (Billingham, Robinson and Gaugas, 1970). One of these components was thought to be IgG (75%) and the other had similar properties to the anti-inflammatory substances found in inflammatory exudates.

One of the few examples of a human inflammatory exudate containing an anti-inflammatory substance was provided by Billingham, Robinson and Robson (1969b) when they investigated the exudate collected from partial gastrectomy patients. It was shown

that this exudate inhibited the formation of carrageenan induced oedema of the rat foot. In addition, the exudate was fractionated using gel filtration, the active component behaving as a macromolecule.

Bee venom of the honey bee (Apis mellifera) has been found to contain a peptide (401) which had a powerful anti-inflammatory action, which compared favourably with both steroidal and non-steroidal drugs (Billingham, Morley, Hanson, Shipolini and Vernon, 1973; Hanson, Morley and Soria-Herrera, 1974). The amino acid sequence for this peptide had been previously established by Vernon, Hanson and Brimblecombe (1969). In addition to being a potent mast cell degranulating factor this peptide was shown to have a vascular effect in that it suppresses the increased vascular permeability caused by the intradermal injection of smooth muscle spasmogens histamine, bradykinin, 5-hydroxytryptophan and prostaglandins (Hanson, Morley and Soria-Herrera, 1974). These workers concluded that the peptide may make the vascular endothelium anergic to phlogistic stimuli. It has been reported recently (Chang and Bliven, 1979) that bee venom may have an immunosuppressive effect. These workers found that a daily subcutaneous dose of bee venom inhibited the formation of adjuvant induced arthritis in the rat. This effect was found to be dose dependant. In addition a single dose of bee venom given at the same time as the adjuvant or, one day after it, suppressed the development of the arthritis by as much as 50%. It was suggested that the bee venom may operate by one of two mechanisms. Firstly the venom caused an alteration of the immune response possible by antigen competition. Secondly the venom operated via endogenous corticosteroids. It was concluded that the immunological role was probably more important.

It was proposed by McArthur, Dawkins, Smith and Hamilton (1971) that human serum contained peptide like substances

in protein bound and free forms. It was suggested that abnormally low concentrations of the free forms were an important factor in the development of rheumatic diseases. A peptide-like substance which possessed anti-inflammatory activity and was capable of binding to circulating proteins was isolated from human serum by McArthur, Smith and Freeman (1972).

Using an improved isolation procedure the substance was shown to exhibit no antagonistic effects towards the inflammatory mediators histamine, 5-hydroxytryptamine, bradykinin and prostaglandins E_2 and $F_{2\alpha}$ when tested on isolated tissue preparations (Elliot, Ford-Hutchinson, Harford, Insley, Smith and Sturgess, 1973). Further work has shown that this substance had an apparent molecular weight of below 1000 daltons, could be partially purified by solvent extraction and was resistant to acid and proteolytic digestion. This plasma fraction exhibits a broad spectrum of anti-inflammatory activity ranging from inhibition of oedema formation to reduction of the infiltration of leucocytes into inflammatory exudates (Smith and Ford-Hutchinson, 1975). It was concluded this fraction must be considered as a specific anti-inflammatory substance and its possible mode of action was on the release of chemotactic factors after the activation of the complement cascade.

The ameliorating effect of hepatic disease on rheumatoid arthritis has been known for some time (Hench, 1933) and although jaundice was thought to be the determining factor in these remissions a satisfactory explanation has never been offered (Hill and Holley, 1966).

Pinals (1973) induced hepatic damage in rats using intraperitoneal injections of the selective hepatotoxin dimethylnitrosamine. He found that adjuvant arthritis of the rat was partially inhibited by the administration of dimethylnitrosamine on the fourth or fifth

day after the injection of the mycobacterial adjuvant. Furthermore, saline extracts of livers from dimethylnitrosamine treated rats were capable of inhibiting the development of adjuvant arthritis, whereas saline extracts from normal rat livers were ineffective. Pinals suggested that some anti-inflammatory material released from damaged liver may have been responsible. Lewis, Best, Bird and Parrott (1978) have shown that this anti-inflammatory activity is contained in the high molecular weight region after Sephadex G-150 fractionation and proteolytic digestion of this fraction may produce a dialysable substance with anti-inflammatory activity.

The hepatoxin carbon tetrachloride has also been found to inhibit the formation of granulation tissue and carrageenan induced oedema (Mielens , Drobeck, Rozitis and Sansone (1969).

One inflammatory exudate which has been extensively studied is that produced by the subcutaneous implantation of polyester sponges into rats. This exudate has been shown to inhibit the formation of granulation tissue (Robinson and Robson, 1964).

It was suggested that this anti-inflammatory factor was not present in normal serum, but may be present in the serum of sponge implanted rats. Further work has shown that the exudate retarded the rate of wound healing, but did not significantly alter the formation of oedema fluid. The active principle was also found to be a non-dialysable (Robinson and Robson, 1966). The exudate was found to be active in other animal models. It inhibited the formation of carrageenan induced oedema of the rat foot and when injected subcutaneously into BCG-vaccinated guinea pigs a significant, though transient, supression of tuberculin reactivity in the skin was noted (Gaugas, Billingham and Rees, 1970).

Billingham, Robinson and Robson (1969a) demonstrated that the exudate could be fractionated by gel filtration and ion exchange

chromatography to produce three fractions, two of which were anti-inflammatory. They further showed that this activity could be destroyed by incubation above 70°C or with pronase. The activity of the exudate was not due to the presence of steroids since the levels in the exudate were lower than in normal serum. The protein/s studied in this work were further purified by preparative polyacrylamide gel electrophoresis (Billingham, and Robinson, 1972). The resultant preparation was claimed to have a high anti-inflammatory activity, but to be no more irritant than saline. Later work has been concerned with the possible mode of action of this preparation, but this has met with little positive success. The inflammatory exudate inhibited the formation of pleural effusion, emigration of both neutrophils and mononuclear cells and the accumulation of β-glucuronidase and lactic dehydrogenase (Doherty and Robinson, 1976b). The same dose of exudate did not, however, inhibit the increased vascular permeability induced in rat skin or rat foot following injection of 5-hydroxytryptamine, histamine, prostaglandin E_1 or bradykinin. Furthermore, sponge exudate did not reduce the haemolytic complement titre of rat serum either *in vitro* or *in vivo*. The exudate had no stabilizing effect of lysosomal preparations (Doherty and Robinson, 1976a). The origin of the anti-inflammatory factor contained within this exudate has been attributed to *de novo* synthesis by the liver following injury to the animal (Billingham, Gordon and Robinson, 1971).

In summary, it appears that polyester sponge induced inflammatory exudate contains an anti-inflammatory factor which is protein-like, synthesized *de novo* by the liver following injury and migrates electrophoretically between the alpha-glycoproteins and transferrin. Little, however, is known regarding its exact nature or mode of action.

The mechanism of action of the potent anti-inflammatory agent dexamethasone is not yet understood, but it has been found that both RNA and protein synthesis appear to be essential for the manifestation of the anti-inflammatory effect (Tsurufuji, Sugio, and Tekemasa, 1979). It has also been shown that glucocorticoids induce the biosynthesis of a protein (or peptide) inhibitor which prevents prostaglandin generation (Flower and Blackwell, 1979).

1.6 The Anti-Inflammatory Activity of Irritants

The relief of inflammation by the use of non-specific irritants has been employed by physicians for many centuries. The mechanism by which a local irritation exerts an additional more remote anti-inflammatory effect is, as yet, an unexplained phenomenon. This counter irritant effect is particularly important when considering any endogenous anti-inflammatory substance since it may be argued that such substances may operate by causing irritation.

Laden, Blackwell and Fosdick (1958) showed that in rats the volume of pleural exudate produced by the injection of an irritant (gum arabic) was reduced by a second irritant (gum arabic or silver nitrate) injected into the knee joint. This anti-inflammatory effect could not be explained by loss of fluid into the injected knee.

The intraperitoneal administration of a wide variety of agents such as hydrogen peroxide, sodium hydroxide, kaolin and talc inhibited the inflammatory oedema of the rat paw induced by formalin, dextran, 5-hydroxytryptamine and polyvinylpyrolidine (Von Buch and Wagner-Jauregg, 1960; Von Buch and Wagner-Jauregg, 1962).

Cygielman and Robson (1963) found that tartar emetic, croton oil and talc caused a significant reduction in the granulation tissue deposited around cotton pellets implanted in intact and adrenalectomised rats. They suggested that there was some limit to the total number of leukocytes which in the course of a few days

could be mobilised to take part in the inflammatory reaction.

Acetic acid, formaldehyde and hypertonic saline inhibited the development of carrageenan induced oedema. This was thought to be related to the extravasation of fluid and proteins into the peritoneal cavity (Jori and Bernardi, 1966).

Atkinson, Boura and Hicks (1969) reported that polyester sponge induced inflammatory exudate possessed local irritant properties. The sponge exudate was found to increase vascular permeability, but its tonicity was found to be unimportant. Atkinson and Hicks (1971) noted that a correlation existed between the anti-inflammatory and irritant properties of sponge exudate. These results were further substantiated using different techniques by Atkinson, Whittle and Hicks (1971). The plasma from sponge bearing rats was claimed to have no anti-inflammatory activity even when the sponges irritancy was augmented by croton oil. These workers (Atkinson and Hicks, 1975b) concluded that the induction of inflammatory lesions in rats did not appear to lead to a detectable release of endogenous anti-inflammatory substances into the circulation.

The mechanism of counter irritation is unknown, but Atkinson and Hicks (1975a) in their review of the subject suggested the following possibilities.

Since the administration of an irritant may be considered to be a stress stimulus it may be that the resultant pituitary-adrenal stimulation is of importance. However, since a number of reports indicate that irritants have a similar effect on adrenalectomized and hypophysectomized rats as normal animals this explanation seems unlikely (Laden, Blackwell and Fosdick, 1958; Cygielman and Robson, 1963).

Another hypothesis is that there may be competition between two inflammatory sites for finite quantities of inflammatory

mediator(s) or their precursors. Atkinson and Hicks (1975a) conclude that this mechanism may be important in acute conditions with finite concentrations of mediator material like the kinin or complement systems. However, competition for cellular and other mediators seems less likely, presumably because given time the system is capable of producing very large numbers of cells and possibly restoring the availability of other mediators.

A possible mechanism which has received much attention is the production and/or release of endogenous anti-inflammatory substances at the site of inflammation. These substances could then enter the blood stream and produce an inhibition of inflammation at a remote site. The evidence supporting this theory comes from a demonstration of anti-inflammatory substances found at inflammatory sites (See Section 1.5) and of anti-inflammatory activity in the blood of animals bearing inflammatory sites. Support for the latter phenomenon has come in part from a study of acute phase reactants. Bogden, Gray and Fuss (1966) while working with alpha-2-glycoprotein found that serum from traumatized animals caused increased alpha-2-glycoprotein synthesis in normal rats. Plasma and serum obtained from rats four days after implantation (s.c.) of polyester sponges has been shown to exert an anti-inflammatory effect (Robinson and Robson, 1964; Billingham, Robinson and Robson, 1969a). These workers have suggested that this anti-inflammatory factor is synthesized by the liver, and indeed, perfused livers from traumatized rats did produce an anti-inflammatory factor (Billingham, Gordon and Robinson, 1971).

The presence of a factor in the plasma of such rats has recently been disputed by Atkinson and Hicks (1975b) who found no evidence of anti-inflammatory activity in their plasma. Atkinson and Hicks (1975a) concluded that no one theory is acceptable.

Rather, it would appear that a variety of mechanisms are evoked by different irritants or inflammatory states.

1.7 The Role of Endotoxins in the Study of Endogenous Anti-Inflammatory Agents

Recently it was concluded that doubt will exist about the presence of anti-inflammatory factors in mammalian body fluids unless stringent precautions are taken to exclude measurable bacterial contamination (Maguire and Wallis, 1977). These workers were investigating the anti-inflammatory properties of anti-lymphocytic serum, which had previously been discovered by Billingham, Robinson and Gaugas (1970). Gel filtration of non-sterile anti-lymphocytic serum yielded two fractions showing anti-inflammatory activity. One fraction eluted with an apparent molecular weight of 200,000 while the other eluted with the serum salts ($\simeq$ 5000) which upon rechromatography behaved as a high molecular weight material. This fraction was contaminated with gram negative bacteria. Sterile anti-lymphocytic serum when chromatographed had only one active fraction (high molecular weight), the lower molecular weight fraction had no detectable anti-inflammatory effect. Bacterial preparations from the non-sterile serum and commercial endotoxin were markedly anti-inflammatory. This lower molecular weight fraction was capable of elevating 11-hydroxycorticosteroid levels and abolishing leukocyte infiltration into the foot injected with irritant, but the serum haemolytic complement levels and the total number of circulating leucocytes were unchanged.

Further evidence concerning the anti-inflammatory role of endotoxin was provided by Smith, Ford-Hutchinson and Walker (1977). These workers found that endotoxin inhibited carrageenan induced foot oedema and reduced the total leucocytes found in an exudate caused by polyester sponge implantation.

This endotoxin preparation did not cause a local irritant action when injected in the foot pad of rats. These workers also investigated the connection between endotoxin and an anti-inflammatory fraction derived from human plasma (Ford-Hutchinson, Insley, Elliot, Sturgess and Smith, 1973). It was found that part of the overall anti-inflammatory activity was due to the presence of material resembling endotoxin. A low molecular weight fraction was also present which possessed properties antagonistic to those of endotoxin.

Endotoxins possess a large number of biological activities some of which are inflammatory. The well known Shwartzman reaction has been described as a model of inflammation (Rosenthale, 1974). Endotoxin has been shown to activate the pathways of complement (Kane, May and Frank, 1973) causing changes in vascular permeability and in neutrophil chemotaxis (Mergenhagen, Snyderman, Gewurz and Shin, 1969). There is an increased release of vasoactive substances, including histamine and the kinins, which may be mediated, at least in part, by activated components of the complement system (Hinshaw, Brake and Emerson, 1965). In contrast it has been reported (Conti, Cluff and Scheder, 1961) that non lethal doses of endotoxin inhibited leucocyte infiltration into areas of rabbit skin infected with staphylococci.

A recent study (Goodman, Way and Irwin, 1979) has shown that the intravenous injection of endotoxin has marked effects. Doses of 50-100 µg per Kg evoked a marked inflammatory response. Within ten minutes there was a decrease in both leucocyte and platelet count. It was suggested that destruction of leucocytes persisted for several hours after the injection. Observation of the vasculature in the rabbit's ear showed that cells stuck to the endothelium leading to occlusion of the blood vessels within one hour.

Thus endotoxin has conflicting properties and its role is unclear.

1.8 The Role of Ologoamines in Inflammation

Oligoamines are synthesized in animal and bacterial cells. The oligoamines, whose trivial names are putrescine, cadaverine, spermine and spermidine, are structurally simple cationic compounds which may be involved in a number of processes. Oligoamines are intimately involved in cell proliferation mechanisms and may have other effects.

1.8.1 Oligoamines and Inflammation

At the present time the evidence for a direct involvement of oligoamines is limited.

The anti-inflammatory effects of dexamethasone have been shown to be mediated by polypeptides synthesized following binding of the steroid to the glucocorticoid receptor and induction of gene expression (Tsurufuji, Sugio and Takemasa, 1979). Bartholeyns, Fozard and Prakash (1980) suggested that the diamine purtescine was necessary for the biosynthesis of these anti-inflammatory mediators. These workers found that dexamethasone induced rises in hepatic ornithine decarboxylase activity (the enzyme responsible for the synthesis of putrescine) and putrescine concentration which was maximal at four hours. The concentrations of spermidine and spermine were unchanged.

Using an irreversible inhibitor of ornithine decarboxylase (α-difluoromethylornithine) the effects of dexamethasone could be inhibited, but the effects of indomethacin were unaltered. Thus these investigators concluded that de novo synthesis of putrescine was an essential factor in the induction of new protein synthesis known to be involved in the anti-inflammatory action of dexamethasone.

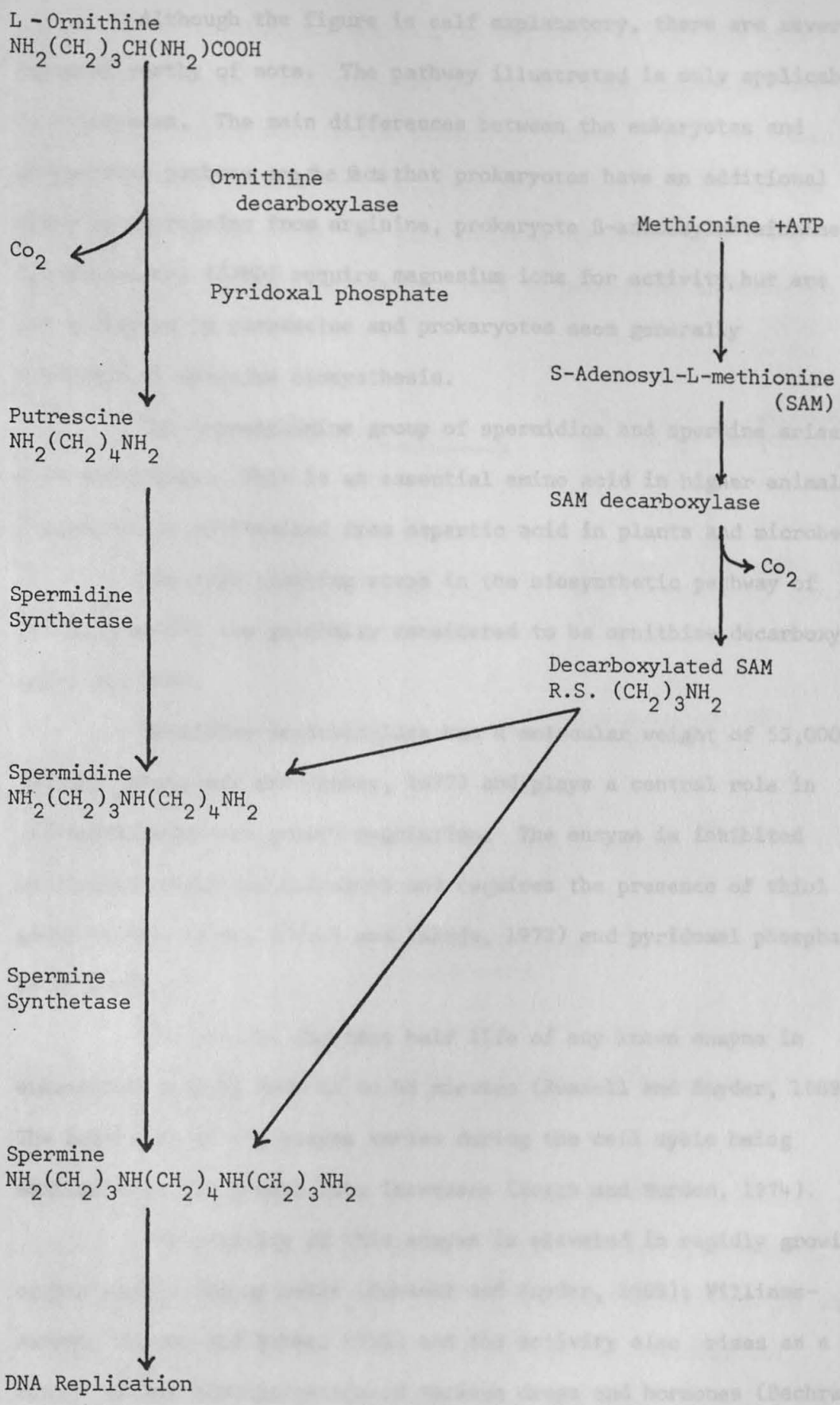
This study was extended by Batholeyns , Fozard and Prakash

(1981) who found that pretreatment with aminoguanidine sulphate, an inhibitor of putrescine catabolism sixteen hours before the administration of α-difluoromethylornithine or actinomycin D, completely prevented blockade of the anti-inflammatory effect of dexamethasone by α- difluoromethylornithine but left unaltered the suppressive effects of actinomycin D. The de novo synthesis was thought to act at the transcriptional level.

The oligoamine levels in the serum from patients suffering from rheumatoid arthritis have been determined by Partsch, Desser and Tausch (1978). There was no significant differences between normal serum and serum from rheumatoid arthritis patients.

1.8.2 The Biosynthesis of Oligoamines

The biosynthetic pathway for oligoamines (collective name for di-; tri-; and tetra-, amines) is outlined in the figure below.


L - Ornithine
$NH_2(CH_2)_3CH(NH_2)COOH$
Ornithine decarboxylase
Co_2
Pyridoxal phosphate
Methionine +ATP
S-Adenosyl-L-methionine (SAM)
SAM decarboxylase
Co_2
Decarboxylated SAM
R.S. $(CH_2)_3NH_2$
Putrescine
$NH_2(CH_2)_4NH_2$
Spermidine Synthetase
Spermidine
$NH_2(CH_2)_3NH(CH_2)_4NH_2$
Spermine Synthetase
Spermine
$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$
DNA Replication

Although the figure is self explanatory, there are several features worthy of note. The pathway illustrated is only applicable to eukaryotes. The main differences between the eukaryotes and prokaryates pathway are the facts that prokaryotes have an additional route to putrescine from arginine, prokaryote S-adenosylmethionine decarbosylases (SAMD) require magnesium ions for activity, but are not activated by putrescine and prokaryotes seem generally incapable of spermine biosynthesis.

The n-propylamine group of spermidine and spermine arises from methionine. This is an essential amino acid in higher animals whereas it is synthesized from aspartic acid in plants and microbes.

The rate limiting steps in the biosynthetic pathway of of oli goamines are generally considered to be ornithine decarboxylase (ODC) and SAMD.

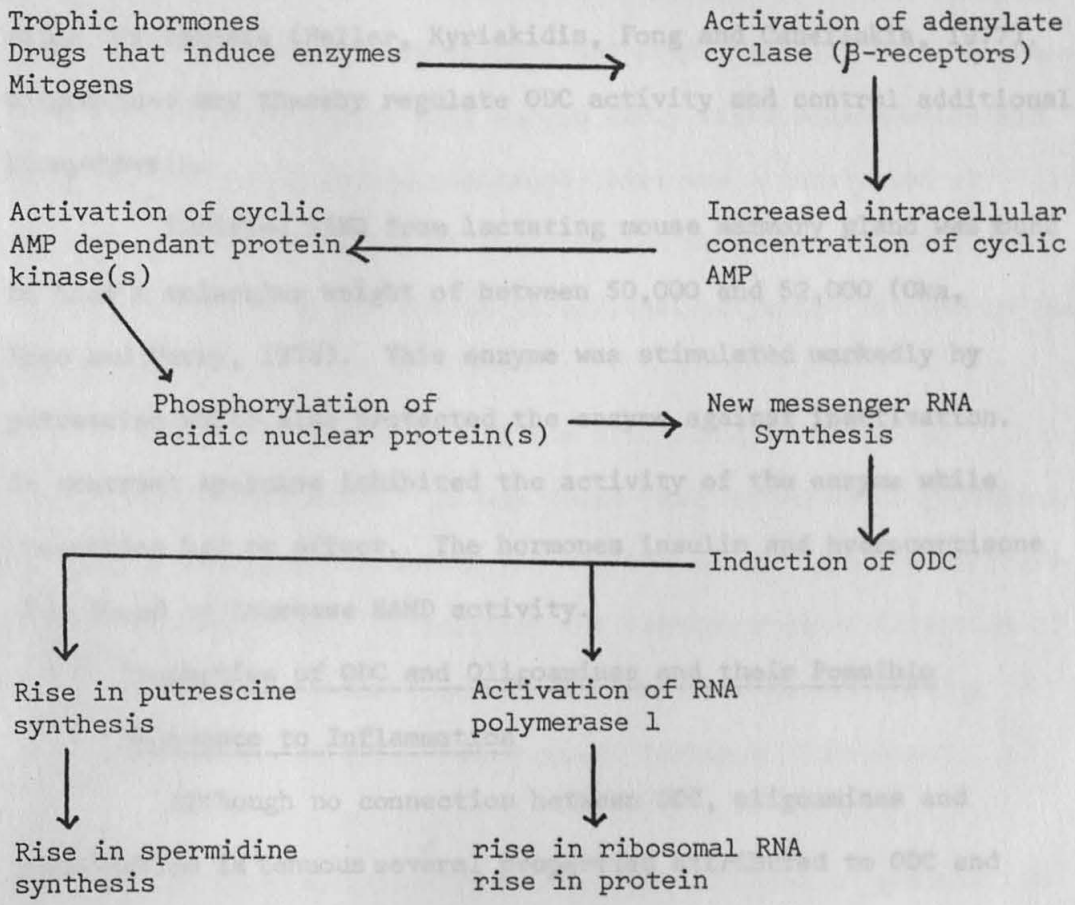
Ornithine decarboxylase has a molecular weight of 55,000 daltons (Obenrader and Prouty, 1977) and plays a central role in differentiation and growth regulation. The enzyme is inhibited by various ornithine analogues and requires the presence of thiol groups (Ono, Inoue, Suzuki and Takeda, 1972) and pyridoxal phosphate as a co-factor.

ODC has the shortest half life of any known enzyme in eukaryotes varying from 15 to 45 minutes (Russell and Snyder, 1969). The half life of the enzyme varies during the cell cycle being maximal when the growth rate increases (Hogan and Murden, 1974).

The activity of this enzyme is elevated in rapidly growing organs and in tumour cells (Russell and Snyder, 1969); Williams-Ashman, Coppoc and Weber, 1972) and the activity also rises as a result of the administration of various drugs and hormones (Bachrach, 1975; Richman, Underwood, Van Wyk and Voina, 1971).

In addition to hormones, cyclic nucleotides or dibutyryl

cyclic AMP stimulated ODC activity in different organs and cells in tissue cultures (Bachrach, 1980). It has been suggested that cyclic nucleotides mediate ODC induction (Russell, Byus and Manen, 1976). It was proposed that ODC, and the products of its action, the oligoamines, might be the link between hormones, cyclic AMP and RNA and protein biosynthesis. This is summarised as shown in the following diagram:-



This mechanism was suggested on the basis of the results from several workers and although there is some conflicting evidence, for example, treatment of rats with ACTH caused an increase in adrenal cyclic AMP without changing ODC activity (Levine, Leaming and Raskin, 1978) the argument remains a powerful one.

One of the most outstanding advances in the study of ODC regulation has been the recent demonstration of ODC antizyme

(Canellakis, Heller, Kyriakidis and Chen, 1978).

This ODC antizyme, which had a molecular weight of 26,500 daltons and could be induced by exposing cells to oligoamines, reacted specifically with ODC, neutralized its activity and had a relatively short half life. It has recently been postulated that ODC antizyme is normally attached to subcellular components (nucleus and ribosomes) and that oligoamines at millimolar concentrations cause its release (Heller, Kyriakidis, Fong and Canellakis, 1977). Oligoamines may thereby regulate ODC activity and control additional biosynthesis.

Purified SAMD from lactating mouse mammary gland was found to have a molecular weight of between 50,000 and 52,000 (Oka, Kono and Perry, 1978). This enzyme was stimulated markedly by putrescine which also protected the enzyme against inactivation. In contrast spermine inhibited the activity of the enzyme while spermidine had no effect. The hormones insulin and hydrocortisone were found to increase SAMD activity.

1.8.3 Properties of ODC and Oligoamines and their Possible Relevance to Inflammation

Although no connection between ODC, oligoamines and inflammation is tenuous several properties attributed to ODC and oligoamines may be relevant.

It has previously been described that liver disease has an ameliorating effect on inflammation (Hench, 1933). ODC and oligoamines have a considerable role in liver damage. Jänne and Raina (1968) observed that after partial hepatectomy there was a marked stimulation of ODC activity which resulted in a rapid accumulation of endogenous

putrescine. This increase in putrescine favours an increase in spermidine since putrescine acts as a substrate in the spermidine synthase reaction and stimulates SAMD, but competitively inhibits spermine synthesis (Hannonen, Raina and Jänne, 1972; Jänne, Pösö and Raina, 1978).

The administration of the hepatotoxin carbon tetrachloride which has been found to inhibit inflammation (Mielens, Drobeck, Rozitis and Sansone, 1969) also causes a marked activation of oligoamine synthesis (Hölttä, Sinervirta and Jänne, 1973). These workers also made the observation that during early liver regeneration and after carbon tetrachloride treatment there was a conversion of spermidine to putrescine. This conversion was suggested to occur due to the action of the enzyme peroxisomal polyamine oxidase in the liver.

The stimulation of ODC was also found to be due to various other treatments. It was found that infusions of hypertonic glucose or mannitol stimulated ODC and DNA labelling while 3.6% w/v saline had no effect. In addition the intraperitoneal injection of celite, a chemically inert mechanical irritant, caused a fifty fold increase in ODC after four hours. (Schrock, Oakman and Bucher, 1970).

Oligoamine synthesis during the formation of granulation tissue induced experimentally by implanting viscose cellulose sponges subcutaneously in the rat has been studied by Raina, Jänne, Hannonen, Hölttä and Ahonen (1973). It was found that the activities of the oligoamine synthesizing enzymes rose steadily during the period of rapid cell proliferation an increase in RNA was also noted. A rapid increase in ODC activity and accumulation of RNA and oligoamines, notably spermidine has also been demonstrated in healing wounds of rat skin (Mizutani, Inoue and Takeda, 1974).

Pregnancy, which is known to cause remissions in rheumatic diseases, (Fagerhol and Laurell, 1970) is also known to alter oligoamine levels. In both the human and rat the levels of oligoamines are elevated during pregnancy. It is notable that in the rat the elevation of putrescine is greatest and spermine shows very little change (Andersson,Henningsson and Rosengren, 1978 ; Russell, Giles, Christian, and Cambell, 1978).

Prostaglandins which are thought to be involved in the inflammatory process (Ferreira and Vane, 1974) also effect ODC levels. Verma and Boutwell (1980) while studying the mechanism of skin tumour promotion by TPA (12-O-tetrdecanoyl phorbol-13-acetate) found that prostaglandins may play an important role in the induction of ODC activity. This induction of ODC activity could be depressed by prior treatment with prostaglandin synthetase inhibitors and this inhibition could be counteracted by treatment with PGE_1 or PGE_2 but not with $PGF_{1\alpha}$ or $PGF_{2\alpha}$.

1.9 Animal Models Used in the Investigation of the Inflammatory Process

Animal models can be useful in the study of inflammation and the screening of potential anti-inflammatory agents. It is necessary, however, to have some understanding of the underlying mechanisms of the models if any conclusions are to be drawn about the test substances.

Satisfactory animal models for most of the rheumatoid diseases are not available, but as Swingle (1974) points out in his excellent review on the subject, animal models are available for different facets of the inflammatory process. Swingle grouped these models into four categories according to the criterion for activity:

1. an interference with the manifestation of one of

the cardinal signs of the inflammation (swelling, redness, heat, pain and loss of function).

2. the modification of one of the events occurring during the inflammatory process (formation of granulation tissue, chemotactic assays in vitro)

3. a biological or chemical characteristic of a class of known anti-inflammatory drugs (inhibition of platelet aggregation in vitro)

4. the modification of those syndromes in laboratory animals which are believed to represent models for various rheumatoid disease states (adjuvant arthritic rat, immune synovitis in the rabbit).

Successful screening programmes can now be designed for potential anti-inflammatory agents. Some models have become more acceptable than others because of their simplicity, economic feasibility and ability to detect known anti-inflammatory drugs.

One of the most popular animal models is the carrageenan induced rats paw oedema originally described by Winter, Risley and Nuss (1962). Swingle (1974) in a study of United States Patents, estimated that 66% of the anti-inflammatory tests carried out used this model. Carrageenan is a mixture of polysaccharides composed of sulphated galactose units and is derived from Irish sea moss (Smith, O'Neill and Perlin, 1955). The test is usually conducted by injecting the carrageenan into the rats hind foot pad and measuring the size of the resultant oedema and comparing this to similar groups of rats that have received drugs as well as the carrageenan. The development of the oedema is considered to be a biphasic event (Vinegar, Shcreiber and Hugo, 1969) with the initial phase attributed to the release of histamine and 5-hydroxytryptamine (5-HT); the plateau phase to kininlike substances; and the second accelerating phase to the release

prostaglanding like substances (Di Rosa and Willoughby, 1971; Di Rosa, Giroud and Willoughby 1971). This model falls into the first category in that one of the cardinal signs of inflammation is being studied.

Another widely used model is the polyarthritis induced in the rat by the injection of Freund's complete adjuvant into the animals tail or hind foot pad (Pearson, 1956). The course of the disease, which is most easily followed by the daily measurement of the rats hind foot volumes, has been described by Pearson and Wood (1959). If the hind foot pad is chosen as the site for injection of the adjuvant the foot swells immediately with a partial subsidence of this swelling about four days after injection. A secondary phase swelling occurs nine to twelve days after injection and this is the expression of the systemic nature of the disease. Recently some work has shown (Liyanage, Currey and Vernon-Robers, 1975) that the severity of the arthritis is dependant on the size of the mycobacterial particles. Further investigation (Vernon-Roberts, Liyanage and Currey, 1976) has shown that there is a rapid and wide-spread dissemination of the mycobacterial particles. Of particular interest is the identification of intact and fragmented tubercle bacilli in the synovial lining cells of joints distant from the injection site. The synovial tissue of the affected joints proliferates and pannus tissue invades the joint space causing severe bone erosion, inflammatory cells, such as mononuclear cells are also present in the synovium (Pearson and Wood, 1963; Pearson, 1963). This syndrome induced in rats has been described as "...the best available model of rheumatoid arthritis" (Walz, DiMartino and Misher, 1971) and "... the nearest experimental approximation to rheumatoid arthritis" (Glenn and Kooyers, 1966) and indeed there are many similarities between it and the human condition. However, in this rat disease rheumatoid factor is

not present (Glenn, Gray and Kooyers, 1965) and also there are few polymorphonuclear leucocytes present at the affected joints (Pearson, 1963).

Granulation tissue is produced during the repair phase of the inflammatory process. The repair begins as a proliferation of fibroblasts and multiplication of small blood vessels by mitoses of connective tissue and endothelial cells. The cellular proliferation penetrates the exudate, producing the highly vascularized granulation tissue. The model used to study this sequence of events is the cotton wool pellet granuloma test. In this model cotton wool pellets of a known weight are implanted into rats and left for seven days after which the animals are sacrificed and the pellets recovered and dried. The amount of granulation tissue can then be measured by reweighing the pellet. Swingle and Shideman (1967) have described three phases of the inflammatory response to subcutaneous implantation of cotton pellets in rats. The first phase (transudative) occurs during the first three hours and represents a "soaking" of the cotton with fluid of low protein content (a non-inflammatory transudate). The second phase of the response occurred between three and seventy-two hours after implantation and was designated the exudative phase, which was measured by assessing the amount of Evans Blue passing from the circulation to the implant region.

The third component of the response occurred on the fourth day after implantation when collagen was first detected in the granuloma also the greatest increase in the number of fibroblasts took place between the third and fifth days (Brunkhorst, 1958).

Thus, by using the animal models described here, and others, it is possibly by using a variety of models to successfully screen out a new anti-inflammatory compound. Since, however, there is no true animal model for the rheumatoid disease of man they are of less

help in an aetiological study of the disease.

1.10 Aims of Research

Two approaches were made in this research. In the first instance it was decided to investigate the role of plasma anti-proteinases in the rheumatic diseases since an anti-proteolytic action on proteinases responsible for joint damage would clearly be anti-arthritic. Many drugs used in the treatment of rheumatic disease are obviously not anti-arthritic, thus a study of the anti-proteinases may prove beneficial. With this aim in mind the levels of various antiproteinases and proteins in human rheumatic synovial fluid samples will be studied. It has been shown that the proteinase inhibitor Trasylol is effective in acute animal models (Förster, 1969) and may be useful in the treatment of rheumatoid arthritis. It is proposed to study the anti-inflammatory activity of Trasylol in some chronic animal models and, using *in vitro* tests, to investigate its possible mode of action.

There is evidence of the existence of endogenous anti-inflammatory factors that are not anti-proteinases. It was decided to probe the identity of these substances further and since evidence exists that these factors may be proteins it was decided to concentrate on this class of substance.

For this work the following samples and techniques were chosen:-

Both *in vivo* and *in vitro* methods will be used to evaluate the anti-inflammatory activity of pooled human rheumatoid synovial fluid both before and after fractionation by gel filtration.

The role of liver damage in inflammation will be explored using liver extracts from animals treated with the hepatotoxin dimethylnitrosamine. This extract and fractions derived from it will be evaluated for their anti-inflammatory activity and possible modes

of action will be considered.

The anti-inflammatory effects of the inflammatory exudate produced by the subcutaneous implantation of polyester sponges are well known, but its mode of action is largely unknown. It is proposed to study several possible modes of action which may be of importance.

The role of oligoamines in inflammation will also be investigated. The relevance of oligoamines to both sponge exudate and liver extracts from dimethylnitrosamine treated rats will be studied. In addition the direct anti-inflammatory effects of oligoamines will be explored using in vivo and in vitro tests. Drugs which are known to promote ODC activity and increase oligoamine levels will also be evaluated.

Finally the role of endotoxin in inflammation and its relevance to the present work will be investigated.

2.1.1 The Induction and Measurement of Adjuvant Arthritis and Carrageenan Oedema in the Rat

Carrageenan oedema was induced in 150-200g male Wistar rats (Bantin and Kingman, Hull) by the method of Winter, Risley and Nuss (1962). A 2% (w/v) solution of the calcium salt of carrageenan (Sigma Chemical Company Limited, U.S.A.) was prepared in 0.9% (w/v) sterile saline. A mixture of 3% halothane in oxygen (flow rate 300 cc/min) and nitrous oxide (flow rate 1,500 cc/min) produced by a Boyles veterinary anaesthetic trolley (British Oxygen Company Limited) was used to anaesthetise the rats. The oedema was induced by injection of 0.05 cm^3 of the carrageenan solution into the plantar aponeurosis of the left hind foot of the rat. Control animals were injected with 0.9% (w/v) sterile saline.

Adjuvant arthritis was induced in 150-200g male Wistar rats using the method employed by Newbould (1963). The adjuvant consisted of human strains of heat killed C, DT and N tubercle bacilli (Ministry of Agriculture Veterinary Laboratories, Weybridge, Surrey). The bacilli were finely ground using a glass pestle and mortar and suspended in liquid paraffin at a concentration of 5 $mg.cm^{-3}$. The rats were anaesthetised, as previously described, and 0.03 $cm.^3$ of the adjuvant suspension was injected into the left hind foot. Control animals were injected with 0.9% (w/v) sterile saline.

The inflammatory response produced by the injection of either the adjuvant or carrageenan was estimated by measuring the increase in volume of the hind feet of the rats. The volume of each paw was measured plethysmographically using a modification of the method described by Harris and Spencer (1962). The hind feet were immersed separately, up to the hair line, in a mercury bath which was linked

to a blood pressure transducer and an oscillograph recorder (Searle Bioscience, Kent). The measurements of the hind feet of the rats were taken just prior to the injection of adjuvant or carrageenan and again at various intervals during the experiments.

2.1.2 Cotton Wool Pellet Granuloma Test in the Rat

This test for anti-inflammatory activity was carried out using a modification of the method of Meir, Schuler and Desaulles (1950). Cotton wool dental pellets (Johnson and Johnson, England) were weighed individually on a torsion balance and only those within 0.25 mg. of the mean were used for any one experiment. The pellets were then sterilized by autoclaving for 20 minutes at a pressure of 15lbs./sq.in. The rats were anaesthetised as previously described, and the sterile pellets implanted subcutaneously, one in each axilla and groin, giving a total of four pellets per animal. On the seventh day after implantation the animals were killed by a blow to the head and the pellets dissected out using a pair of forceps. The pellets were dried at 60°C for 24 hours and reweighed on the torsion balance. The increase in weight of the pellets was used as an index of the amount of granulation tissue deposited. Hence, the anti-inflammatory effect was calculated as the reduction of the increase in weight of the cotton wool pellets in the treated groups as compared with the untreated control group of animals.

2.1.3 The Effect of Substances on Pinnal Anaphylaxis in the Mouse

The effect of various substances upon the development of a local anaphylactic reaction in the mouse pinna was investigated using the method first described by Church, James and Miller (1974).

Male BKW mice (15-20g) were sensitised to horse serum by subcutaneous injection of 0.1 $cm.^3$ of a 1/20 dilution and 14 days later 0.1 $cm.^3$ of a 1/50 dilution. Six to eight days after the second dose of horse serum the mice were taken to a warm room and 1 hour

later were injected intravenously with 0.25 $cm.^3$ of a 0.5% (w/v) Evans blue solution. The test substance was given intraperitoneally immediately after the dye solution. Forty minutes later the mice were challenged by piercing each ear with a hypodermic needle (23 gauge) which had previously been dipped into horse serum to a known depth. The area of blueing around the site of challenge was measured at 30 minutes and 60 minutes after piercing by observation through a dissecting microscope, the eyepiece of which had been fitted with a measuring graticule. The mice were anaesthetized using sodium pentabarbitone (60 $mg.Kg.^{-1}$) for the duration of the experiment.

2.1.4 Production of Inflammatory Exudate Using Polyester Sponge

The method employed was similar to the one used by Robinson and Robson (1964 and 1966). Recently, Maguire and Wallis (1977) working on anti-lymphocytic serum (ALS) suggested that bacterial contamination during the collection and subsequent fractionation of ALS was responsible for the anti-inflammatory activity of the lower molecular weight fractions.

This finding conflicts with the earlier work of Billingham, Robinson and Gaugas (1970) who attributed the anti-inflammatory activity of the low molecular weight fraction to the presence of an anti-inflammatory protein. It must be noted that these workers took no apparent sterile precautions and, except for sterilization of the sponges, neither did Robinson and Robson (1964, 1966). To try and resolve these difficulties every effort was made to ensure the integrity of the exudate produced.

Strips of polyester sponge (F.W. Woolworth and Company) 25 x 50 x 5 mm. were inserted subcutaneously in the backs of intact, adrenalectomised or sham adrenalectomised rats, one piece per rat. These acted as the stimulus for exudate formation, and before

implantation, were sterilized in an oven for two hours at 150°C. Adrenalectomy was performed on 150-200g, male Wistar rats which were anaesthetised as previously described. The operation area was closely shaved and swabbed with Betadine Alcoholic solution (Napp Laboratories Limited, Watford), an established germicidal and bacteriacidal agent. Adrenalectomy was carried out through a median dorsal skin incision. A cut was made in the body wall, over the anterior pole of each kidney, and the adrenal glands carefully removed by means of angled forceps. The body wall incisions were sutured with sterile silk thread (AR306, Armour Pharmaceuticals Limited, England), the sponges inserted, and the wound closed with sterile 12 mm. metal suture clips. The wound was sprayed with Nobecutane (Astra Chemicals Limited, Watford), a plastic wound dressing, to prevent infection. The surgical instruments used throughout the operation had previously been sterilized by autoclaving for 20 minutes at a pressure of 15lbs./sq.in. and the operation carried out under a microbiological screen. The animals were allowed to recover and maintained on normal laboratory diet, (Pilsbury's, England) and given 0.9% (w/v) saline ad Libitum . Four days after implantation of the sponges the animals were killed by a blow to the head, the sponges removed and the exudate collected in sterile centrifuge tubes by manual squeezing. This procedure was carried out under sterile conditions, as previously described. The exudate was centrifuged at 2000 r.p.m. for 30 minutes in an MSE bench centrifuge to remove cells, fibrin clots and other debris. The fluid was dialysed against distilled water at 4°C with at least three changes of the external fluid. The exudate was then freeze dried and stored in a sealed, sterile bottle at -30°C until required. All glassware in contact with the exudate and dialysis tubing were previously sterilized. Also all transfer operations were carried out under a microbiological screen.

2.1.5 Production of Liver Extracts from Dimethylnitrosamine treated rats.

Liver extracts were prepared from livers of rats that had received intra-peritoneal injections of 0.5 $cm.^3$ dimethylnitrosamine in 0.9% saline (25mg/kg body weight) three days earlier. The livers from these animals were homogenized for 30 seconds in ice cold 0.9% saline (40 $cm.^3$ 0.9% saline for every 20g. of freshly excised liver). The cell debris were removed by centrifugation at 100,000g for 30 minutes in an MSE superspeed ultra centrifuge. The clear supernatents were then pooled, dialysed overnight at $4^{\circ}C$ against 0.9% saline, freeze dried and stored at $-30^{\circ}C$ until required.

2.2.1 The Fractionation of Pooled Human Rheumatic Synovial Fluid

The process of gel filtration was used to separate the anti-inflammatory substances present in human rheumatic synovial fluid.

The pooled human rheumatic synovial fluid was partially fractionated by eluting 5 $cm.^3$ of the pooled synovial fluid through a Pharmacia (Uppsala) column (SR25/100) 2.5 cm. in diameter and 100 cm. long packed with Sephadex G-150 (Pharmacia), using a 0.1M phosphate buffer pH7.4 as eluant. The technique of upward elution was utilized with flow rate of 20 $cm.^3 h^{-1}$.

The column eluate was monitored at 280 mm. using a Uvicord II flow through cell (L.K.B. Instruments, England) connected to a strip chart recorder and collected in 10 $cm.^3$ fractions by an Ultrorac 7000 automatic fraction collector (L.K.B.). The fractions were then pooled with reference to the elution pattern, dialysed against distilled water for 24 hours at $4^{\circ}C$, freeze dried and stored at $-30^{\circ}C$ until required for use.

2.2.2 Fractionation of sponge induced inflammatory exudate

The method used in this case was the same as that used to fractionate human rheumatoid synovial fluid except for minor changes. In this case the eluant was 0.5M sodium chloride containing a 0.003M sodium azide (to prevent bacterial growth) and 500 mg. of material was applied to the column dissolved in 5 cm^3 of eluant. This method is the same as previously described by Billingham, Robson and Robinson (1969).

2.2.3 Fractionation of Liver extracts from Dimethylnitrosamine treated rats.

A freeze dried extract of livers from dimethylnitrosamine treated rats (see Section 2.1.5) was fractionated by gel filtration.

The sample (300 mg. in 2 $cm.^3$ elution buffer) was applied to the top of a column (K26/40 Pharmacia), 2.6 cm. in diameter, 40 cm. long packed with Sephadex G-150. The elution buffer was 0.1M phosphate buffered, 0.9% saline (pH7.4). The eluate was monitored at 280 mm. and collected in 10 $cm.^3$ fractions. The proteolytic activity of each fraction was determined by incubating 1 $cm.^3$ of each fraction with azure blue hide powder (see section 2.5.4).

The 10 $cm.^3$ fractions were pooled on the basis of protease activity and possible irritancy since proteases are irritants when injected into animals. The new fractions were then dialysed for 24 hours, at 4^oC, against water, freeze dried and stored at -30^oC until required.

2.3 The Induction and Partial Purification of Rabbit Antibodies to Rat Proteins

An attempt was made to raise rabbit antisera against the exudate obtained from adrenalectomised donor rats and the proteins

contained within the exudate that interact with lysosomal membranes. These proteins are of interest since stabilisation of membranes may explain their anti-inflammatory activity. The antisera was produced using the method described by Lewis, Capstick and Cosh (1975).

Lysosomes were isolated from rabbit liver by the method of Lewis, Symons and Ancill (1969). Fifteen grams of fresh rabbit liver was chopped into fine pieces and homogenized with chilled 0.25M sucrose in 0.05M tris-acetate buffer, pH7.4 so as to give a 10% (w/v) homogenate. This was centrifuged at 700g for 10 minutes in an M.S.E. bench centrifuge. The supernatent was retained and centrifuged at 15,000g for 20 minutes in an M.S.E. Superspeed 50 ultracentrifuge. The resultant pellet was resuspended in fresh buffered sucrose, and centrifuged again, as above. The pellets were then resuspended in 30 cm^3 of buffered sucrose solution.

Three flasks were then incubated for 90 minutes at 37°C each one containing 5 cm^3 of the lysosome suspension, 2 cm^3 of a 100 $mg.cm^{-3}$ solution of the exudate from adrenalectomised donors and 1 cm^3 of 0.25M sucrose in 0.05M Tris-acetate buffer, pH7.4. After incubation the mixtures were centrifuged at 20,000g for 30 minutes in an M.S.E. Superspeed 50 ultracentrifuge. The pellets were then washed with large excess of saline (0.9% w/v) frozen and thawed three times and centrifuged again at 40,000g for 30 minutes. The pellets were stored at -30°C until needed for injection.

One third of the material isolated above was resuspended in 1.5 cm^3 of sterile saline (0.9% w/v) and added to 1.5 cm^3 of an adjuvant (0.5 mg. of finely ground human heat-killed tubercle bacilli per cm^3 of liquid paraffin). A stable water in oil emulstion was formed by repeatedly drawing the mixture through a narrow gauge syringe needle. New Zealand White rabbits, 1-2 Kg (Hylyne Rabbits,

Cheshire) were injected intramuscularly at three different sites with 0.5 cm^3 of the emulsion. The treatment was repeated after two weeks and after four weeks to ensure a high circulating level of antibody.

A similar procedure was adopted for the production of an antisera to the exudate. In this case, however, 10 mg. of the protein was resuspended in 1.5 cm^3 of sterile saline (0.9% w/v) and treated as described for the pellets.

After four weeks 20-30 cm^3 of blood was taken from the rabbits by cardiac puncture; this was allowed to clot at 4°C and centrifuged for 10 minutes at 2000 r.p.m. in an M.S.E. bench centrifuge. Following this the immunoglobulin fraction of the rabbit serum was partially purified by ion-exchange chromatography on DEAE cellulose (Sigma Chemical Company, U.S.A.). A chromatography column (30cm. x 3 cm. diameter) was packed with 15g of DEAE cellulose taken up in and equilibriated at pH7.5 with 0.01M phosphate buffer, pH7.5. A 10 cm^3 sample of serum was dialysed overnight at 4°C against 0.01M phosphate buffer, pH7.5 and applied to the column, the same buffer being used to elute the column. The eluate from the column was monitored at 280mm using an LKB Uvicord II and collected in 5 cm^3 fractions by an LKB Ultrorac 7000 fraction collector. The fractions of the first protein peak contained mainly immunoglobulins and these were pooled, and dialysed against aquacide (flake polyethylene glycol, Calbiochem, U.S.A.) to reduce the volume to about 3 cm^3. The fraction was then dialysed for 24 hours against a solution of 0.1M sodium chloride and 15mM sodium azide (to inhibit bacterial growth) and stored at 4°C until required for use.

The column was restored by eluting it with 200 cm^3 of a solution composed of 1.5M sodium chloride and 0.3M phosphate buffer pH7.0, to release the remaining bound protein. The column was then

re-equilibriated with 0.01M phosphate buffer, pH7.5, for further use.

2.4.1 Immunoelectrophoresis of Proteins

1% (w/v) agarose (Sigma Chemical Company, U.S.A.) in 6mM sodium barbitone-sodium acetate buffer, pH8.6, was liquified in a water bath at 100°C. When fully liquified, and free from lumps, the agarose solution was allowed to cool to 55°C and poured onto an immunoelectrophoresis tray (Shandon, Cheshire) fitted with microscope slides. A perspex scraper (Shandon) was then passed over the tray to remove the surplus liquid agarose. The agarose gel was allowed to set leaving a 1 mm. thick layer of gel covering the slides. A slot 30 mm. x 1 mm. was cut out of the agarose gel down the centre of each slide. Two holes, 1 mm. diameter, were cut opposite each other either side of the slot 3 mm. from its edge and about 10 mm. from one end. 1 µl of serum or protein solution was applied to each hole with a microsyringe (Hamilton Micromeasure, Switzerland.). The slide tray was then placed in a Shandon electrophoresis tank which contained 6 mM sodium barbitone-sodium acetate electrode buffer, pH8.6. A voltage of 100 volts was applied across the agarose gel such that the protein migrated parallel with the edge of the slot towards the anode. The migration of the protein was visualised by a drop of bromophenol blue, the electrophoresis was stopped when the bromophenol blue reached the end of the slot, the dye having travelled 20 mm. The slot was filled with the appropriate antiserum, which was allowed to diffuse into the gel for 12 hours. In this manner the antibody and antigen are brought together within the agarose gel to form an antigen-antibody complex which becomes visible as a white precipitin arc. This precipitin arc may then be examined by dark ground illumination (Photopol lamp, Shandon) or may be visualised by staining with amidoblack. The staining was

accomplished by first immersing the agarose gel in 0.9% (w./v) saline for 12 hours to remove any non-precipitated proteins. The arcs were stained by immersing the gels in a solution of 1% (w/v) amido-black in 7% (v/v) acetic acid for one hour. The background staining in the agarose gel was then removed by washing the gel in 7% (v/v) acetic acid until the stained arcs were visible against a clear background.

Rabbit immunoglobulins against whole rat serum, whole human serum, human alpha-2-macroglobulin and human alpha-1-antitrypsin were obtained from Dako-immunoglobulins, Denmark.

2.4.2 Quantitative Estimation of Specific Proteins by Electroimmuno Assay

The method of "rocket" immunoelectrophoresis developed by Laurell (1966) was used. In this technique the antigen under investigation was induced, by an electric field, to migrate into an agarose gel layer, containing a monospecific antibody.

The migration of this antigen, corresponding to the antibody, towards the anode ceased when its concentration reached the equivalence point (i.e. when all the antigen has formed complexes with antibody in the gel). The height of the resultant "rocket" is proportional to the amount of antigen present.

1% (w/v) agarose gel in 0.07M barbital buffer, pH8.6, containing 2mM calcium lactate was liquified in a water bath at 100°C. The agarose solution was allowed to cool to 40°C and the requisite amount of antiserum added. The antiserum concentration used was the minimum one that had previously been found to form a distinct precipitin line with the antigen. The antisera to human alpha-1-antitrypsin and human alpha-2-macroglobulin (Dako immunoglobulins, Denmark) was mixed with agarose in the ratios of 1:50 and 1:75 respectively. A 15 cm^3 portion of agarose containing the antiserum

was then poured into a mould consisting of two identical rectangular glass plates 20 cm. x 10 cm. spaced 1 mm. apart by a U-shaped former manufactured from 1 cm. wide polythene strips. The mould was clamped and held upright until the gel had set. The clamps were removed and one glass plate slid away to leave a slab of gel 18 cm. x 8 cm. x 1 mm. Holes 4 mm. in diameter were cut in the gel along the longest edge 2 cm. from the edge with the centres of the holes a minimum of 8 mm. apart. The gel was placed in a Shandon electrophoresis tank with the line of holes perpendicular to the electric field and furthest from the anode. Eight microlitres of the solution to be tested were pipetted into separate holes. For this procedure human synovial fluid and serum was diluted 1:15 and 1:10 to assay alpha-1-antitrypsin and alpha-2-macroglobulin respectively, the dilutions being made with electrode buffer which consisted of 0.07M barbital buffer, pH8.6. A power pack was then used to apply a voltage of 70-80 volts across the gel. The electrophoresis was allowed to proceed for 18 hours by which time the height of the "rockets" remained constant. The voltage was then switched off and the height of the "rockets" measured from the centre of the holes to the peak tips using a dark field illuminator to easily visualise the "rockets". Standard antigen solutions were examined during each electrophoretic run so that the results for different samples on different runs could be directly compared.

2.4.3. The Testing and Use of Antisera for the Evalation of Anti-Inflammatory Proteins in Inflammatory Exudate

The antiserum produced in section 2.3 was first tested for purity by immunoelectrophoresis (see section 2.4.1), inflammatory exudate from sponge implanted, adrenalecomised rats being used as the antigen.

The antiserum was also tested by immunoelectrophoresis using

normal rat plasma as antigen in order to establish if proteins in the inflammatory exudate which stabilised lysosomal membranes were normally present in plasma.

Also, 0.5 cm^3 of anti-whole exudate and 0.5 cm^3 of anti-lysosomal membrane antisera were each mixed with 1 cm^3 of normal rat plasma, allowed to stand at 4°C overnight, centrifuged to remove precipitated proteins and concentrated to about 0.5 cm^3 using Aquacide (Calbiochem). These modified antisera were then tested by immuno-electrophoresis using normal rat plasma and sponge induced inflammatory exudate as antigens.

2.5 Methods used in the assay of Plasma and Tissue Samples

2.5.1 Trypsin Inhibitory Capacity (TIC)

The TIC activity of various samples was measured by the method described by Dietz, Rubinstein and Hodges (1974), using the inhibitory action of the sample on the breakdown of alpha-N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) (Sigma Chemical Company, U.S.A.) to p-nitroaniline by trypsin (from bovine pancreas, twice crystallised) (Sigma Chemical Company, U.S.A.).

A 2 cm^3 portion of trypsin solution (20 $\mu g\ cm^{-3}$ in 0.1M Tris-HCl buffer, pH8.2, and 0.02M calcium chloride) was added to 10 µl of the sample. An aliquot of trypsin solution (2 cm^3) was also added to 10 µl of a 40 $mg.cm.^{-3}$ bovine serum albumin solution (Fraction V) (Sigma Chemical Company, U.S.A.) as a control. The solutions were then allowed to stand at room temperature for 10-15 minutes to activate the trypsin inhibitory proteins. 0.5 cm^3 of this mixture were then added to 2.5 cm^3 of the BAPNA substrate (0.45 $mg.cm^{-3}$ in 0.1M Tris-HCl buffer, pH8.2 and 0.02M calcium chloride) and incubated for 10 min. in a shaking water bath at 37°C. The reaction was stopped by the addition of 0.5 cm^3 of 30% (v/v) acetic acid. The samples were read against a blank at 400 mm. on a Pye-Unicam SP500

spectrophotometer. The blanks were prepared by adding the sample-trypsin solution to the BAPNA substrate after the incubation and addition of the 30% (v/v) acetic acid. A standard curve of the absorbance at 400 mm. of p-nitroaniline was also prepared.

2.5.2 Trypsin-Protein Esterase (TPE) Activity

The method used to determine the T.P.E. activity of various samples was developed by Ganrot, P.O. (1966), which enables the concentration of certain large molecular weight proteinase inhibitors to be determined. These inhibitors can bind and inhibit trypsin so that it cannot breakdown large molecular weight substrates, such as proteins, but it can still degrade small molecular weight substrates, such as BAPNA. The activity of the bound trypsin is unaffected by soybean trypsin inhibitor. A 0.5 cm^3 portion of trypsin solution (100 µg trypsin per cm^3 of 0.1M Tris-HCl buffer, pH8.2, and 0.02M calcium chloride) was added to 25µl of sample fluid, 0.5 cm^3 of Tris-HCl buffer alone was added to 25 µl of a 40 $mg.cm^{-3}$ bovine serum albumin solution as a blank. After 10 seconds 1.5 cm^3 BAPNA solution containing soybean trypsin inhibitor (Type II-S, Sigma Chemical Company, U.S.A.)(0.9 mg. BAPNA and 66.8 µg. soybean trypsin inhibitor per cm^3 0.1M Tris-HCl buffer, pH8.2, and 0.02M calcium chloride) was also added. The mixture was incubated for 10 minutes at 37°C in a shaking water bath. The reaction was stopped by the addition of 0.5 cm^3 of 30% (v/v) acetic acid. The samples were read against the blank at 400 nm in a Pye-Unicam SP 500 spectrophotometer.

2.5.3 Estimation of Protein Concentration

Protein concentrations were estimated by the biuret procedure of Gornall, Bardawill and David (1949). The biuret reagent consisted of a solution of 6mM copper sulphate, 2.1mM sodium potassium tartrate, 0.8M sodium hydroxide and 6mM potassium iodide. 5 cm^3 of

this reagent was added to 0.1 cm^3 sample, 0.1 cm^3 standard protein solution (80 mg. protein cm^{-3}) (Sigma Chemical Company, U.S.A.) and 0.1 cm^3 of distilled water to act as blank. After standing for 30 minutes at room temperature the absorbance of the solutions were read against the blank at 540 nm. on a Pye-Unicam SP 500 spectrophotometer and compared with the standard protein solution.

2.5.4 Proteinase Activity

Proteinase Activity was determined by a method similar to that used by Rinderknecht, Geokas, Silverman, Lillard and Haverback (1968). A 0.5 cm^3 portion of sample and 2 cm^3 of 0.1M Tris-HCl buffer, pH7.4, were incubated with 20 mg. of Azure blue impregnated hide powder (Calbiochem, U.S.A.) for twenty four hours at 37°C in a shaking water bath. The solution was then cooled in ice and centrifuged for ten minutes, at 3500 g. to remove any residual hide powder. The supernatent, containing the released blue dye, was recovered and the absorbance at 595 nm determined using a Pye-Unicam SP 500 spectrophotometer. A suitable blank for each sample was prepared by incubating the sample alone and adding it to the hide powder and buffer suspension immediately prior to centrifugation. A standard curve of the absorbance at 595 nm. of completely dissolved Azure blue impregnated hide powder was also prepared.

2.5.5 Quantitative Estimation of Putrescine and Cadaverine

The levels of putrescine and cadaverine were determined using a modification of the method first described by Seiler and Askar (1971).

This method involves the reaction of the diamine with 1-dimethylaminonapthalene-5-sulphonyl chloride (DANS-Cl, Sigma Chemical Co.) followed by two-dimensional thin-layer chromatographic separation of the dansylated derivatives, and

fluorimetry of the DANS-putrescine and DANS-cadaverine extracted from the carrier.

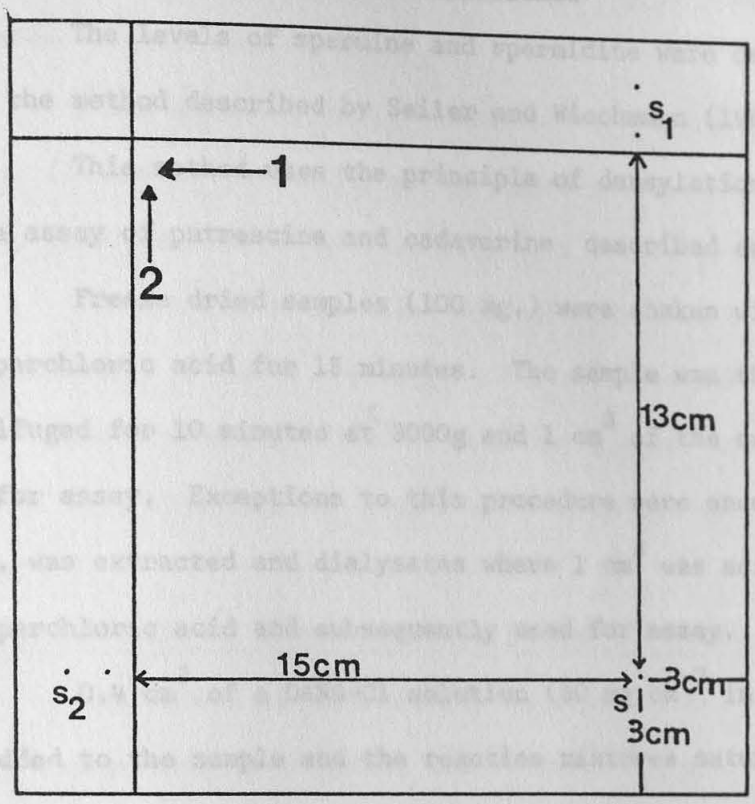
Freeze dried samples (200 mg.) were shaken with 2 $cm.^3$ of 0.2N perchloric acid for 15 minutes. The sample was then centrifuged for 10 minutes at 3000 g and 1 cm^3 of the supernatent used for assay. Exceptions to this procedure were endotoxin, where 50 mg. was extracted, and dialysates where 1 cm^3 was acidified with 0.2M perchloric acid and subsequently used for assay.

2.5 cm^3 of a DANS-Cl solution (4 $mg.cm^{-3}$ in acetone) was added to the sample and the reaction mixtures saturated with sodium carbonate. After completion of the dansylation (by reaction overnight, in the dark, at room temperature), 10 mg. of D-proline (Sigma Chemical Co.) dissolved in 0.2 cm^3 of water were added in order to react with the excess DANS-Cl. After two hours, the dansylated amines were extracted from the reaction mixture with 2 x 10 cm^3 of toluene. The toluene was removed by evaporation under reduced pressure and the resulting residues dissolved in 0.5 $cm.^3$ of toluene/ethyl acetate (7:3).

Each sample was quantitatively (20 µl or 40 µl) applied onto a separate 20 x 20 cm. thin layer plate consisting of 250 µm layers of Silica Gel G (BDH Chemicals). Standards were also applied to the plate as overleaf.

The plates were developed in two dimensions, the solvent system for the first dimension was trichlorethylene-methanol (19:1) followed by benzene-methanol (19:1) and the solvent for the second dimension was chloroform-triethylamine (10:1) which was prepared immediately before use.

After development, the thin layer plates were dried and examined under an ultra-violet lamp, where, the spots corresponding to putrescine and cadaverine were marked by comparison with the



S = Sample; S_1 = Standards, first direction; S_2 = Standards, second direction.

standards. These plate areas were scraped out and the dansylated amines extracted from the carrier by shaking with 5 cm^3 of benzene-acetic acid (99:1).

After standing for 5 minutes the carrier settled out and the fluorescent intensity of the extracts were measured at 500 nm; fluroescence was excited at a wavelength of 365 nm.

The amounts of putrescine and cadaverine were determined from previously prepared calibration curves. The calibration curves were obtained by taking solutions containing known amounts of putrescine and cadaverine and treating these in the same way as the samples.

2.5.6 Estimation of Spermine and Spermidine

The levels of spermine and spermidine were determined using the method described by Seiler and Wiechmann (1967).

This method uses the principle of dansylation as employed in the assay of putrescine and cadaverine described earlier.

Freeze dried samples (100 mg.) were shaken with 2 cm^3 of 0.2N perchloric acid for 15 minutes. The sample was then centrifuged for 10 minutes at 3000g and 1 cm^3 of the supernatent used for assay. Exceptions to this procedure were endotoxin, where 50 mg. was extracted and dialysates where 1 cm^3 was acidified with 0.2N perchloric acid and subsequently used for assay.

0.4 cm^3 of a DANS-Cl solution (30 $mg.cm^{-3}$ in acetone) was added to the sample and the reaction mixtures saturated with sodium carbonate. After completion of the dansylation (by reaction overnight, in the dark, at room temperature) 0.1 cm^3 of a proline solution (100 $mg.cm.^{-3}$) was added to remove the excess DANS-Cl. After 30 minutes the dansylated amines were extracted using 5 cm^3 of benzene.

Each sample was quantitatively (20 µl) applied onto a thin layer plate consisting of a 250 µm layer of Silica Gel G. The samples were appled 3 cm. from the bottom of the plate and 2 cm. apart, each 20 x 20 cm. plate consisted of four samples and five standards.

The plates were developed using ethyl acetate-cyclohexane (1:1) with 1% triethylamine as solvent.

Two runs, using this solvent system, were needed to get a good separation. After development, the thin layer plates were dried and examined under an ultra-violet lamp, where, spots corresponding to spermine and spermidine were marked by comparison with the standards. These plate areas were scraped out and the

dansylated amines extracted from the carrier by shaking with 5 cm^3 of benzene-acetic acid (99:1).

After standing for five minutes the carrier settled out and the fluorescent intensity of the extracts was measured at 500 nm; fluorescence was excited at a wave length of 365 nm.

The amounts of spermine and spermidine were determined from previously prepared calibration curves. The calibration curves were obtained by taking solutions containing known amounts of spermine and spermidine and treating these in the same way as the samples.

2.6 Determination of Degree of Stabilization of Guinea-Pig Macrophages

The effect of inflammatory exudates (human rheumatic synovial fluid, sponge induced exudate), their fractions, and various other agents (endotoxin, oligoamines) on guinea-pig macrophages was investigated using the method of Lewis, Best and Bird (1977). The macrophages were isolated by injecting guinea pigs intraperitoneally with 100 cm^3 of saline (0.9 w/v). 12 hours later this dose was repeated and the peritoneal cavity drained of fluid. The macrophages were harvested by centrifugation for 10 minutes at 1000g. in an MSE bench centrifuge. The macrophages were then washed in cold saline ($4^{\circ}C$) and resuspended in 0.1M phosphate buffered saline, pH7.4 at a protein concentration of 7 $mg.cm.^{-3}$ as determined by the Biuret method. The following flasks were set up for each test:-

a) Test

2 cm^3 macrophage suspension

1 cm^3 test solution

b) Control

2 cm^3 macrophage suspension

1 cm^3 phosphate buffered saline, pH 7.4

c) Blank

2 cm^3 phosphate buffered saline, pH7.4

1 cm^3 test solution.

All test solutions were prepared using 0.1M phosphate buffered saline, pH7.4 and each test was duplicated. The flasks were then incubated for 90 minutes at 37°C in a shaking water bath, centrifuged at 2000g. for 10 minutes and the supernatents assayed for acid phosphatase activity (Symons, Lewis, and Ancill, 1969).

Portions of the supernatents (0.1 cm^3) were incubated with 0.5 cm^3 of p-nitrophenyl phosphate (0.015M) and 0.5 cm^3 of 0.09M citrate buffer, pH4.8 for 30 minutes at 37°C in a shaking water bath. The reaction was stopped by addition of 5 cm^3 of 0.1M sodium hydroxide solution and the results read at 410 nm in Pye Unicam SP 500 spectrophotometer, all tests being duplicated.

2.7 The Evaluation of Trasylol as a Potential Anti-Inflammatory Drug

2.7.1 The Effect of Trasylol in the Cotton Wool Pellet Granuloma Test

Rats which had previously been implanted with four cotton wool pellets (see section 2.1.2) were given intra-peritoneal injections of 5,000 KIU and 10,000 KIU per Kg. body weight per day for the duration of the experiment. Control rats were given injections of 1.0 cm^3 of 0.9% (w/v) saline alone per day. The experiment was repeated using doses of 20,000 KIU and 40,000 KIU per Kg. per day, also using a ten times more concentrated solution of Trasylol doses of 50,000 KIU and 100,000 KIU per Kg. per day were used. (Trasylol donated by Bayer U.K.Limited).

2.7.2 Trasylol in the Adjuvant Arthritic Rat

Adjuvant arthritic rats were given daily intra-peritoneal injections of 50,000 KIU and 100,000 KIU per Kg. bodyweight from the day of the injection of adjuvant until the end of the experiment. Adjuvant arthritic control rats were given daily injections of 1.0 cm^3

of 0.9% (w/v) saline alone.

2.7.3 The Inhibition of Neutral Proteinases in Human Rheumatoid Synovial Fluid by Trasylol

A modification of the method described in section 2.5.4 was used to determine the inhibitory effect of Trasylol on proteinase activity in human rheumatoid synovialfluids. In this instance 1 ml. of Trasylol (10,000 K IU per ml.)was substituted for 1 cm^3 of the buffer, the assay being identical in all other respects.

2.8. The Levels of Proteinases and Anti-proteinases in Human Rheumatoid Synovial Fluid

Synovial fluids were aspirated from the knee joints of patients with a rheumatic disease. The fluids were centrifuged at 7,500 r.p.m. for 25 minutes at 4°C to remove the cells and debris. The cell-free fluids were then stored at -30°C, in airtight containers, until required for use. The following tests were then carried out on the fluid samples:-

a) Total Protein Levels (see Section 2.5.3)

b) Proteinase Levels (see Section 2.5.4)

c) TIC Levels (see Section 2.5.1)

d) T.P.E. Levels (see Section 2.5.2)

e) Alpha-1-antitrypsin levels (see Section 2.4.2)

f) Alpha-2-macroglobulin levels (see Section 2.4.2)

The results were subjected to a linear regression analysis using a computer, t-values for coefficient significance were also calculated.

2.9 The Anti-Inflammatory Activity of Human Rheumatoid Synovial Fluid

2.9.1 The Effect of Human Rheumatoid Synovial Fluid on the Carrageenan Oedema in the Rat

One hour prior to injection of carrageenan rats were given intra-peritoneal injections of 1.0 cm^3 of a human rheumatoid synovial

fluid. The rats received an additional dose of 1.0 cm^3 of the synovial fluid at the time of injection of the carrageenan. Control rats were given injections with 1.0 cm^3 of 0.9% (w/v) saline alone instead of the synovial fluid.

2.9.2 The Evaluation of Human Rheumatoid Synovial Fluid using the Cotton Wool Pellet Granuloma Test

Rats which had previously been implanted with four cotton wool pellets were given intra-peritoneal injections of 1.0 cm^3 of a human rheumatoid synovial fluid per day for the duration of the experiment. Control rats were injected with 1.0 cm^3 of 0.9% (w/v) saline alone per day.

2.9.3 Anti Inflammatory activity of Pooled Human Rheumatic Synovial Fluid and Fractions

The resulting fractions prepared from pooled human rheumatic synovial fluid in section 2.2.1 were assayed for anti-inflammatory activity using the carrageenan oedema test. Each group of rats received 1 cm^3 of a 100 $mg.cm^{-3}$ solution of one fraction intraperitoneally, this being compared with rats receiving intra-peritoneal injections of 1 cm^3 saline (controls) and 1 cm^3 of the pooled human rheumatic synovial fluid.

2.9.4 The Irritant Effects of Human Rheumatic Synovial Fluid and Its Fractions

The irritant effects of the synovial fluid used in section 2.9.1 and of the pooled synovial fluid and fractions from it (section 2.9.3) were assessed by a method similar to the one first described by Atkinson and Hicks (1971). In this case the irritant activity was evaluated by measuring the increase in paw volume following a subplantar injection of 0.05 cm^3 of the synovial fluid, or a solution of its fractions (see above), into the left hind foot. Control animals being injected with 0.05 cm^3 of 0.9% (w/v) saline alone.

2.9.5 Elucidation of the Mechanism of Action of Human Rheumatic Synovial Fluid

It was decided to investigate whether the anti-inflammatory effect of this exudate was due to large molecular weight substances or to small molecules or fragments of larger ones.

To accomplish this a modification of the carrageenan oedema test was used.

Ten days prior to injection of carrageenan rats were anaesthetized using a Boyles veterinary anaesthetic trolley as previously described. Using a sterile operating procedure (see section 2.1.4) a median dorsal skin incision was made and a dialysis sac, (5 cm. long, 1 cm. diameter)(Gallenkamp and Company Limited, Birmingham) which was sealed at both ends, was inserted along the back. The wound was closed with 10 mm. metal suture clips. The dialysis sacs had previously been sterilized by autoclaving for 20 minutes at a pressure of 15lbs. per sq. in. and had been filled with the appropriate solution under a microbiological screen, the solutions having been sterilized by filtration using a 0.22 μ m membrane filter (Millipore (U.K.) Limited, London).

Three groups of rats received one of the following treatments:-

a. Sham operated controls.

b. Dialysis sacs containing 1 cm^3 of 0.9% (w/v) saline alone.

c. Dialysis sacs containing 1 cm^3 of a 150 $mg.cm.^{-3}$ solution of human rheumatic synovial fluid.

Ten days after this treatment a carrageenan test was carried out as previously described.

2.9.6 Determination of the Degree of Stabilization of Guinea-Pig Macrophages by Human Rheumatic Synovial Fluid and its Fractions.

Using the method previously described (section 2.6) the effect of human rheumatic synovial fluid solutions (200,100,10 and 1 mg. cm.$^{-3}$) and the fractions produced by gel filtration (200,100, 10 and 1 mg.cm.$^{-3}$) on the stability of guinea-pig macrophages was investigated.

2.10 The Anti-inflammatory Activity of Liver Extracts from Dimethylnitrosamine Treated Rats

2.10.1 The Effect of Fractions of Liver Extracts from Dimethylnitrosamine Treated Rats on the Carrageenan Oedema Model

The samples prepared by the gel filtration of dimethyl nitrosamine (DMNO) liver extracts (section 2.2.3) were assayed for anti-inflammatory activity using the carrageenan oedema test. Each group of rats received 1 cm^3 of a 100 mg.cm^{-3} solution of one fraction intraperitoneally, this being compared with rats receiving intraperitoneal injections of 1 cm^3 saline alone.

The irritancy of these fractions was also measured (see section 2.9.4) and compared to saline.

2.10.2 The Release of Dialysable Molecules by DMNO Liver Extract and Fractions

It was decided to determine if DMNO liver extract or the anti-inflammatory fractions self degraded in any way to release small dialysable molecules. This was accomplished by setting up four conical flasks each containing 50 cm^3 of 0.01M phosphate buffered saline, pH7.4. Each flask also contained an identical dialysis bag (5 cm. long, 1 cm. diameter), which contained one of the following solutions:-

Flask 1 - 1 cm^3 of 0.9% (w/v) saline

Flask 2 - 1 cm^3 of a 100 $mg.cm^{-3}$ solution of the test material

Flask 3 - as flask 2 plus 10 mg. of trypsin

Flask 4 - 1 cm^3 of 10 $mg.cm^3$ solution of trypsin

The flasks were incubated at 37°C in a shaking water bath and the bathing fluid sampled at 30 minute intervals for three hours and read at 280 mm. using a spectrophotometer.

2.10.3 Elucidation of the Mechanism of Action of DMNO Liver Extract Fraction

In order to determine if the release of dialysable material was important in vivo an experiment similar to the one described in section 2.7.5 was carried out.

Dialysis sacs were implanted subcutaneously into rats.

Three groups of rats received one of the following treatments:-

a. Sham operated controls

b. Dialysis sacs containing 1 cm^3 of 0.9% saline

c. Dialysis sacs containing 100 mg. of the fraction and 10 mg. trypsin in 1 cm^3 of saline.

Ten days later a carrageenan test was performed.

2.10.4 The Role of Oligoamines in the Mechanism of Action of DMNO Liver Extracts

Using the methods previously described (2.5.5 and 2.5.6) liver extracts from DMNO treated and normal rats were assayed for the presence of spermine, spermidine, cadaverine and putrescine.

In addition, in an experiment similar to the one carried out above (2.10.2) the dialysate of a DMNO extract was examined for oligoamines. This experiment used the same method as before, but in this case aseptic techniques were employed throughout. The bathing

fluid consisted of 100 cm^3 of buffer and the sealed flasks were incubated for seven days, 1 cm^3 of the dialysate was then used for analysis.

2.11 The Anti-inflammatory Properties of Exudate from Sponge Implanted Rats

2.11.1 Determination of the Degree of Stabilization of Guinea-pig Macrophages by Sponge Exudate and Its Fractions

Using the method previously described (section 2.6) the effect of sponge exudate solutions (100,50,10 and 1 $mg.cm^{-3}$) and the fractions produced by gel filtration (100,50, 10 and 1 $mg.cm^{-3}$) on the stability of guinea-pig macrophages was investigated.

2.11.2 The Proteolytic Activity of Sponge Exudate

Proteinase activity was determined using a modification of the method previously described (2.5.4). In this case the sample was 1 cm^3 of a 100 mg.cm-3 solution of sponge exudate and the quantities of buffer and substrate were doubled.

It was also decided to determine if the sponge exudate self degraded in any way and what was released during this process. This was investigated using the method described in section 2.10.2.

2.11.3 Elucidation of the Mechanism of Action of Sponge Exudate

Using the method previously described (2.9.5) dialysis sacs were implanted subcutaneously into rats to determine the importance of the release of dialysable material.

Five groups of rats received one of the following treatments:-

a. Sham operated controls
b. Dialysis sacs containing 1 cm^3 of 0.9%(w/v) saline
c. Dialysis sacs containing 1 cm^3 of a 100 $mg.cm^{-3}$ solution of sponge exudate

d. Dialysis sacs containing 1 cm^3 of a 100 $mg.cm^{-3}$ solution of sponge exudates plus 5 mg. of trypsin

e. Dialysis sacs containing 1 cm^3 of a 5 $mg.cm^{-3}$ solution of trypsin.

Ten days later a carrageenan oedema test was carried out.

2.11.4 The Anti-inflammatory Activity of Sponge Exudate Dialysate

Dialysate molecules released by sponge exudate were tested for their anti-inflammatory activity.

The dialysate was collected by dissolving 1.5g of aseptically derived exudate in 50 cm^3 of water and placing this in a sterile dialysis sac. The dialysis sac was then immersed in 100 cm^3 of water in a flask using aseptic techniques. The sterile water was changed every two days until the end of the ten day incubation at 37°C.

After removal the dialysates were freeze-dried, pooled and stored at -15°C until required.

The freeze-dried powder was dissolved in 15 cm^3 of water and used in the following carrageenan oedema test.

Three groups of rats were treated as follows:-

Group 1 — 1 cm^3 saline intraperitoneally one hour prior to the carrageenan.

Group 2. — 1 cm^3 of the dialysate solution intraperitoneally one hour prior to the carrageenan.

Group 3. — 0.1 cm^3 of the dialysate solution, subcutaneously starting ten days prior to the carrageenan.

The dialysate was also tested for irritancy and the residue left after dialysis was tested both for anti-inflammatory and irritant activity, the dose being 100 mg. per rat.

2.11.5 The Role of Oligoamines in the Mechanism of Action of Sponge Exudate

The levels of spermine, spermidine, cadaverine and putrescine were determined in sponge exudate, normal serum, serum from sponge implanted rats and dialysates of sponge exudate (2.10.4).

2.12 The Role of Oligoamines in Inflammation

2.12.1 The Anti-inflammatory Activity of Oligoamines in the Carrageenan Oedema Test

Using doses of 50 µg, 500 µg and 5,000 µg per Kg body weight given intraperitoneally in 1 cm^3 of 0.9%(w/v) saline the four oligoamines spermidine, spermine, cadaverine and putrescine were tested using the carrageen oedema model, the compounds being given one hour before the carrageenan.

The same solutions were also used to test the irritancy of each oligoamine.

2.12.2 The Inhibitory Effect of Oligoamines on Pinnal Anaphylaxis in the Mouse

Using a fixed dose of 50 µg per Kg bodyweight given intraperitoneally in 0.1 cm^3 of saline the four oligoamines were tested for their effect upon the development of a local anaphylactic reaction in the mouse pinna.

2.12.3 The Anti-inflammatory Action of Putrescine in the Adjuvant Arthritic Rat

The effect of daily subcutaneous injections of 50 µg and 500 µg per Kg body weight (dose volume 0.5 cm^3) upon the development of adjuvant arthritis in the rat was investigated.

2.12.4 Determination of the Degree of Stabilization of Guinea-pig Macrophages by Oligoamines

Solutions of the oligoamines spermine, spermidine, cadaverine and putrescine at concentrations of 10 µg, 100 µg,

1 mg. and 10 mg.cm^{-3} were made up using phosphate buffered saline and their effect on the stability of guinea-pig macrophages measured.

2.12.5 The Anti-inflammatory Activity of Thioacetamide and Theophylline in the Carrageenan Oedema Test

A single dose of thioacetamide (150 mg Kg^{-1}) given by intraperitoneal injection twenty four hours prior to the carrageenan was evaluated for its anti-inflammatory action. Theophylline given orally (15 mg.Kg^{-1}) for the previous two days was also evaluated.

2.13 The Role of Endotoxin in Inflammation

2.13.1 The Anti-inflammatory Properties of Endotoxin

The anti-inflammatory effect of bacterial endotoxin (E. Coli: serotype 0111: B4, Sigma Chemical Co., U.S.A) on the development of carrageenan oedema in the rat was investigated. Doses of 5 µg, 20 µg and 100 µg per Kg. bodyweight were administered intraperitoneally one hour prior to the carrageenan.

The irritancy of a 100 µg $cm.^{-3}$ solution of the endotoxin was also measured.

2.13.2 Determination of the Degree of Stabilization of Guinea-pig Macrophages by Endotoxin

The effect of bacterial endotoxin solutions (100, 50, 10 and 1 µg.$cm.^{-3}$) on the stability of guinea-pig macrophages was investigated as previously described (2.6).

The bacterial endotoxin used in the above experiments was also assayed for the levels of spermine, spermidine, cadaverine and putrescine present.

3. RESULTS

3.1 The Evaluation of Trasylol as a Potential Anti-Inflammatory Agent

For methods see section 2.

3.1.1 The Effect of Trasylol in the Cotton Wool Pellet Granuloma Test

Trasylol when given in daily doses of 5,000, 10,000, 20,000,40,000, 50,000 and 100,000 KIUper Kg body weight, by intraperitoneal injection, to rats implanted with cotton wool pellets showed no tendency to inhibit the formation of granulation tissue around these pellets.

3.1.2 The Action of Trasylol in the Adjuvant Arthritic Rat

In this model doses of 50,000 and 100,000 $KIU\ Kg^{-1}$ of Trasylol were ineffective in inhibiting the inflammatory response caused by the adjuvant. The animals treated with Trasylol showed a significantly ($p < 0.05$) greater increase in foot volume of the injected foot when compared to the controls after day 12 of the experiment.

3.1.3 The Inhibition of Neutral Proteinases in Human Rheumatic Synovial Fluid by Trasylol

Table 3.1 shows to what extent Trasylol inhibited the proteolytic action of human synovial fluid samples from patients both male and female, with various rheumatic conditions.

Table 31.

Disease State	Sex	% Inhibition of Proteinase Activity by Trasylol
Rheumatoid	M	33.8
Arthritis	M	61.4
seropositive	M	8.1
	F	9.2
	F	70.0
	F	22.9
	F	38.8
Rheumatoid	M	38.9
Arthritis	F	27.8
seronegative	F	89.5
Osteoarthritis (OA)	M	2.2
	F	87.8
	F	77.8
OA + Gout	M	95.9
Chondocalcinosis	F	93.2
Unknown	F	92.95
Unknown	M	43.8

Table 3.1. The percentage inhibition of proteinases in human rheumatic synovial fluid samples by Trasylol.

3.2 The Levels of Proteinases and Anti-proteinases in Human Rheumatic Synovial Fluid Samples

Table 3.2 shows the levels of alpha-1-antitrypsin, alpha-2-macroglobulin, trypsin inhibiting capacity (TIC), trypsin-protein esterase activity (TPE), proteinase activity and total protein in samples of synovial fluid withdrawn from patients of both sexes, suffering from a variety of rheumatic conditions. A total of 21 samples were used in this study.

The results from the study carried out above were entered into a computer which was programmed to analyse groups of data by linear regression and calculate the significance of these results. Table 3.3 shows the significance levels calculated by the computer.

In addition to the data presented in this table the ratio of alpha-1-antitrypsin levels to alpha-2-macroglobulin levels against proteinase activity was also subjected to analysis. The significance level for this calculation was found to be 90.2%.

Table 32. The levels of [illegible] and anti-proteinase in human rheumatic-synovial fluid samples.

alpha-1-antitrypsin	alpha-2-macroglobulin	TIC Levels umol $min^{-1} cm^{-3}$	TPE Levels	Protease Levels *	Total Protein $g/100\ cm^3$	Sex/ Disease State
20	6	0.96	0.095	0.29	5.28	M,RA+
13	4	0.43	0.051	6.16	5.68	F,RA+
18	4	0.52	0.063	0.58	4.44	F,RA+
14	5	0.56	0.082	0.71	5.26	M,RA+
27	9	1.04	0.017	7.12	7.49	F,RA-
22	4.5	0.59	0.094	14.45	7.18	M,RA-
11.5	7	0.42	0.030	0.87	6.12	F,
6.5	2	0.17	0.039	0.54	3.36	M,
9	2	0.42	0.039	0.40	5.19	M,OA
11.5	3	0.65	0.000	0.80	5.35	F,RA+
13	4	0.61	0.000	8.84	8.53	F,RA+
8	2	0.32	0.043	0.70	5.52	F,CH
8.5	3	0.27	0.045	0.93	5.08	F,
9	3	0.37	0.000	-	4.38	F,OA
7	6	0.01	0.000	2.01	4.73	F,RA+
4	4	0.00	0.000	2.01	5.19	M,RA+
9	3	0.06	0.000	1.43	5.97	
12	6	0.00	0.000	2.15	6.78	F,RA-
9	3	0.35	0.029	2.71	4.18	F,RA+
7	2	0.32	0.000	5.46	5.33	F,
10	13	0.47	0.080	2.24	7.18	M,

M, Male; F, female; RA+, Rheumatoid arthritis serapositive; RA-, Rheumatoid arthritis seranegative; OA, osteoarthritis; CH, chondrocalcinosis.
* mg. of substrate broken down in 24 hours.

Table 3.3 The Significance levels between various parameters as determined by computer analysis.

	alpha-1-anti-trypsin	alpha-2-macro-globulin	TIC	TPE	Proteinase	Total Protein
alpha-1-antitrypsin		90.5%			96.9%	
alpha-2-macroglobulin	90.5%		81.4%	73.7%	29.8%	97.9%
TIC		81.4%			78.5%	
TPE		73.7%			24.6%	
Proteinase	96.9%		78.5%	24.6%		99.6%
Total Protein		97.9%			99.6%	

Values over 95.0% are taken as being statistically significant.

3.3 The Anti-inflammatory Activity of Human Rheumatic Synovial Fluid

3.3.1 The Effect of Human Rheumatic Synovial Fluid on the Carrageenan Oedema in the Rat

Figure 3.1 shows the effect of intraperitoneal injections of a human rheumatic synovial fluid on carrageenan induced oedema in rats. This treatment significantly reduced the carrageenan oedema when compared to control animals treated with saline alone.

The rheumatic synovial fluid chosen for this experiment had the following biochemical features:-

alpha-1-antitrypsin level	20
alpha-2-macroglobulin level	6
TIC Level	0.96µmole $min^{-1}cm^{-3}$
TPE Level	0.095
Proteinase Level	0.29 mg broken down in 24 hours
Total Protein	5.28g per 100 cm^3

This synovial fluid was also tested for its irritant properties by injecting 0.05 $cm.^3$ into the subplantar region of the left hind foot of rats. There was no significant difference between these animals and animals injected with saline only (see Figure 3.2).

3.3.2 The Evaluation of Human Rheumatic Synovial Fluid using the Cotton Wool Pellet Granuloma Test

Daily intraperitoneal injections of 1.0 cm^3 of human rheumatic synovial fluid had no effect on the formation of granulation tissue around cotton wool pellets as can be seen in table 3.4.

Treatment	Mean Increase in pellet weight (mg) ± SEM
Control	10.12 ± 0.44
Human Synovial Fluid	10.37 ± 0.41

Table 3.4

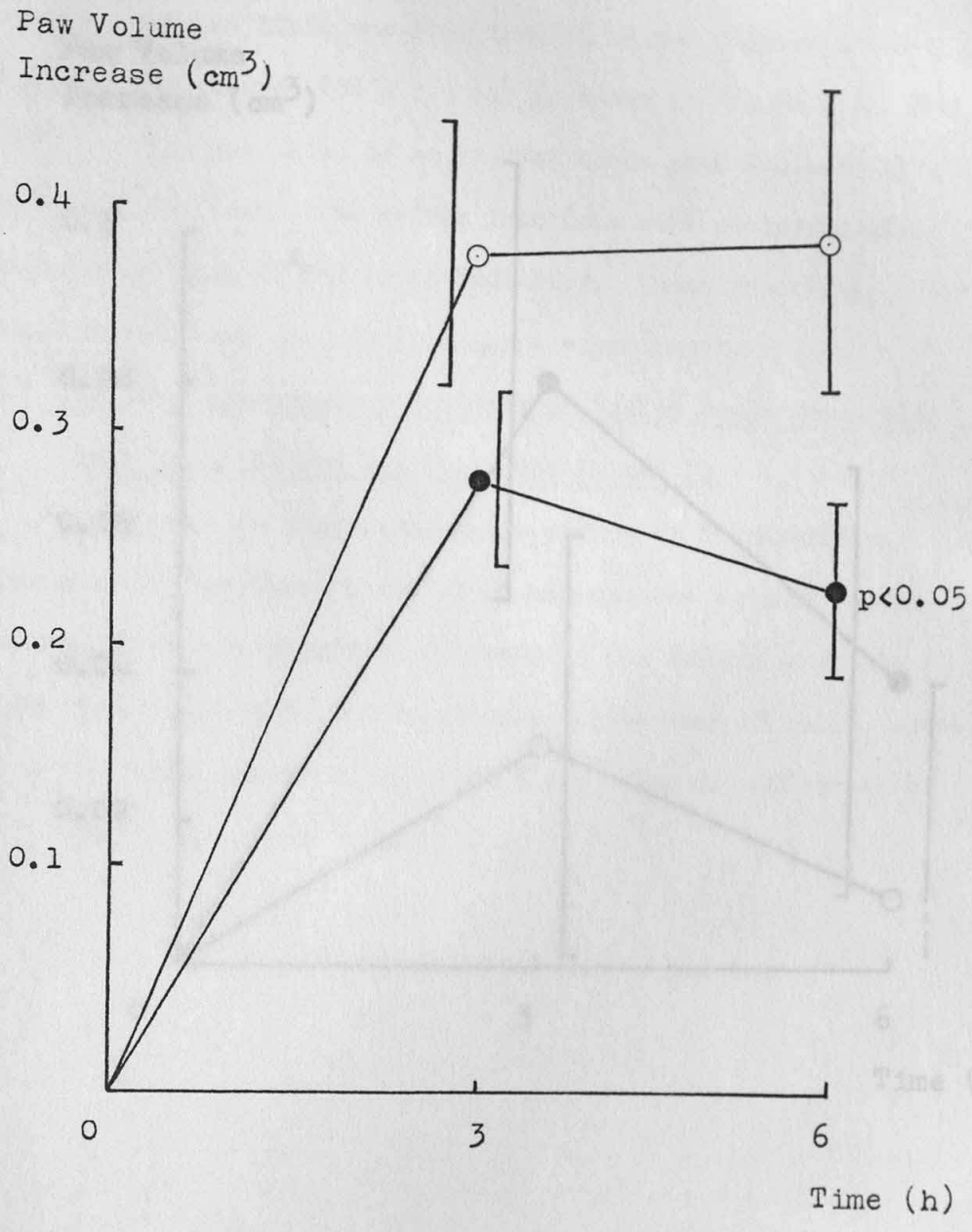


Figure 3.1 Increase in foot volume of rats injected with carrageenan one hour after administration of 1 cm^3 of human rheumatic synovial, which was repeated at the time of injection of the carrageenan ●——●. Increase in foot volume of control rats injected with carrageenan ○——○.

Each result represents the mean of six animals ±SEM

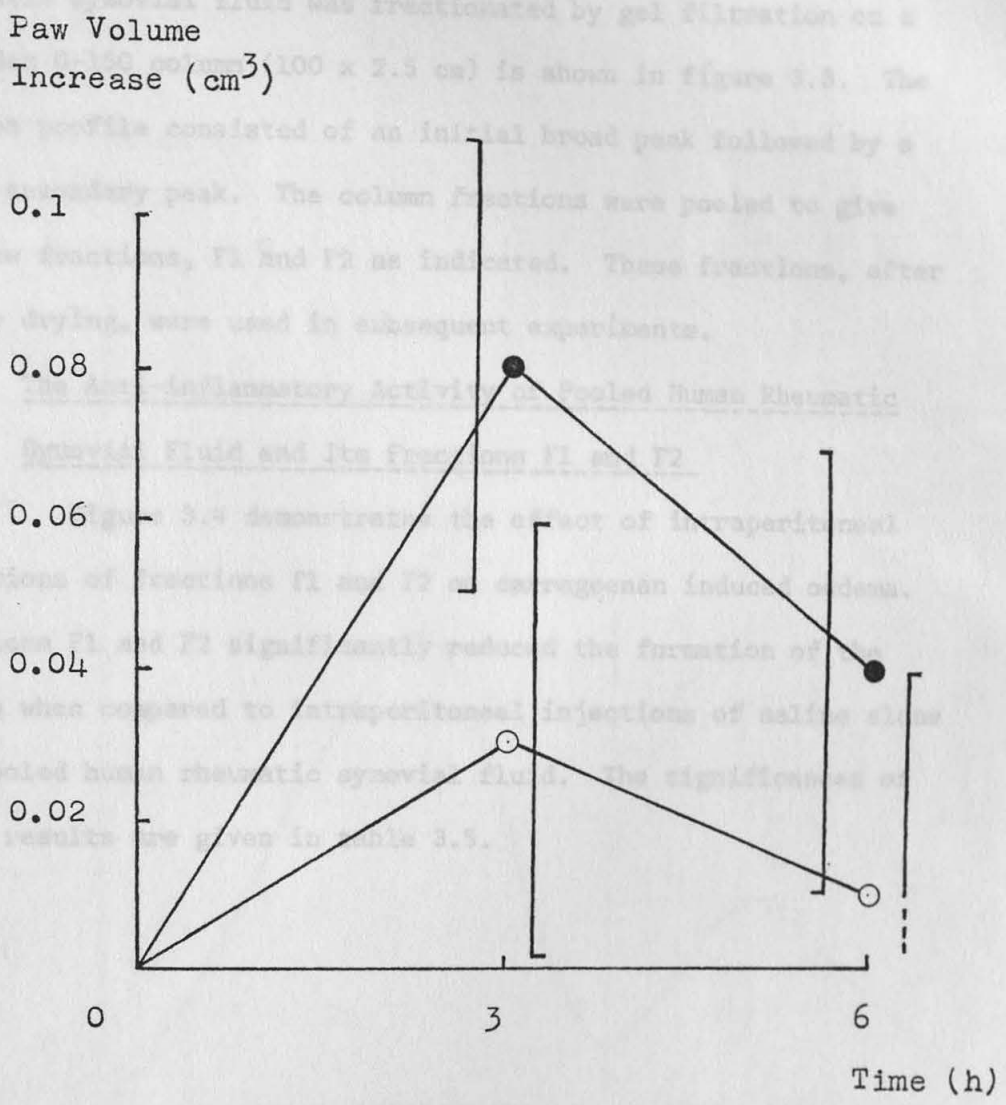


Figure 3.2 Increase in foot volume of rats injected with 0.05 cm^3 of human rheumatic synovial fluid into the left hind foot pad ●—● compared with controls injected with saline alone ○—○ . Each result represents the mean of six animals ± SEM

3.3.3 The Fractionation of Pooled Human Rheumatic Synovial Fluid

The elution pattern obtained when 5 cm^3 of pooled human rheumatic synovial fluid was fractionated by gel filtration on a Sephadex G-150 column (100 x 2.5 cm) is shown in figure 3.3. The elution profile consisted of an initial broad peak followed by a small secondary peak. The column fractions were pooled to give two new fractions, F1 and F2 as indicated. These fractions, after freeze drying, were used in subsequent experiments.

3.3.4 The Anti-inflammatory Activity of Pooled Human Rheumatic Synovial Fluid and Its Fractions F1 and F2

Figure 3.4 demonstrates the effect of intraperitoneal injections of fractions F1 and F2 on carrageenan induced oedema. Fractions F1 and F2 significantly reduced the formation of the oedema when compared to intraperitoneal injections of saline alone and pooled human rheumatic synovial fluid. The significances of these results are given in table 3.5.

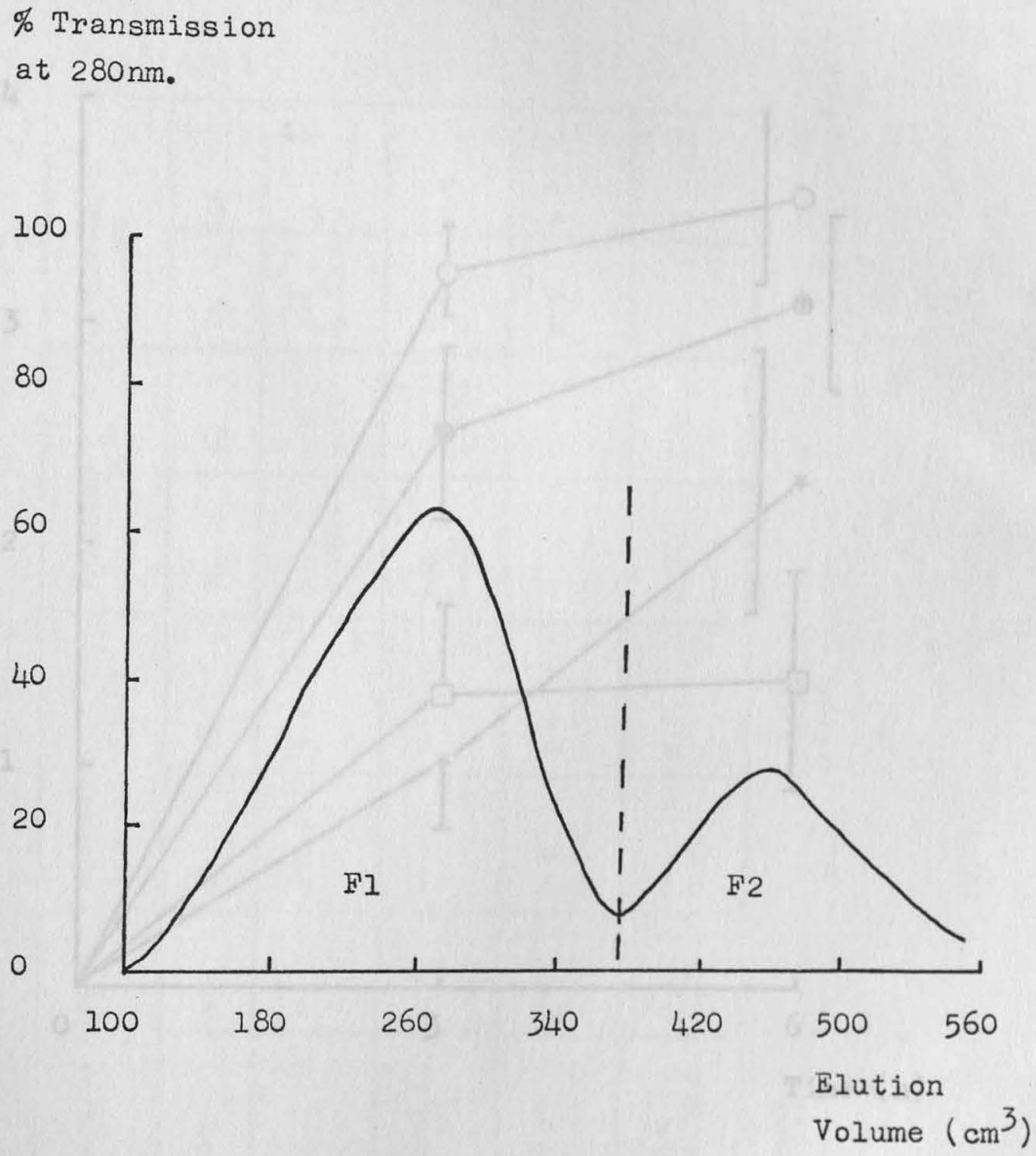


Figure 3.3 The protein elution pattern of human rheumatic synovial fluid through a Sephadex G-150 column in 0.1M phosphate buffer, pH7.4. The absorbance at 280 nm of the eluted proteins was measured. Fractions pooled are indicated by F1 and F2.

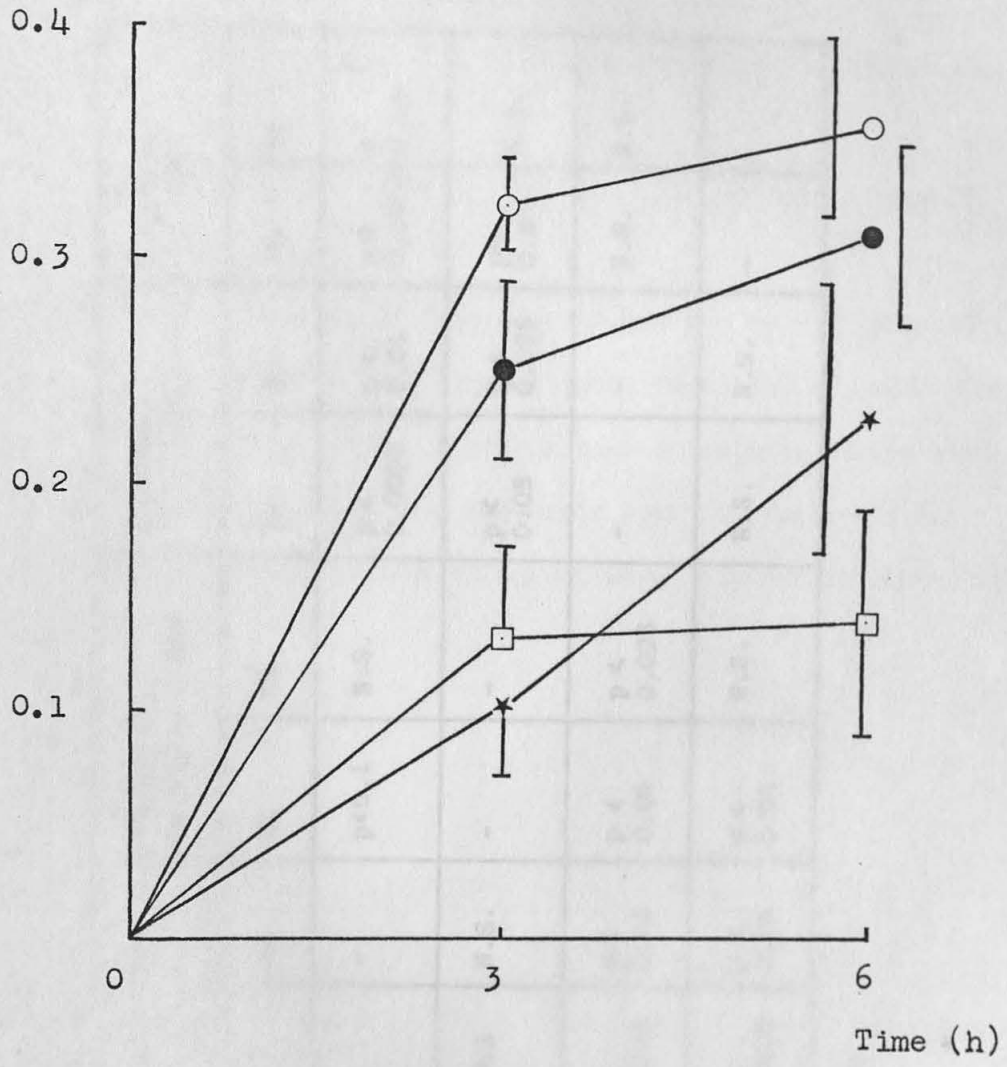


Figure 3.4. Increase in foot volume of rats injected with carrageenan and treated with human rheumatic synovial fluid ●——● , fraction F1 □——□ , fraction F2 ✶——✶ and saline alone ○——○.

Each result represents the mean of six animals ± SEM

Table 3.5

Treatment	Saline Alone		Rheumatic Synovial Fluid		Fraction F1		Fraction F2	
	3h	6h	3h	6h	3h	6h	3h	6h
Saline Alone	-	-	$p<0.1$	N.S.	$p < 0.0025$	$p < 0.01$	$p < 0.0005$	p 0.05
Rheumatic Synovial Fluid	$p<0.1$	N.S.	-	-	$p < 0.05$	$p < 0.025$	$p < 0.01$	N.S.
Fraction F1	$p < 0.0025$	$p < 0.01$	$p < 0.05$	$p < 0.025$	-	-	N.S.	N.S.
Fraction F2	$p < 0.0005$	$p < 0.05$	$p < 0.01$	N.S.	N.S.	N.S.	-	-

N.S. = not significant

3.3.5 The Irritant Activity of Pooled Human Rheumatic Synovial Fluid and Fractions F1 and F2

The irritant effects of subplantar injections of human rheumatic synovial fluid, fraction F1 and fraction F2 are shown in figure 3.5.

All of the test substances are significantly more irritant than saline alone and are also significantly different from each other.

3.3.6 Elucidation of the Mechanism of Action of Human Rheumatic Synovial Fluid

The effect on carrageenan induced oedema of implanted dialysis sacs containing 150 mg. of human rheumatic synovial fluid can be seen in figure 3.6. Dialysis sacs containing saline alone and the sacs containing human rheumatic synovial fluid did not significantly inhibit oedema formation when compared to the untreated controls.

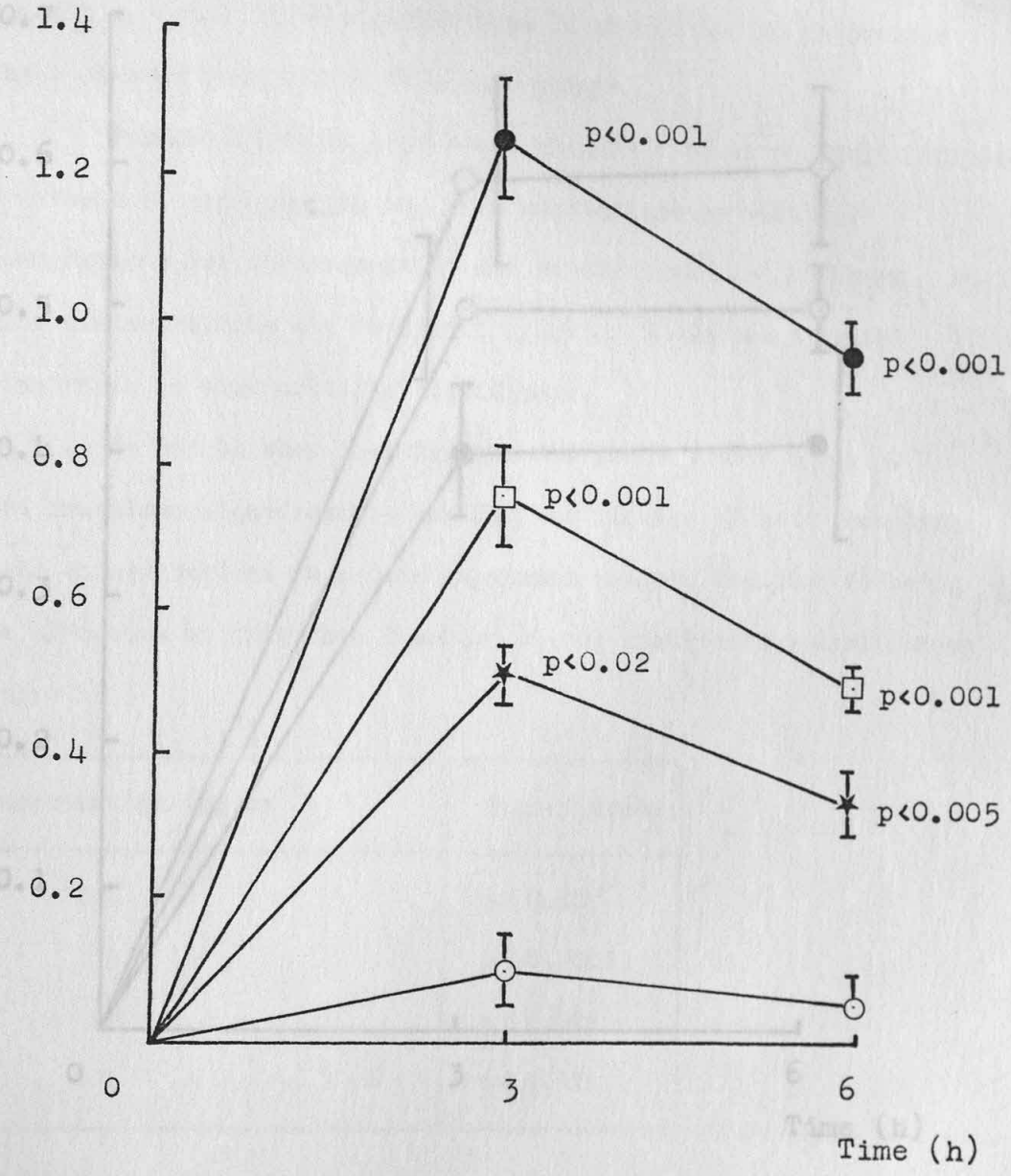


Figure 3.5 Increase in foot volume of rats injected with human rheumatic synovial fluid ●——●, fraction F1 □——□, and fraction F2 ✶——✶ compared with controls injected with saline alone ○——○.

Each result represents the mean of six animals ± SEM.

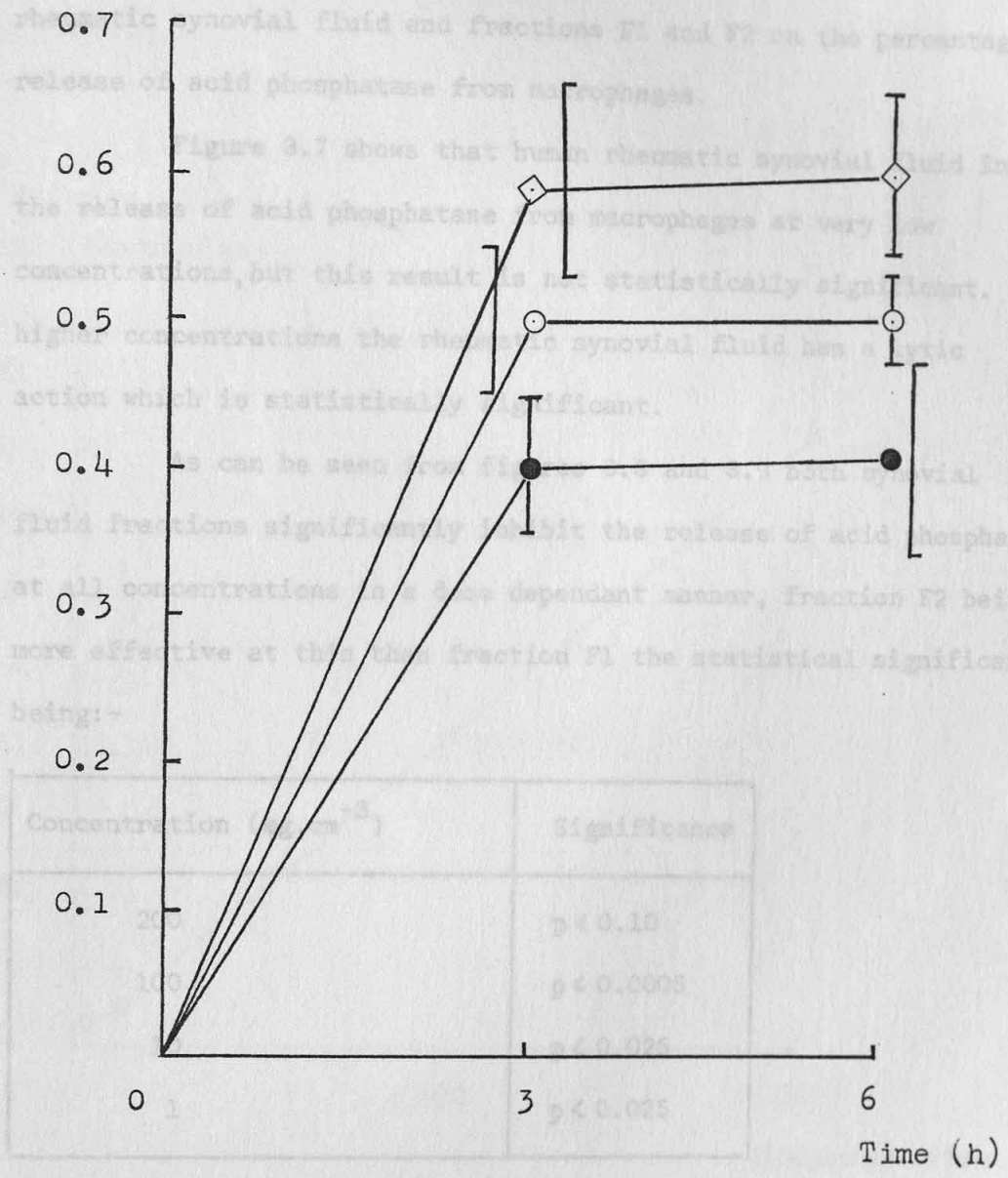


Figure 3.6 The effect on carrageenan induced oedema of implanted dialysis sacs containing human rheumatic synovial fluid ●——●, and saline alone ○——○.

Sham operated controls are also shown ◇——◇.

Each result represents the mean of seven animals ± SEM.

3.3.7 The Effect of Human Rheumatic Synovial Fluid and Fractions F1 and F2 upon Acid Phosphatase release from Macrophages

Figures 3.7, 3.8, and 3.9 show the effect of human rheumatic synovial fluid and fractions F1 and F2 on the percentage release of acid phosphatase from macrophages.

Figure 3.7 shows that human rheumatic synovial fluid inhibited the release of acid phosphatase from macrophages at very low concentrations, but this result is not statistically significant. At higher concentrations the rheumatic synovial fluid has a lytic action which is statistically significant.

As can be seen from figures 3.8 and 3.9 both synovial fluid fractions significantly inhibit the release of acid phosphatase at all concentrations in a dose dependant manner, fraction F2 being more effective at this than fraction F1 the statistical significance being:-

Concentration ($mg.cm^{-3}$)	Significance
200	$p < 0.10$
100	$p < 0.0005$
10	$p < 0.025$
1	$p < 0.025$

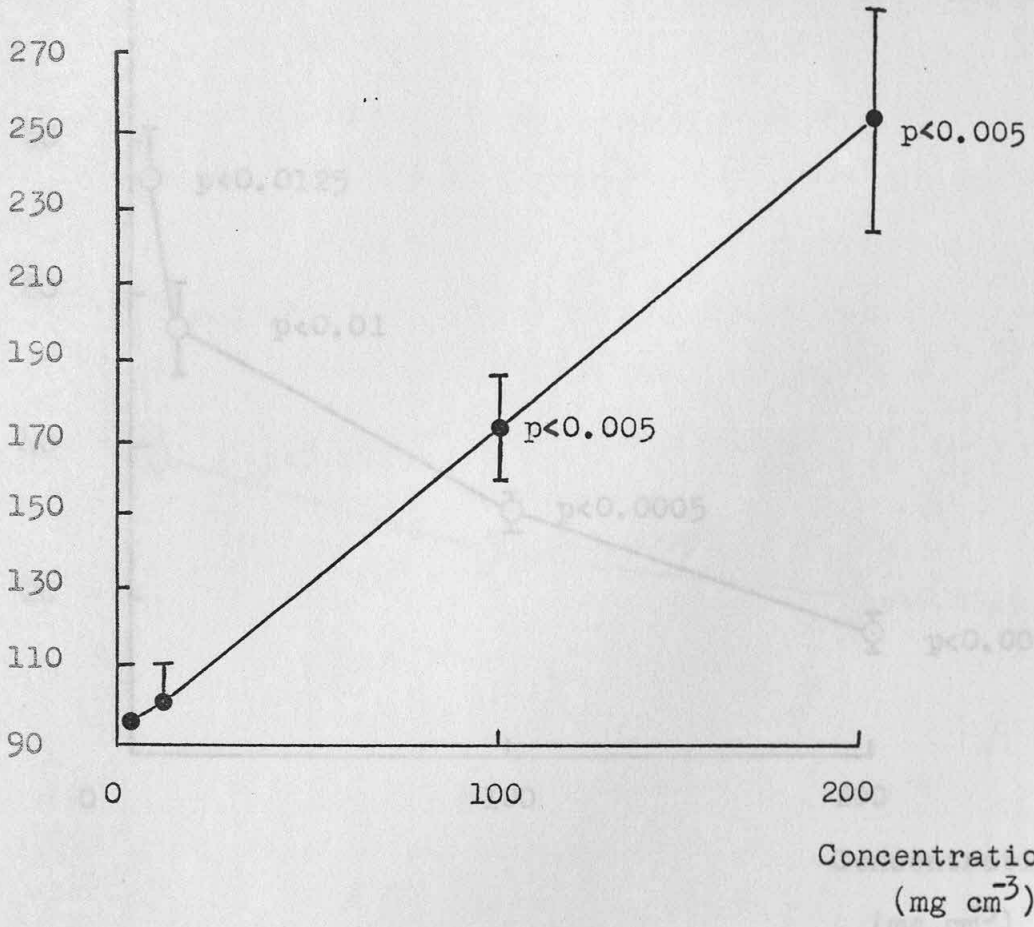


Figure 3.7 The effect of rheumatic synovial fluid on the release of acid phosphatase from macrophages.

Each result represents the mean of four observations ± SEM.

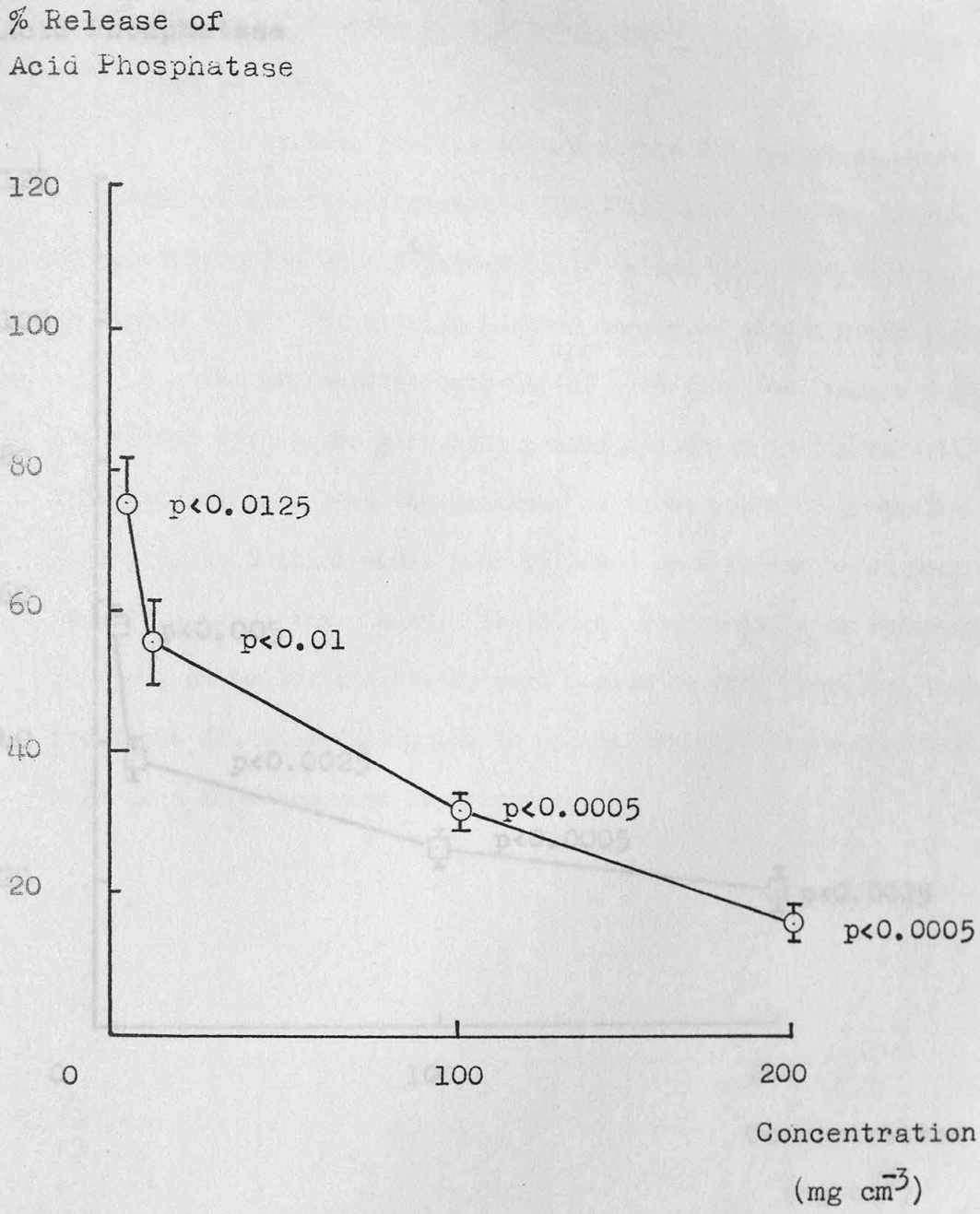


Figure 3.8 The effect of fraction F1 on the release of acid phosphatase from macrophages.

Each result represents the mean of four observations ± SEM.

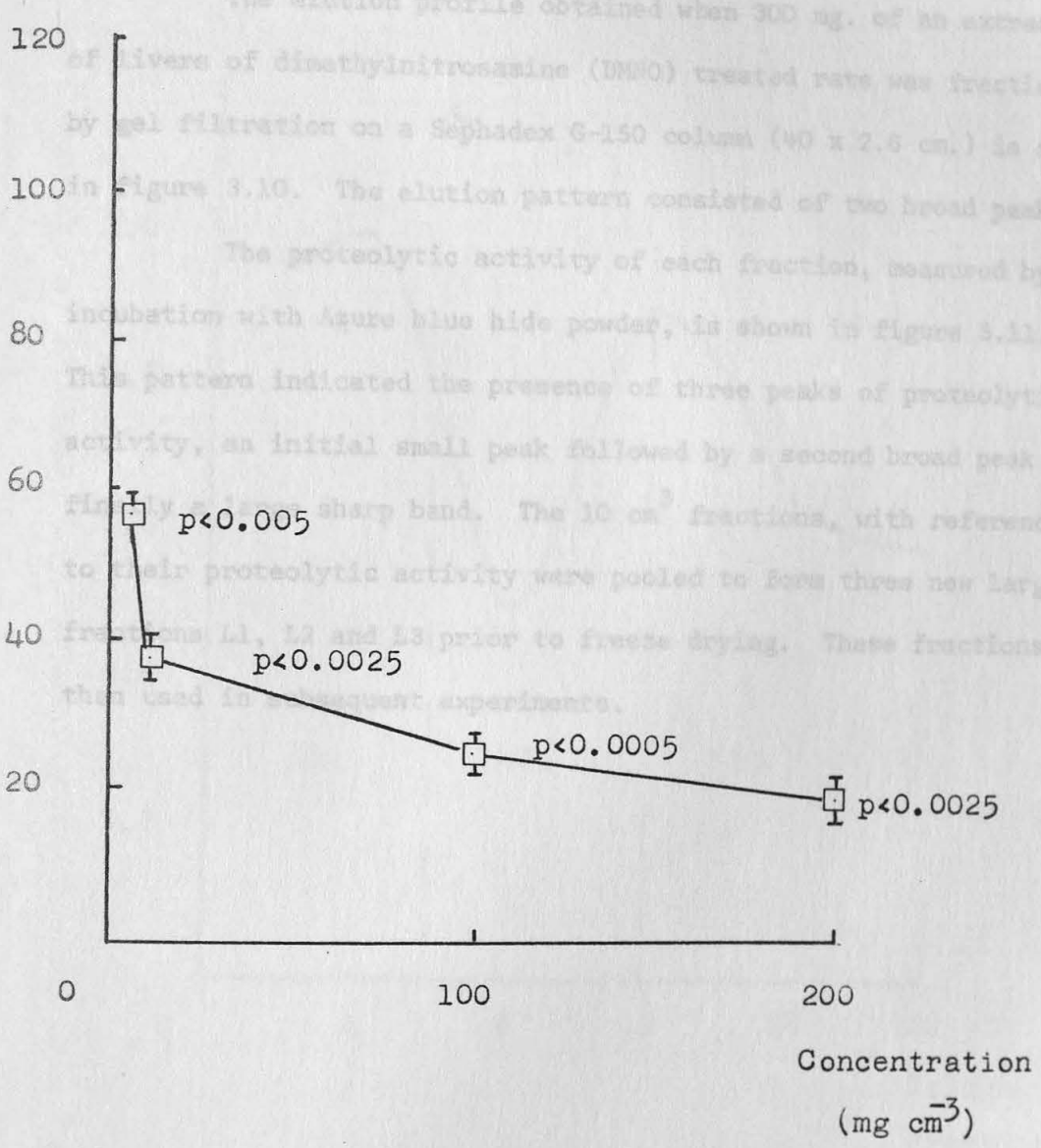


Figure 3.9 The effect of fraction F2 on the release of acid phosphatase from macrophages.

Each result represents the mean of four observations ± SEM.

3.4 The Anti-inflammatory Activity of Liver Extracts from Dimethylnitrosamine Treated Rats

3.4.1 The Fractionation of a Liver Extract from Dimethylnitrosamine Treated Rats

The elution profile obtained when 300 mg. of an extract of livers of dimethylnitrosamine (DMNO) treated rats was fractionated by gel filtration on a Sephadex G-150 column (40 x 2.6 cm.) is shown in figure 3.10. The elution pattern consisted of two broad peaks.

The proteolytic activity of each fraction, measured by incubation with Azure blue hide powder, is shown in figure 3.11. This pattern indicated the presence of three peaks of proteolytic activity, an initial small peak followed by a second broad peak and finally a large sharp band. The 10 cm^3 fractions, with reference to their proteolytic activity were pooled to form three new larger fractions L1, L2 and L3 prior to freeze drying. These fractions were then used in subsequent experiments.

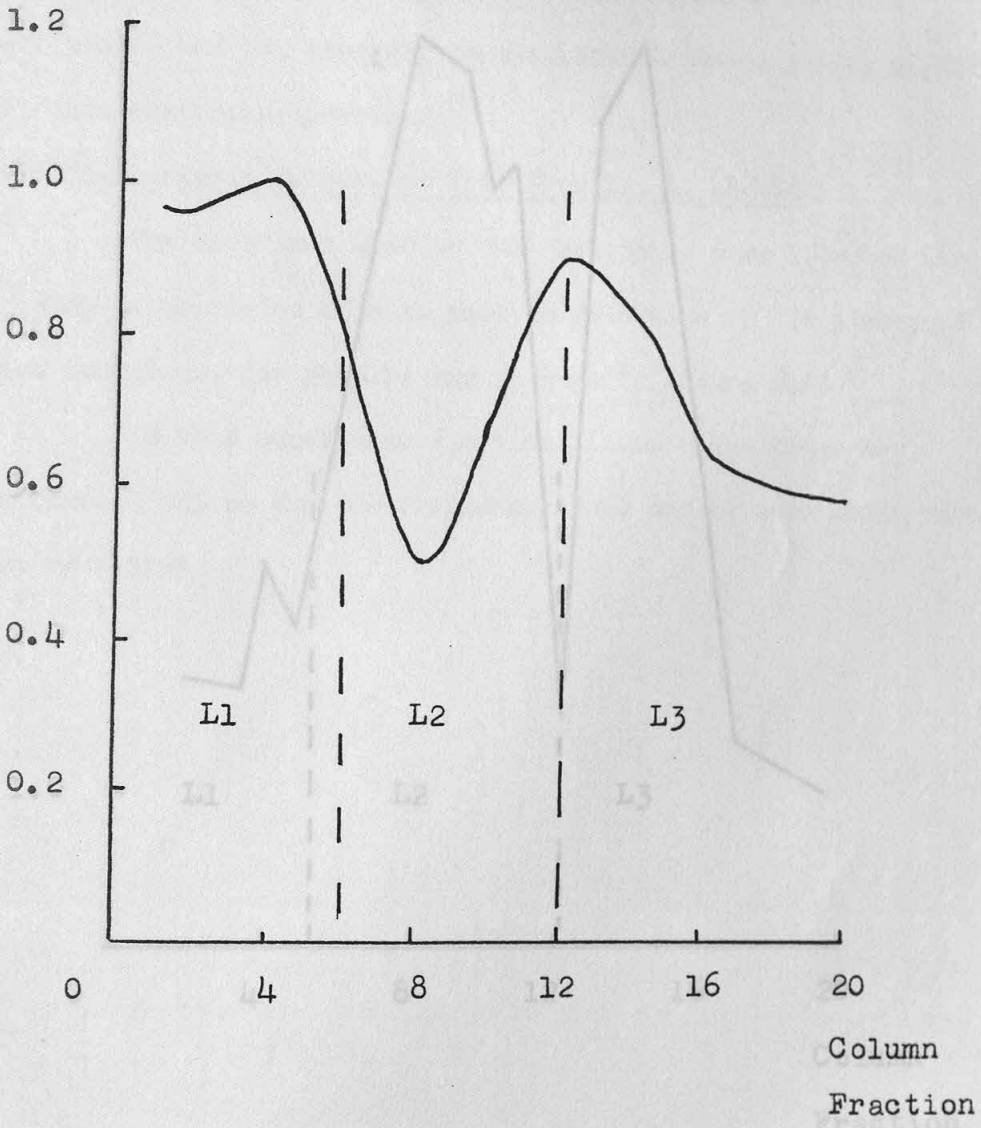


Figure 3.10 The protein elution profile of a liver extract from DMNO treated rats using a Sephadex G-150 column. The absorbance at 280 nm of the eluted proteins was measured. The column fractions were pooled to form L1, L2 and L3.

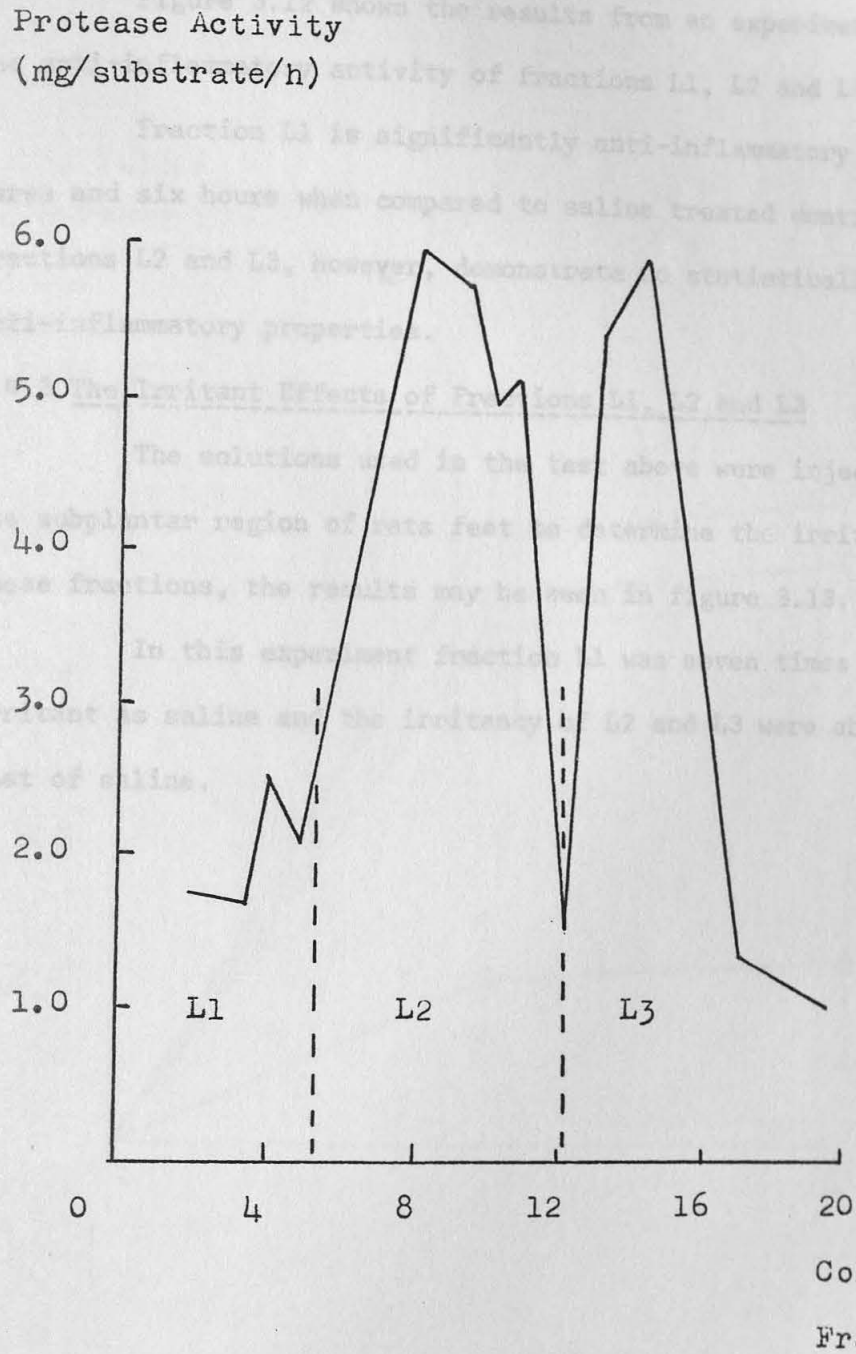


Figure 3.11 Distribution of proteolytic activity in the eluate from a Sephadex G-150 column to which a liver extract from DMNO treated rats had been applied. The column fractions were pooled to form L1, L2 and L3.

3.4.2 The Effect of Fractions L1, L2 and L3 on the Carrageenan Oedema Model

Figure 3.12 shows the results from an experiment to assay the anti-inflammatory activity of fractions L1, L2 and L3.

Fraction L1 is significantly anti-inflammatory at both three and six hours when compared to saline treated controls. Fractions L2 and L3, however, demonstrate no statistically significant anti-inflammatory properties.

3.4.3 The Irritant Effects of Fractions L1, L2 and L3

The solutions used in the test above were injected into the subplantar region of rats feet to determine the irritancy of these fractions, the results may be seen in figure 3.13.

In this experiment fraction L1 was seven times as irritant as saline and the irritancy of L2 and L3 were about twice that of saline.

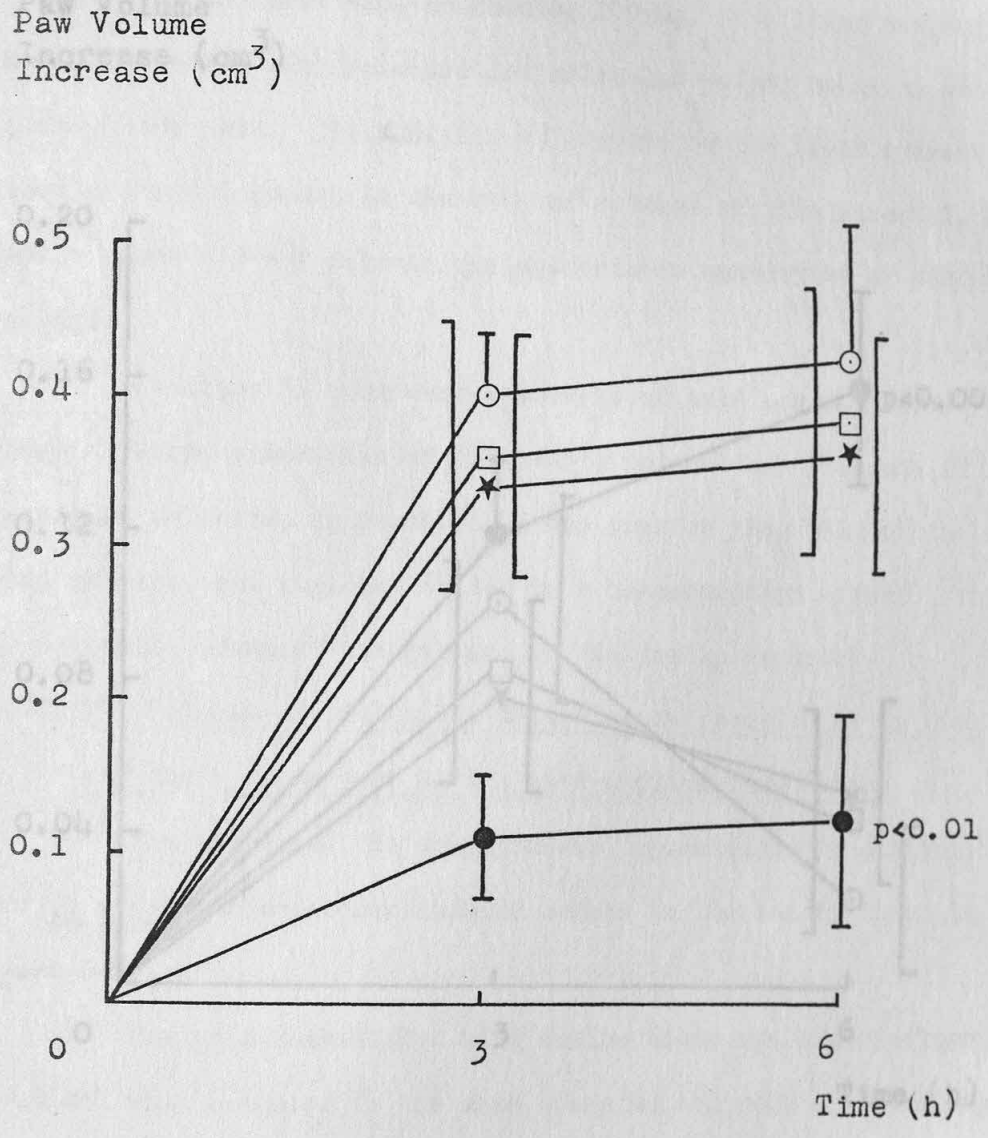


Figure 3.12 The effect of fractions L1 ●—●, L2 □—□ and L3 ✶—✶ (all 500mg. Kg^{-1}) on the increase in foot volume of rats injected with carrageenan. Controls were treated with saline only ○—○.

Each result represents the mean of seven animals ± SEM.

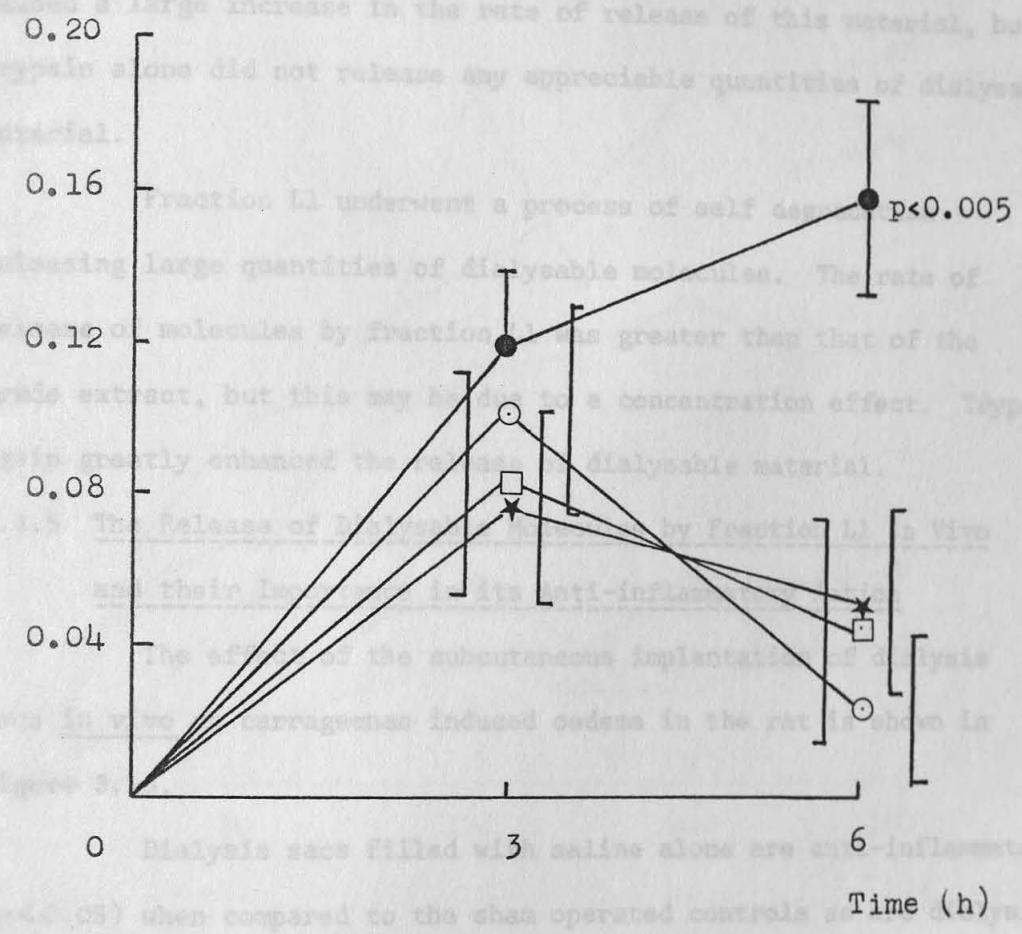


Figure 3.13 Increase in foot volume of rats injected with, fraction L1 ●—●, fraction L2 □—□ and fraction L3 ✶—✶ compared with controls injected with saline alone ○—○ .

Each result represents the mean of five animals ± SEM.

3.4.4 The Release of Dialysable Molecules by DMNO Liver Extract and Fraction L1

The release of low molecular weight material from dialysis sacs over a period of six hours is displayed in figure 3.14.

The dialysis sacs containing 100 mg. of a liver extract from DMNO treated rats released low molecular weight material at a slow steady rate. The addition of trypsin to the liver extract caused a large increase in the rate of release of this material, but trypsin alone did not release any appreciable quantities of dialysable material.

Fraction L1 underwent a process of self degradation releasing large quantities of dialysable molecules. The rate of release of molecules by fraction L1 was greater than that of the crude extract, but this may be due to a concentration effect. Trypsin again greatly enhanced the release of dialysable material.

3.4.5 The Release of Dialysable Molecules by Fraction L1 in Vivo and their Importance in its Anti-inflammatory Action

The effect of the subcutaneous implantation of dialysis sacs *in vivo* on carrageenan induced oedema in the rat is shown in figure 3.15.

Dialysis sacs filled with saline alone are anti-inflammatory ($p < 0.05$) when compared to the sham operated controls as are dialysis sacs containing 100 mg. of fraction L1 and 10 mg. of trypsin ($p < 0.025$).

There was, however, a significant difference ($p < 0.05$) between these two groups indicating that material released by the protein fraction caused an anti-inflammatory effect.

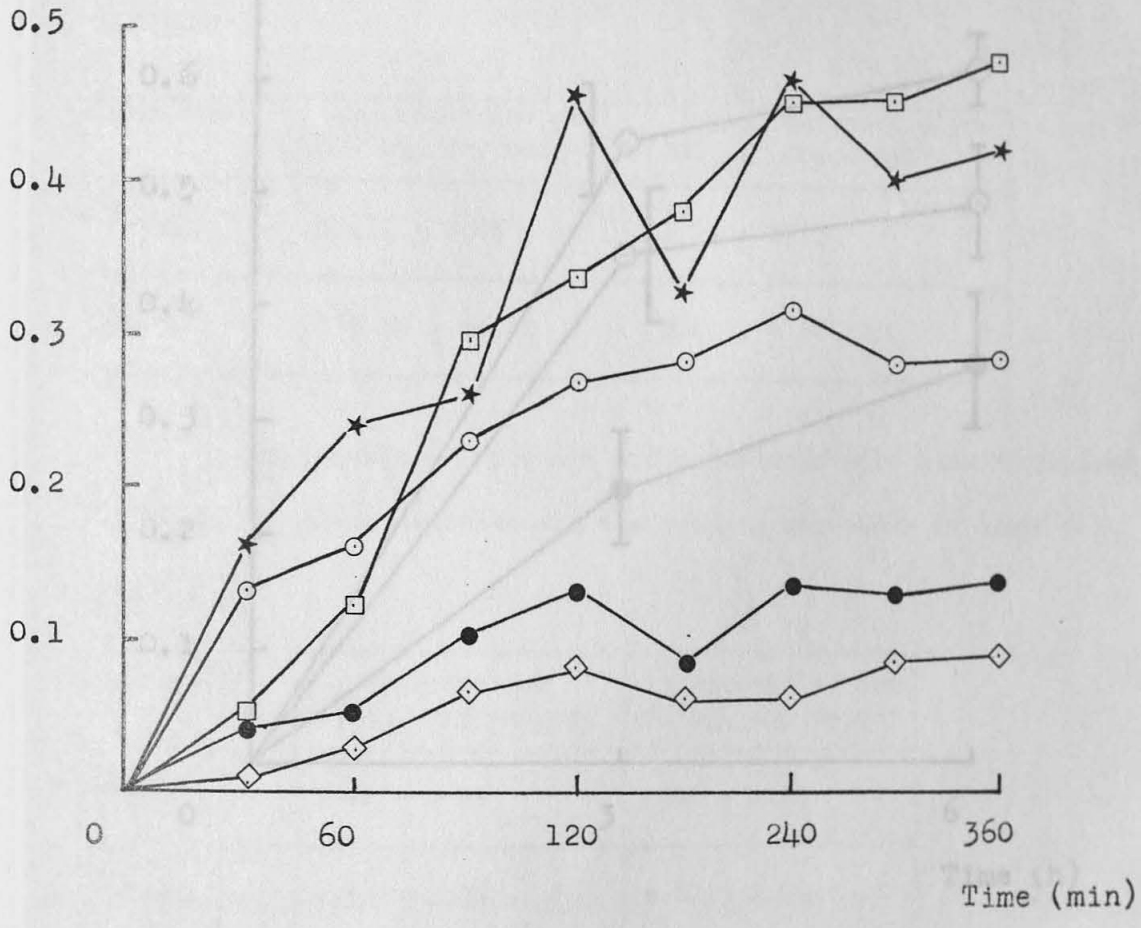


Figure 3.14 The Release of dialysable substances (as measured by absorbance) from dialysis sacs containing:-

100 mg. Liver extract from DMNO treated rats ●—●,

100 mg. Liver extract from DMNO treated rats and 5 mg. of trypsin □—□,

100 mg of fraction L1 ○—○,

100 mg of fraction L1 and 5 mg. of trypsin ✶—✶,

and 5 mg. of trypsin alone ◇—◇.

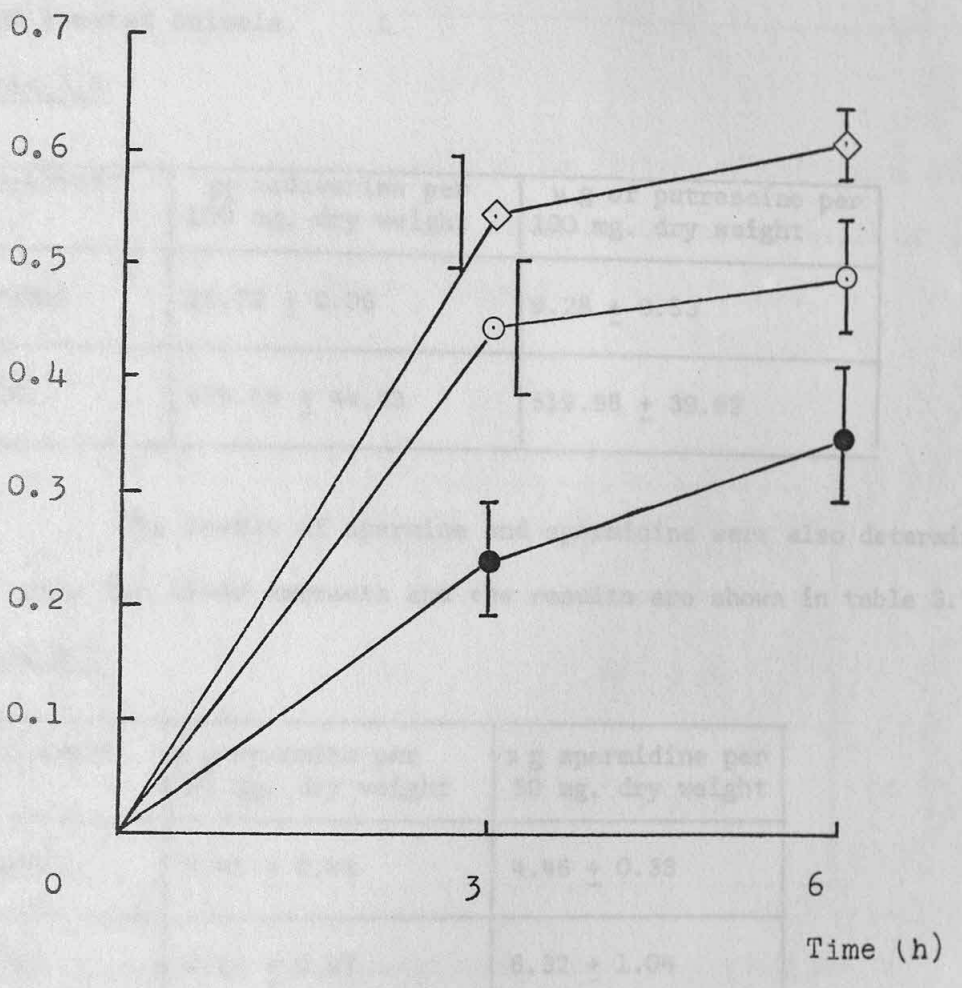


Figure 3.15 The effect of carrageenan induced oedema of implanted dialysis sacs containing saline alone O—O and 100 mg. of fraction L1 and 10 mg. trypsin ●—● compared to sham operated controls ◇—◇.

Each result represents the mean of seven animals ± SEM.

3.4.6 The Role of Oligoamines in the Mechanism of Action of DMNO Liver Extracts

Table 3.6 shows the levels of putrescine and cadaverine found in liver extracts from normal and DMNO treated rats.

The levels of both putrescine ($p < 0.001$) and cadaverine ($p < 0.001$) are significantly higher in the liver extracts from the DMNO treated animals.

Table 3.6

Treatment	µg cadaverine per 100 mg. dry weight	µ g of putrescine per 100 mg. dry weight
NORMAL	26.22 ± 0.05	9.28 ± 0.53
DMNO	576.69 ± 44.43	519.98 ± 39.62

The levels of spermine and spermidine were also determined in these two liver extracts and the results are shown in table 3.7.

Table 3.7

Treatment	µ g spermine per 50 mg. dry weight	µ g spermidine per 50 mg. dry weight
NORMAL	8.45 ± 0.49	4.46 ± 0.33
DMNO	4.15 ± 0.97	6.32 ± 1.04

Although the levels of spermidine are elevated by treatment with DMNO the result is not statistically significant. Spermine is significantly higher ($p < 0.01$) in normal liver extracts.

The dialysate of a DMNO extract (100 mg.) alone and of DMNO extract (100 mg.) plus trypsin (10 mg.) were examined for the presence of putrescine and cadaverine, the results are shown in table 3.8.

Contents of Dialysis sac	µg cadaverine per cm^3 of dialysate	µg putrescine per cm^3 of dialysate
DMNO EXTRACT	34.09 ± 3.98	22.12 ± 1.21
DMNO EXTRACT + TRYPSIN	40.09 ± 2.78	22.50 ± 1.94

Table 3.8

The effects of trypsin on the release of cadaverine and putrescine are not significant. Table 3.9 shows the levels of the other polyamines spermine and spermidine in dialysates.

Table 3.9

Contents of Dialysis sac	µg spermine per cm^3 of dialysate	µg spermidine per cm^3 of dialysate
DMNO EXTRACT	0.96 ± 0.68	3.54 ± 0.34
DMNO EXTRACT + TRYPSIN	0.85 ± 0.28	4.52 ± 0.32

From the data presented in table 3.9 trypsin has no significant effect on the release of spermine and spermidine from a DMNO liver extract.

3.5 The Anti-inflammatory Properties of Sponge Induced Inflammatory Exudate

3.5.1 The Fractionation of Sponge Exudate by Gel filtration

The elution profile obtained when sponge exudate was fractionated on a Sephadex G-150 column (100 x 2.5 cm.) is illustrated in figure 3.16. The fractions were pooled with reference to this profile to form two new fractions S1 and S2.

3.5.2 The Effect of Sponge Induced Inflammatory Exudate and Fractions S1 and S2 on Acid Phosphatase Release from Macrophages

Figures 3.17, 3.18 and 3.19 illustrate the effect of sponge induced inflammatory exudate and fractions S1 and S2 upon the percentage release of acid phosphatase from macrophages. Figure 3.17 indicates that the unpurified exudate significantly inhibits the release of acid phosphatase at all the concentrations used. However, at a concentration of 100 $mg.cm^{-3}$ of exudate there is a significant ($p < 0.005$) decrease in the inhibition of acid phosphatase release. Figure 3.18 shows that fraction S1 significantly inhibits the release of acid phosphatase upto a concentration of 50 $mg.cm^{-3}$ of exudate. Above this concentration the inhibition rapidly decreases and at 100 $mg.cm^{-3}$ of exudate lysis of the cells is quite pronounced.

Fraction S2 exhibits similar properties to S1 (see Figure 3.19), but, as can be seen the range over which S2 exerts an inhibitory effect is quite small. Indeed at concentrations greater than 10 mg. cm^{-3} the inhibitory effects are either decreased or completely abolished, that is, a lytic effect is exerted.

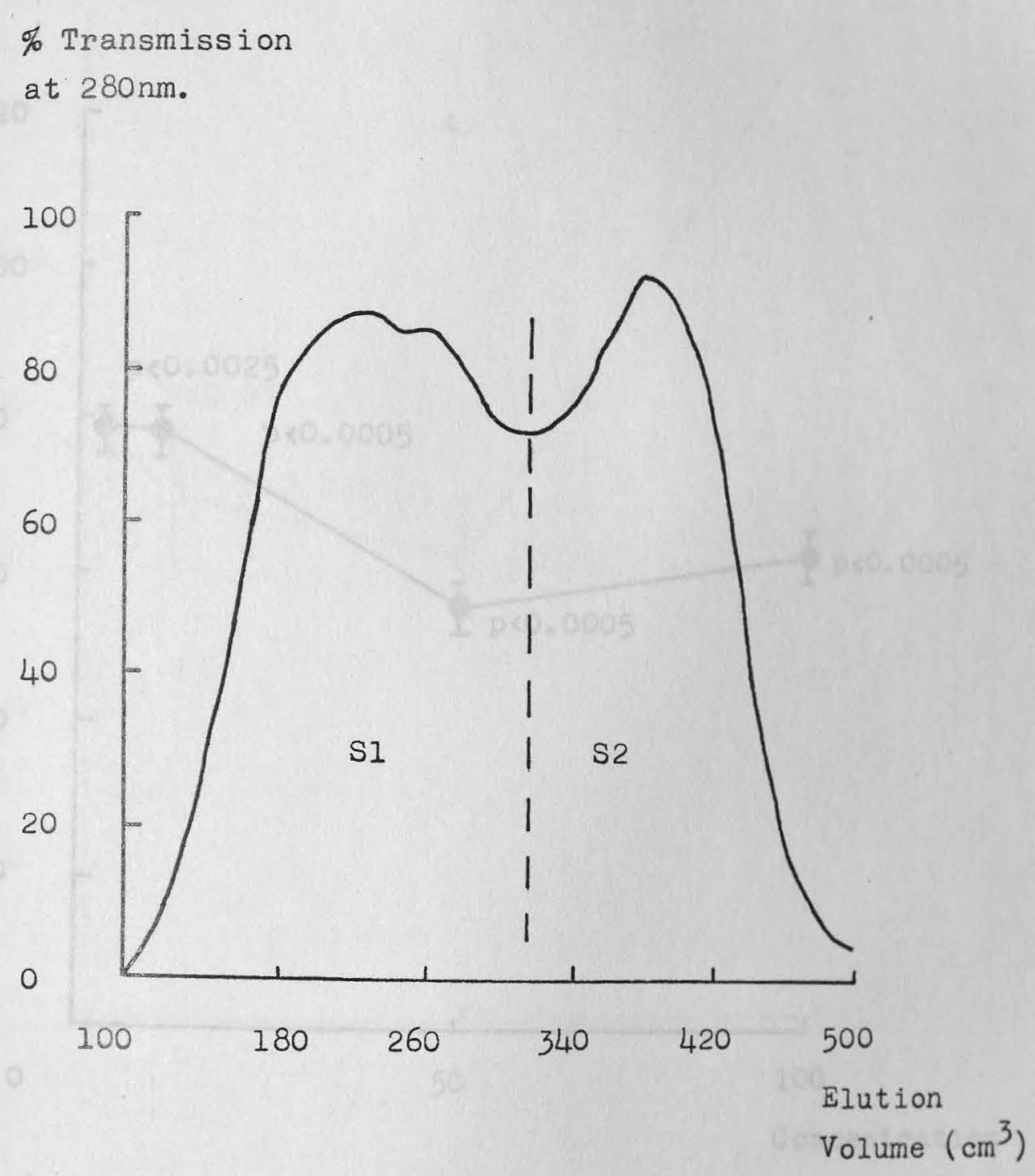


Figure 3.16 The protein elution profile of inflammatory exudate through a Sephadex G-150 column in 0.5M saline. The absorbance at 280 nm. of the eluted proteins was measured. Fractionspooled are indicated by S1 and S2.

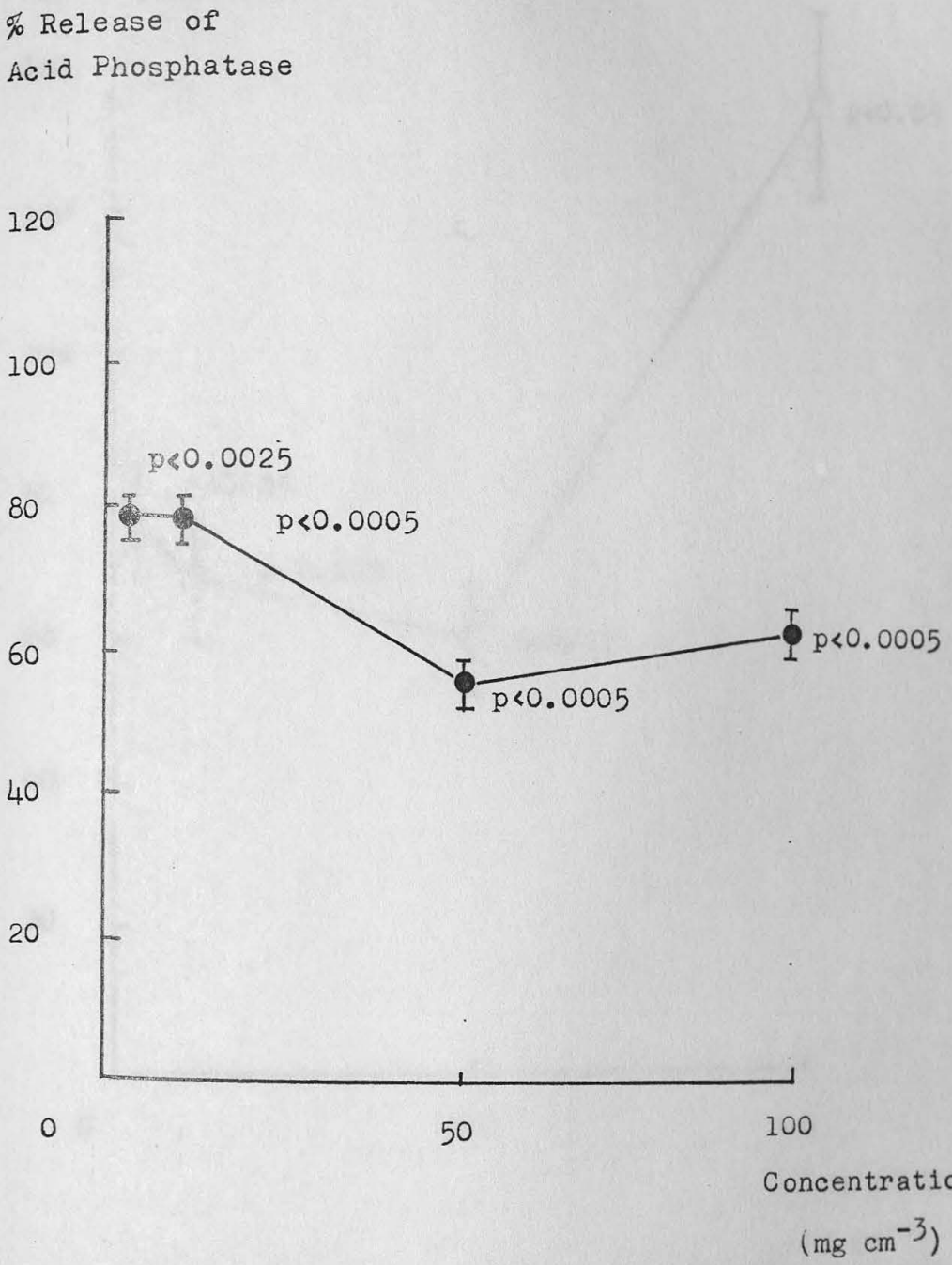


Figure 3.17 The effect of inflammatory exudate on the percentage release of acid phosphatase from macrophages.

Each result represents a mean of four observations ± SEM.

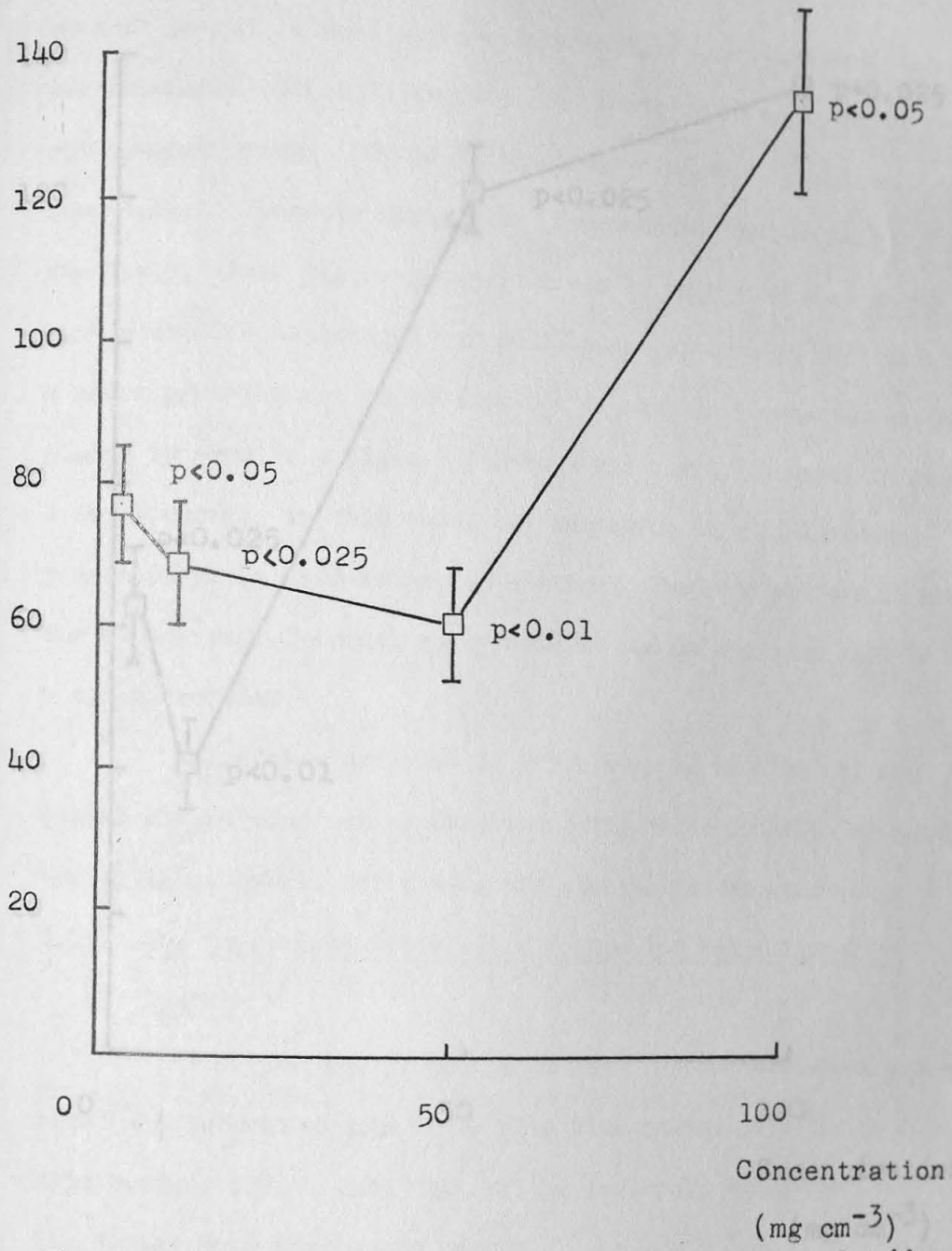


Figure 3.18 The effect of fraction S1 on the release of acid phosphatase from macrophages.

Each result represents a mean of four observations ± SEM.

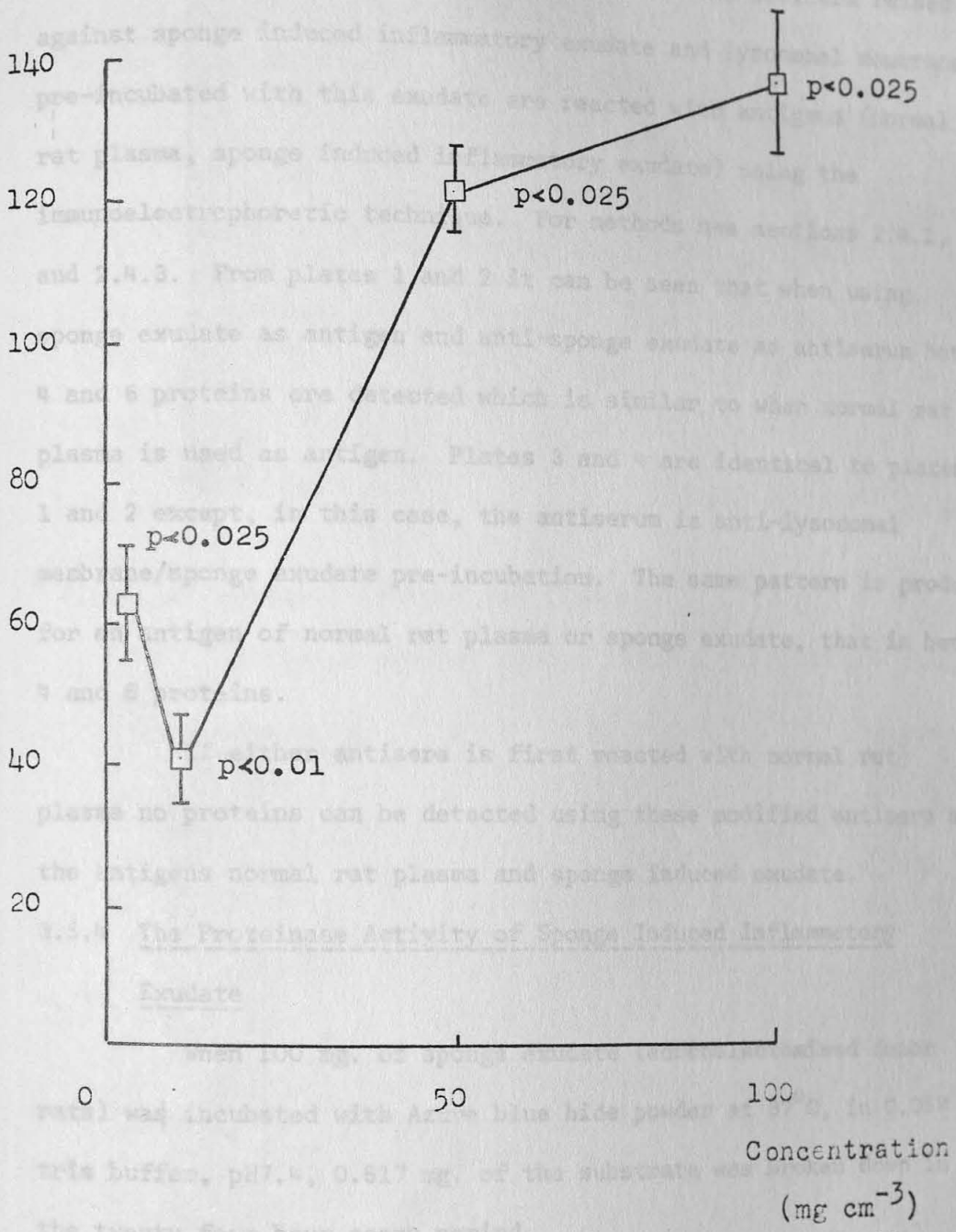


Figure 3.19 The effect of fraction S2 on the release of acid phosphatase from macrophages.

Each result represents a mean of four observations ± SEM.

3.5.3 Production and Use of an Antisera to Sponge Induced Inflammatory Exudate

Plates 1 to 4 show the results when the antisera raised against sponge induced inflammatory exudate and lysosomal membranes pre-incubated with this exudate are reacted with antigens (normal rat plasma, sponge induced inflammatory exudate) using the immunoelectrophoretic technique. For methods see sections 2.4.1, 2.4.2 and 2.4.3. From plates 1 and 2 it can be seen that when using sponge exudate as antigen and anti-sponge exudate as antiserum between 4 and 6 proteins are detected which is similar to when normal rat plasma is used as antigen. Plates 3 and 4 are identical to plates 1 and 2 except, in this case, the antiserum is anti-lysosomal membrane/sponge exudate pre-incubation. The same pattern is produced for an antigen of normal rat plasma or sponge exudate, that is between 4 and 6 proteins.

If either antisera is first reacted with normal rat plasma no proteins can be detected using these modified antisera and the antigens normal rat plasma and sponge induced exudate.

3.5.4 The Proteinase Activity of Sponge Induced Inflammatory Exudate

When 100 mg. of sponge exudate (adrenalectomised donor rats) was incubated with Azure blue hide powder at 37°C, in 0.05M tris buffer, pH7.4, 0.617 mg. of the substrate was broken down in the twenty four hour assay period.

When compared with pooled human rheumatoid synovial fluid this activity is below average (3.02 mg. in 24 hours), but the sponge exudate has a low, but appreciable amount of proteolytic activity.

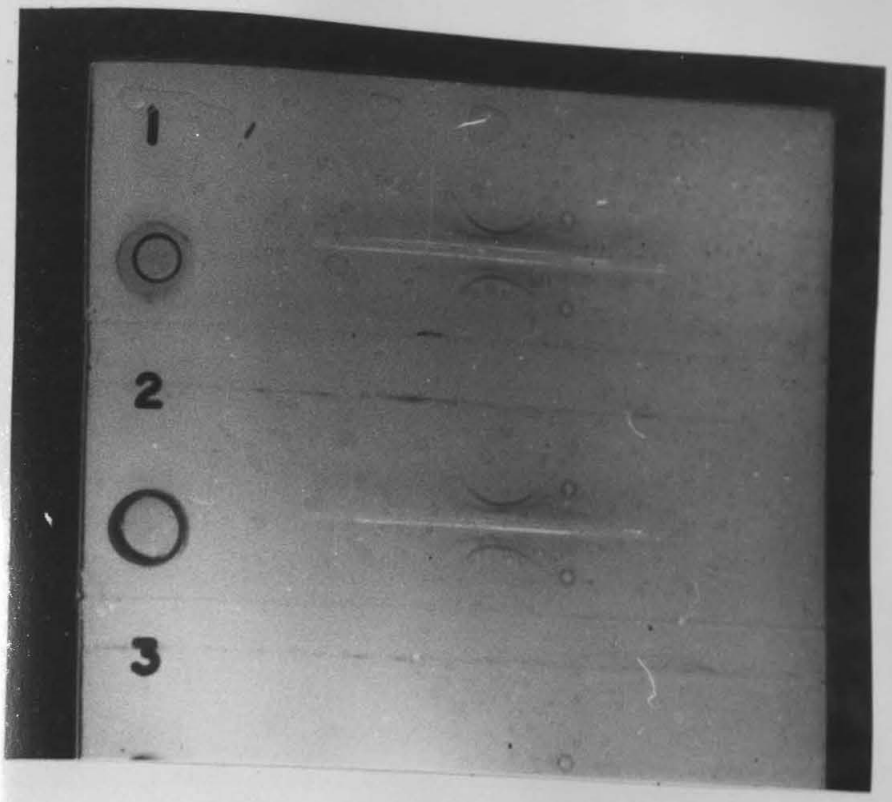


Plate 1. Antigen - Normal Rat Plasma.

Antiserum against sponge induced inflammatory exudate.

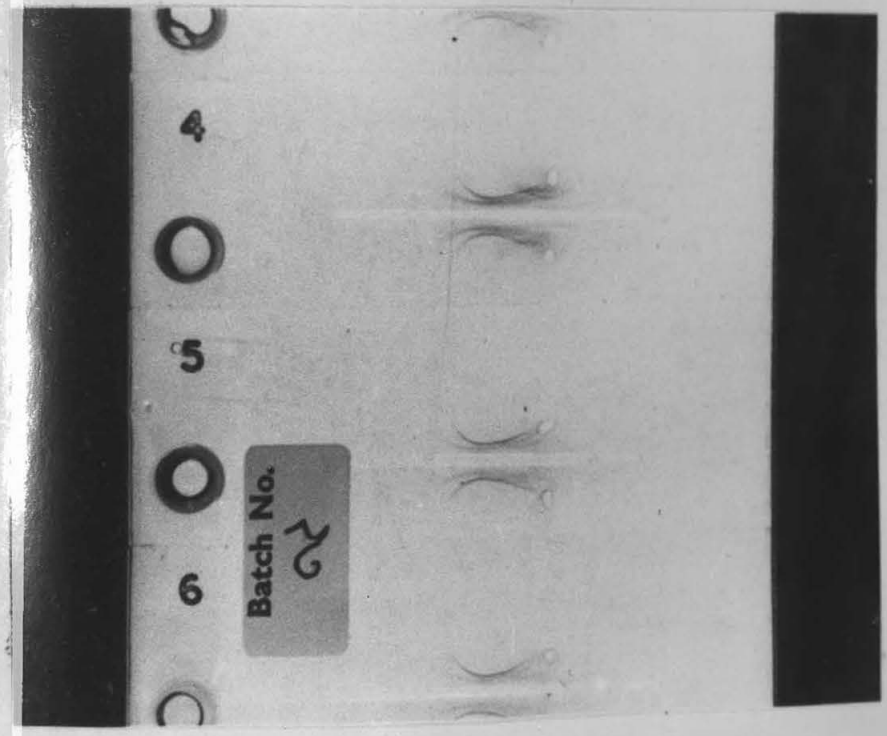


Plate 2. Antigen - Sponge induced inflammatory exudate.

Antiserum - as in Plate 1.

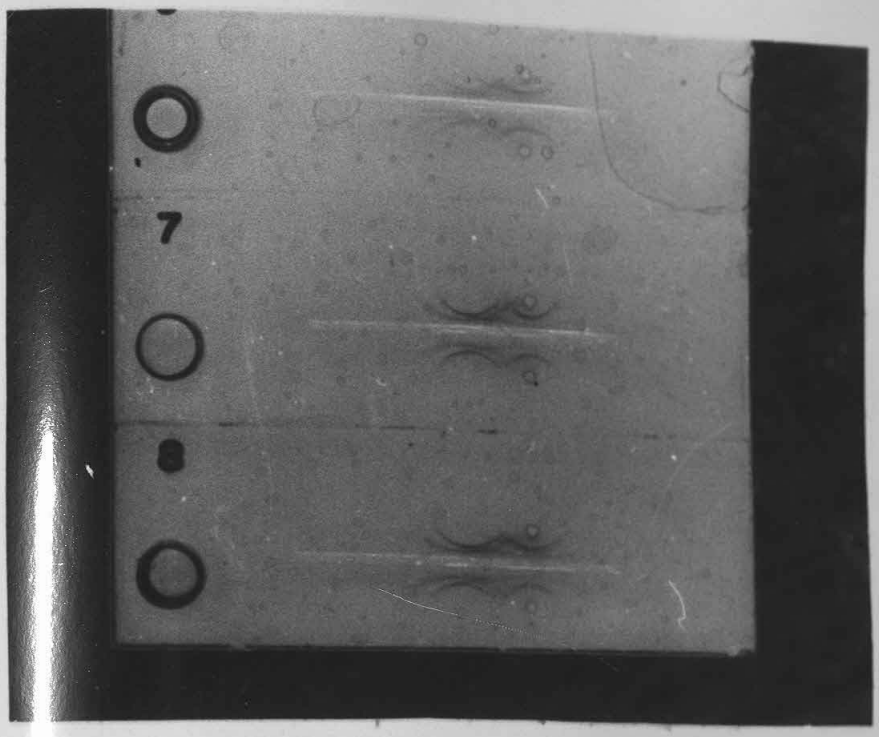


Plate 3. Antigen - Normal Rat Plasma.

Antiserum - Anti-lysosomal membrane/inflammatory exudate.

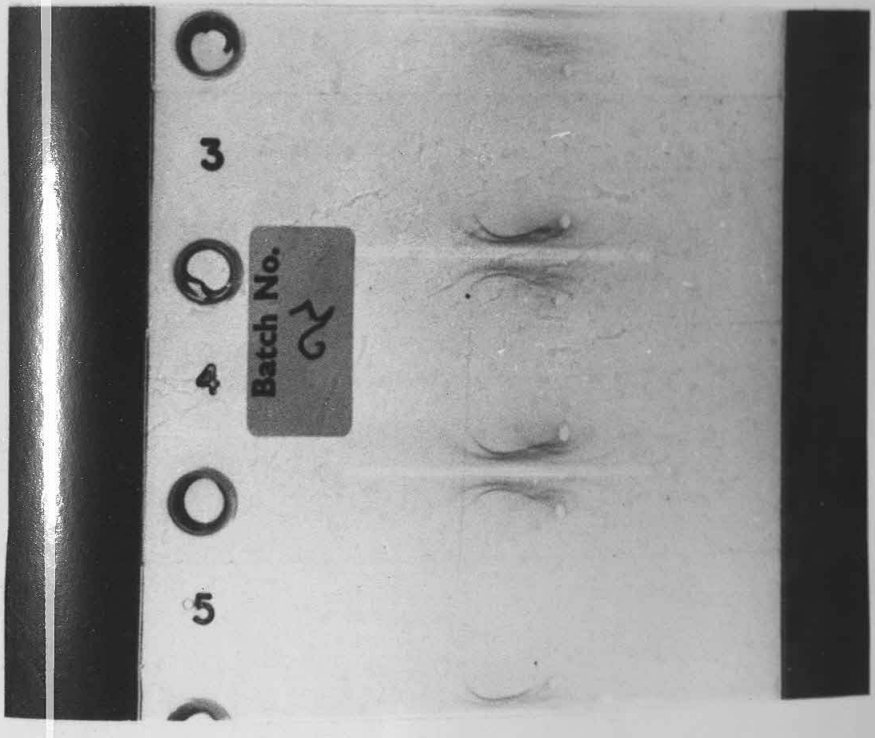


Plate 4. Antigen - Sponge Induced Inflammatory Exudate.

Antiserum - as above in Plate 3.

3.5.5 The Self-digestion of Sponge Induced Inflammatory Exudate In Vitro

Figure 3.20 displays the release of low molecular weight material from dialysis sacs over a period of six hours. The dialysis sacs containing inflammatory exudate alone released low molecular weight fragments at a slow steady rate. The addition of trypsin to the exudate caused a large increase in the rate of release of these fragments, but it must be noted that trypsin itself undergoes some degradation.

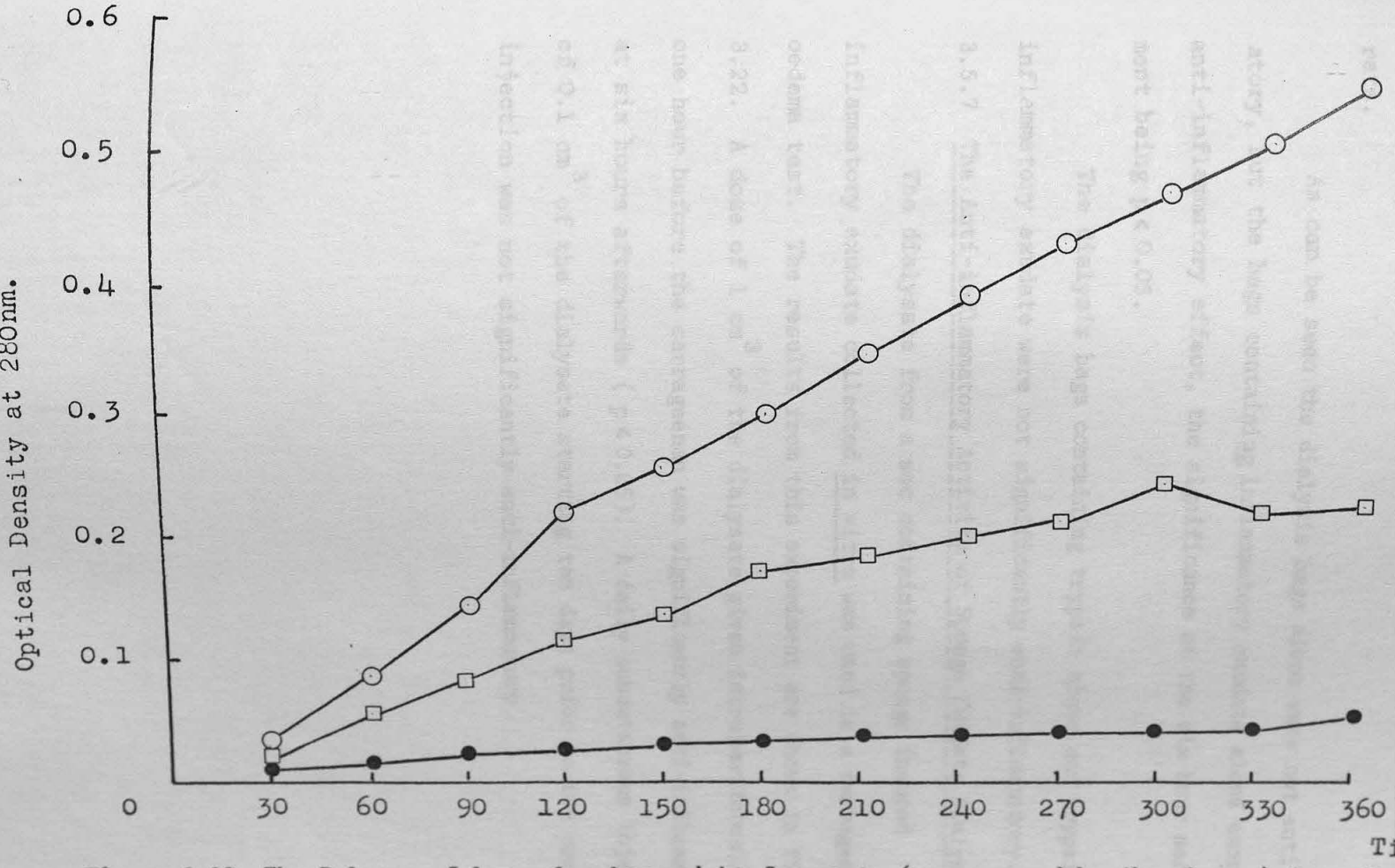


Figure 3.20 The Release of low molecular weight fragments (as measured by Absorbance) from dialysis sacs containing inflammatory exudate alone ●—●, inflammatory exudate and trypsin ○—○, and trypsin alone □—□.

3.5.6 The Release of Dialysable Molecules in Vivo by Sponge Induced Inflammatory Exudate

Figure 3.21 shows the effect of implanted dialysis bags, containing varous substances on carrageenan induced oedema in rats.

As can be seen the dialysis bags alone were not anti-inflammatory, but the bags containing inflammatory exudate alone exerted an anti-inflammatory effect, the significance at the six hour measurement being $p < 0.05$.

The dialysis bags containing trypsin alone and trypsin plus inflammatory exudate were not significantly anti-inflammatory.

3.5.7 The Anti-inflammatory Activity of Sponge Exudate Dialysate

The dialysate from a sac containing sponge induced inflammatory exudate collected *in vitro* was used in a carrageenan oedema test. The results from this experiment are shown in figure 3.22. A dose of 1 cm^3 of the dialysate given intraperitoneally one hour before the carrageenan was significantly anti-inflammatory at six hours afterwards ($p < 0.05$). A daily subcutaneous injection of 0.1 cm^3 of the dialysate starting ten days prior to the carrageenan injection was not significantly anti-inflammatory.

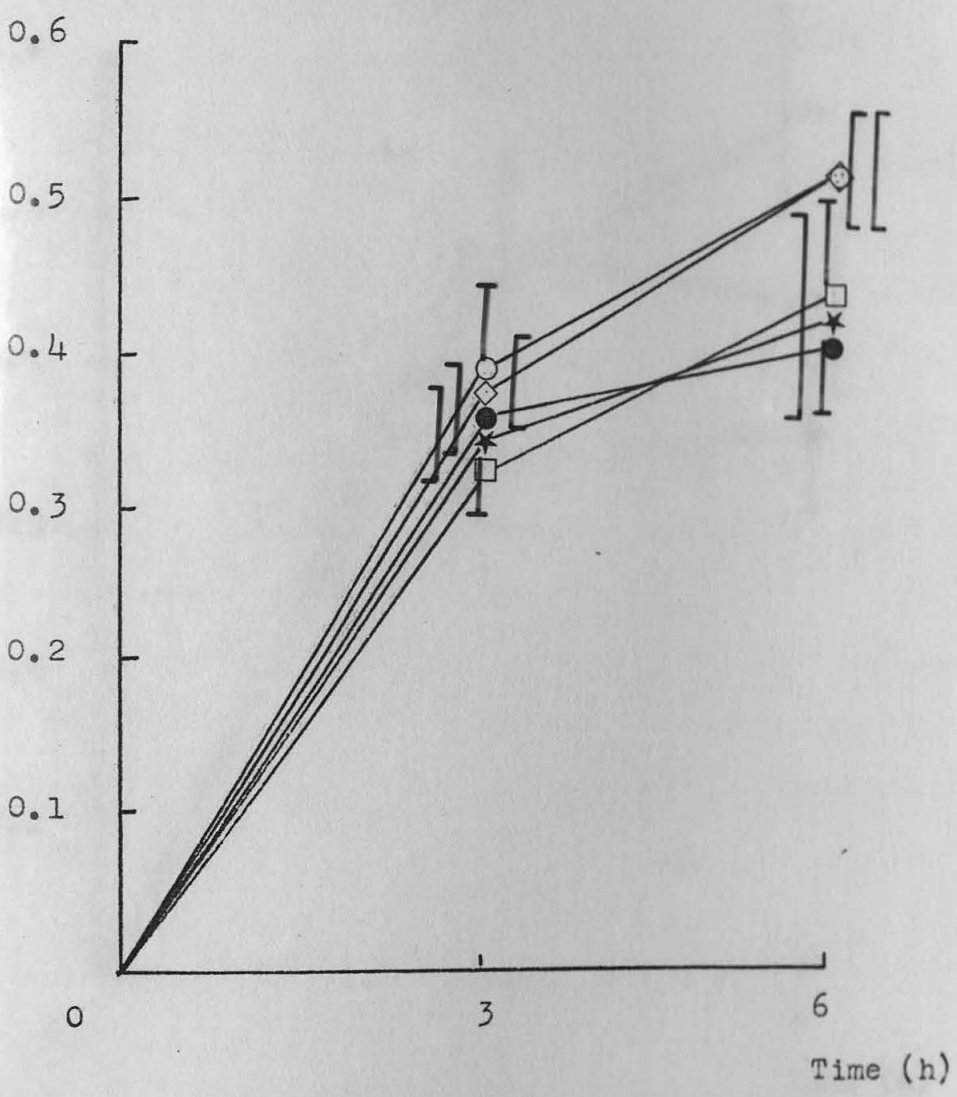


Figure 3.21 The effect on carrageenan induced oedema of implanted dialysis bags containing inflammatory exudate alone ●—●, inflammatory exudate and trypsin ✱—✱, trypsin alone □—□, and saline alone ○—○. Sham operated controls are also shown ◇—◇.

Each results represents the mean of six animals ± SEM

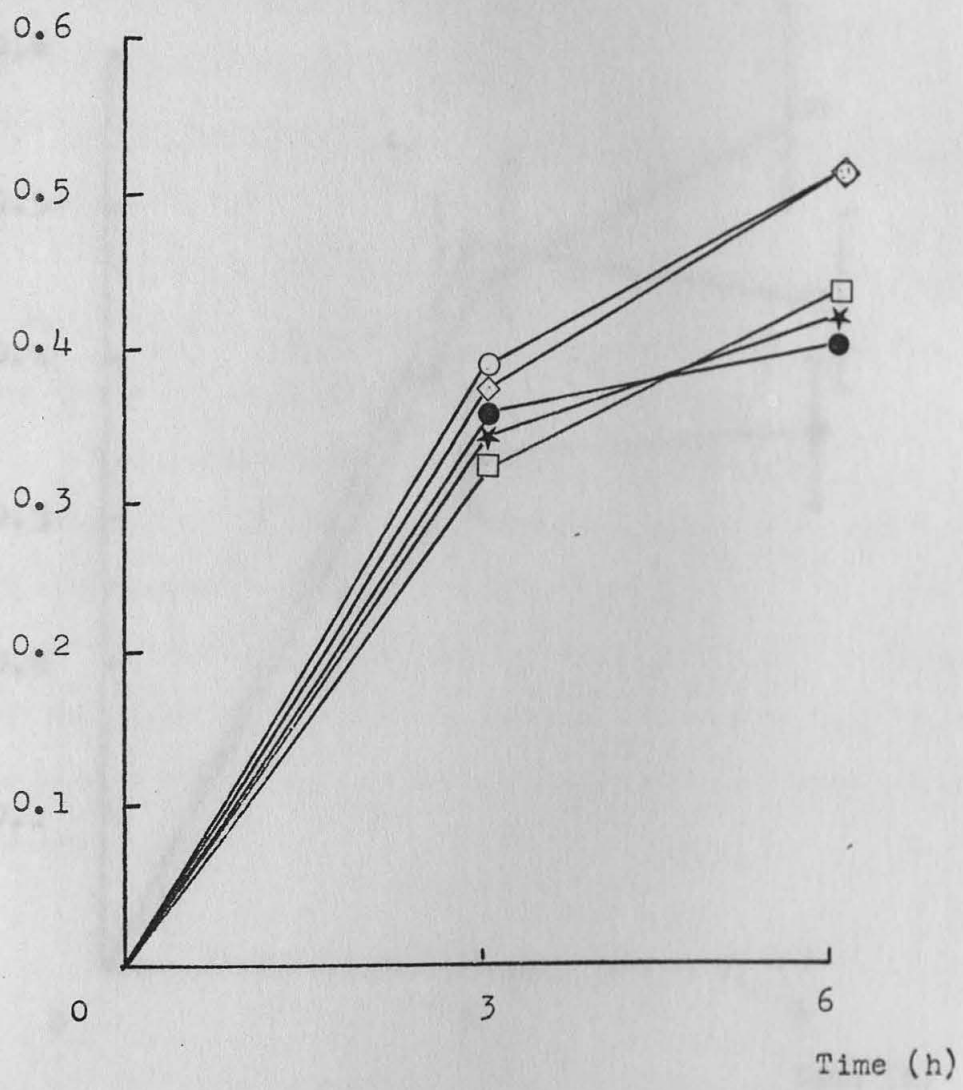


Figure 3.21 The effect on carrageenan induced oedema of implanted dialysis bags containing inflammatory exudate alone ●—●, inflammatory exudate and trypsin ✱—✱, trypsin alone □—□, and saline alone ○—○. Sham operated controls are also shown ◇—◇.

Each results represents the mean of six animals ± SEM

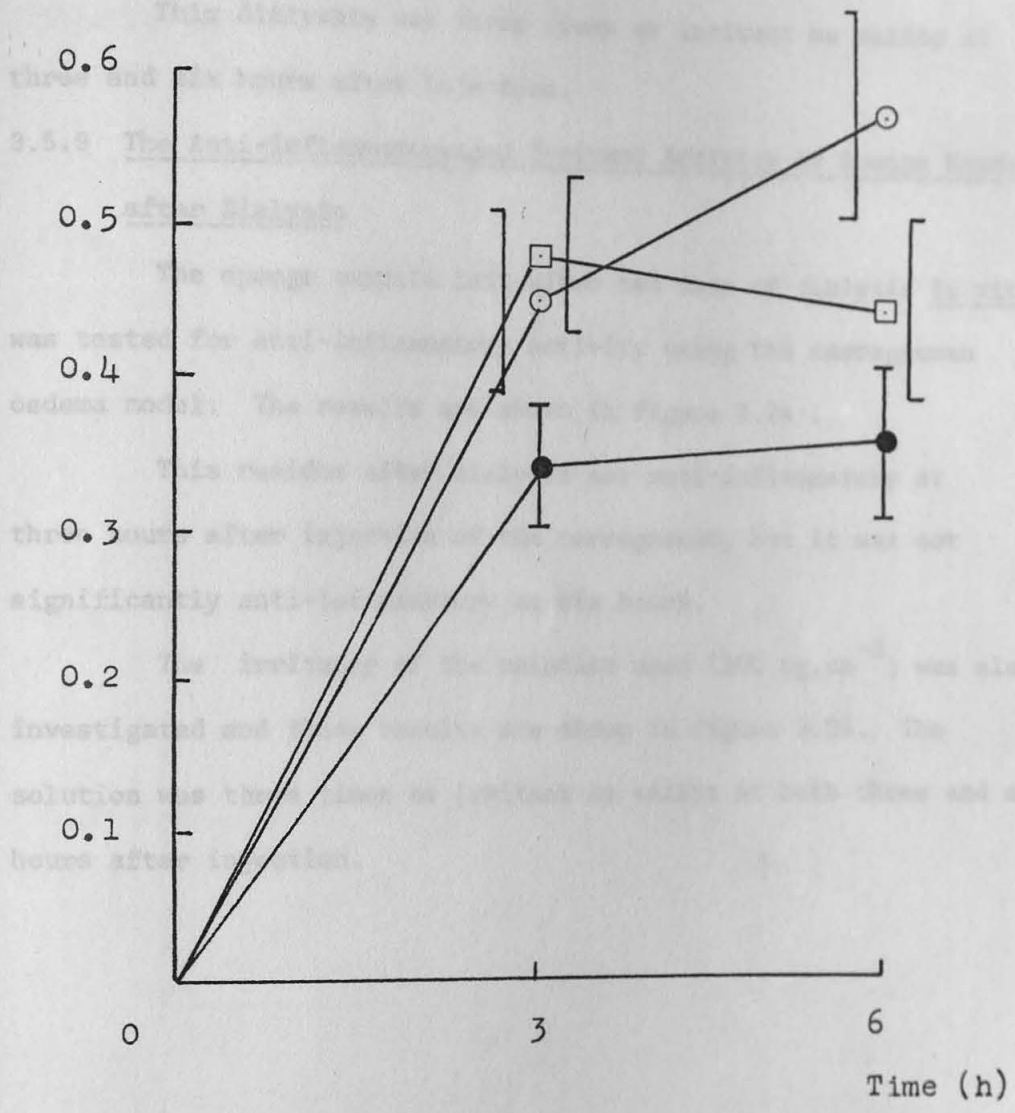


Figure 3.22 The effect on carrageenan induced oedema of a single dose of sponge exudate dialysate ●—● and a multiple dose of dialysate □—□, compared to untreated controls ○—○. Each result represents the mean of seven animals ± SEM.

3.5.8 The Irritancy of Sponge Induced Exudate Dialysate

The sponge exudate dialysate used in the previous experiment was also tested for its irritant activity. The results from this experiment are shown in figure 3.23.

This dialysate was three times as irritant as saline at three and six hours after injection.

3.5.9 The Anti-inflammatory and Irritant Activity of Sponge Exudate after Dialysis

The sponge exudate left after ten days of dialysis *in vitro* was tested for anti-inflammatory activity using the carrageenan oedema model. The results are shown in figure 3.24.

This residue after dialysis was anti-inflammatory at three hours after injection of the carrageenan, but it was not significantly anti-inflammatory at six hours.

The irritancy of the solution used (100 $mg.cm^{-3}$) was also investigated and these results are shown in figure 3.25. The solution was three times as irritant as saline at both three and six hours after injection.

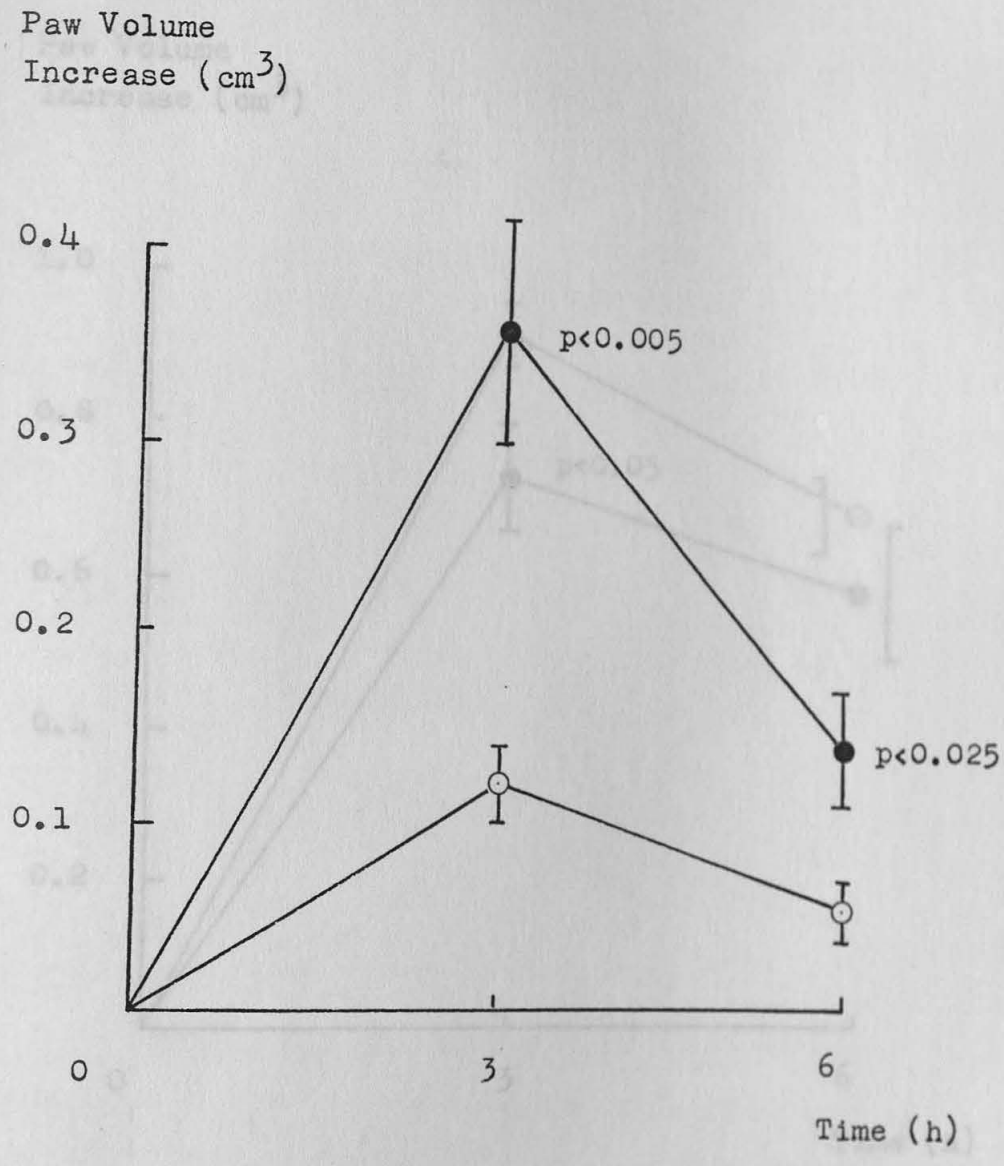


Figure 3.23 Increase in foot volume of rats injected with sponge exudate dialysate ●—● compared to controls injected with saline alone O—O.

Each result represents the mean of seven animals ± SEM.

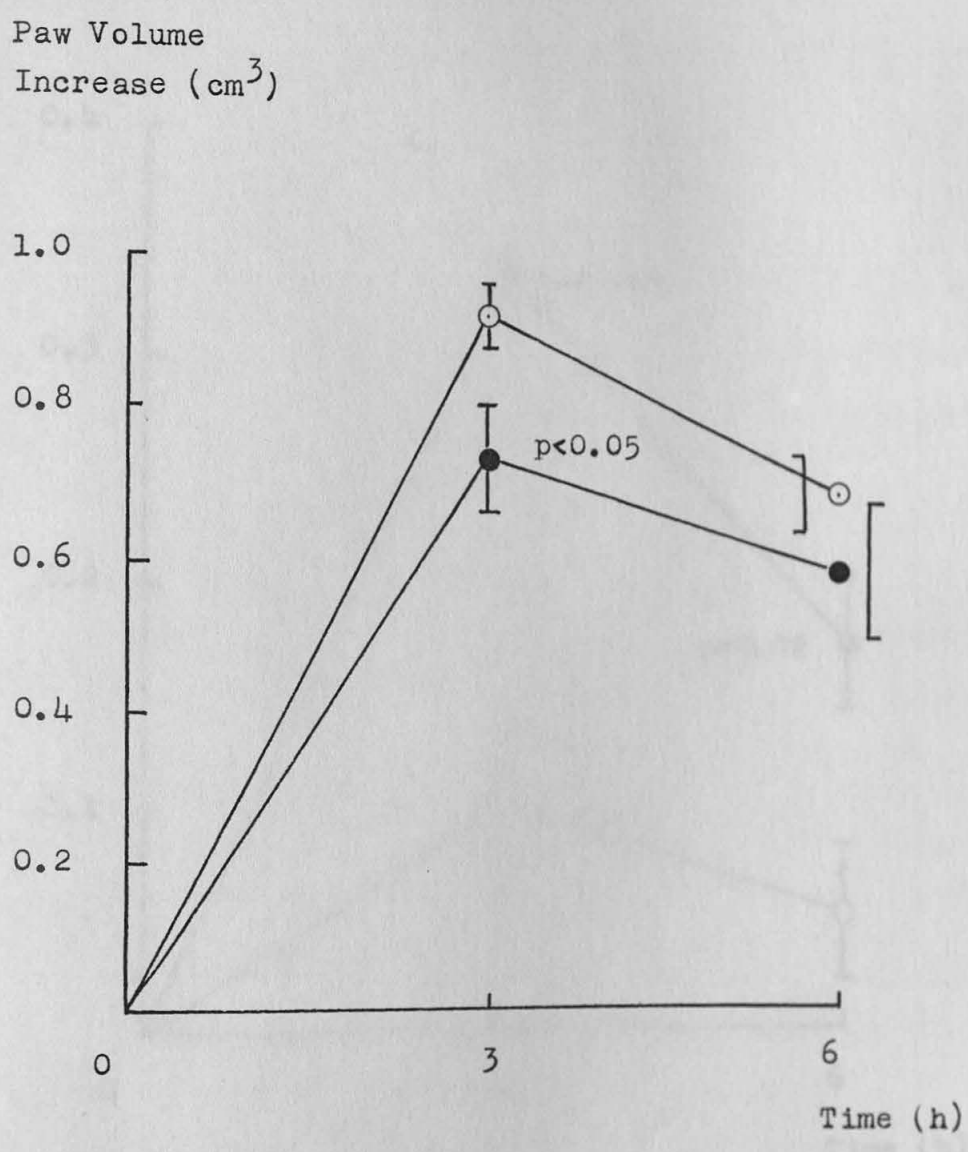


Figure 3.24 The effect on carrageenan induced oedema of dialysed sponge exudate ●—●, compared to saline treated controls ○—○.

Each result represents the mean of six animals ± SEM.

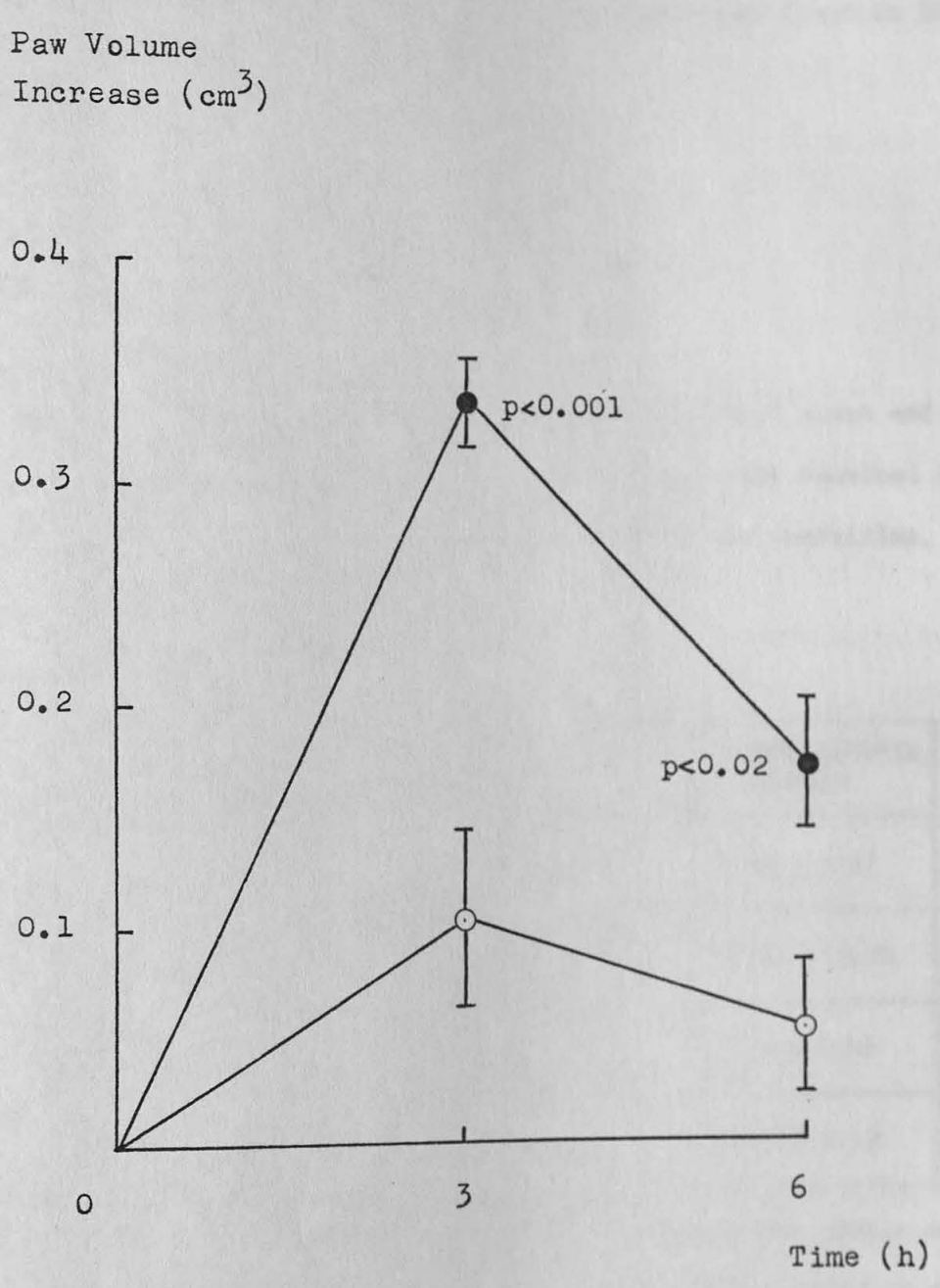


Figure 3.25 The increase in foot volume of rats injected with dialysed sponge exudate ●—●, compared to controls injected with saline alone ○—○.

Each result represents the mean of six animals ± SEM.

3.5.10 The Levels of Oligoamines in Sponge Exudate and Sponge Exudate Dialysate

The following levels of oligoamines were found in 100 mg. dry weight of sponge exudate:-

putrescine 6.76 ± 0.65 µg

cadaverine 19.60 ± 0.14 µg

spermine 17.60 ± 3.91 µg

spermidine 12.70 ± 1.10 µg

The dialysate of sponge exudate (100 mg.) alone and of sponge exudate plus trypsin (100 + 10 mg.) were examined for the presence of putrescine, cadaverine, spermine and spermidine, the results are shown in table 3.10.

Table 3.10.

Amount of oligoamines per cm^3 dialysate	SPONGE EXUDATE	SPONGE EXUDATE + TRYPSIN
µg putrescine	4.21 ± 0.03	5.43 ± 0.82
µg cadaverine	16.19 ± 0.18	17.18 ± 0.19
µg spermine	1.69 ± 0.56	1.16 ± 0.44
µg spermidine	3.03 ± 0.49	3.46 ± 0.19

From table 3.10 trypsin has no significant effect on the levels of putrescine, spermine and spermidine found in the dialysate. The level of cadaverine, however, is significantly elevated ($p < 0.005$).

3.6 The Role of Oligoamines in Inflammation

3.6.1 The Anti-inflammatory Action of Oligoamines on Carrageenan Induced Oedema

The effect of carrageenan induced oedema of the four oligoamines spermine, spermidine, cadaverine and putrescine at dose levels of 50 µg, 500 µg and 5000 µg per Kg. body weight are shown in figures 3.26, 3,27, 3.28 and 3.29 respectively.

Spermine (figure 3.26) shows no significant difference from the saline treated controls at any of the doses used.

Spermidine (figure 3.27) at 50µg per Kg depressed swelling, but the result was not statistically significant. When the dose was increased to 500 µg per Kg. a greater degree of inhibition of oedema was observed, the five hour measurement being significant ($p < 0.05$). A dose of 5000 µg per Kg. was significantly anti-inflammatory at both three ($p < 0.05$) and five ($p < 0.05$) hours.

Cadaverine exhibits slight anti-inflammatory activity. This activity is not dose related and the only statistically significant point is the three hour ($p < 0.05$) measurement using a dose of 50 µg per Kg.

Putrescine was intensely anti-inflammatory against carrageenan induced oedema. At all the doses used, at both three and five hours after injection of the carrageenan, oedema formation was significantly inhibited.

The inhibitory effect of putrescine was not dose dependant.

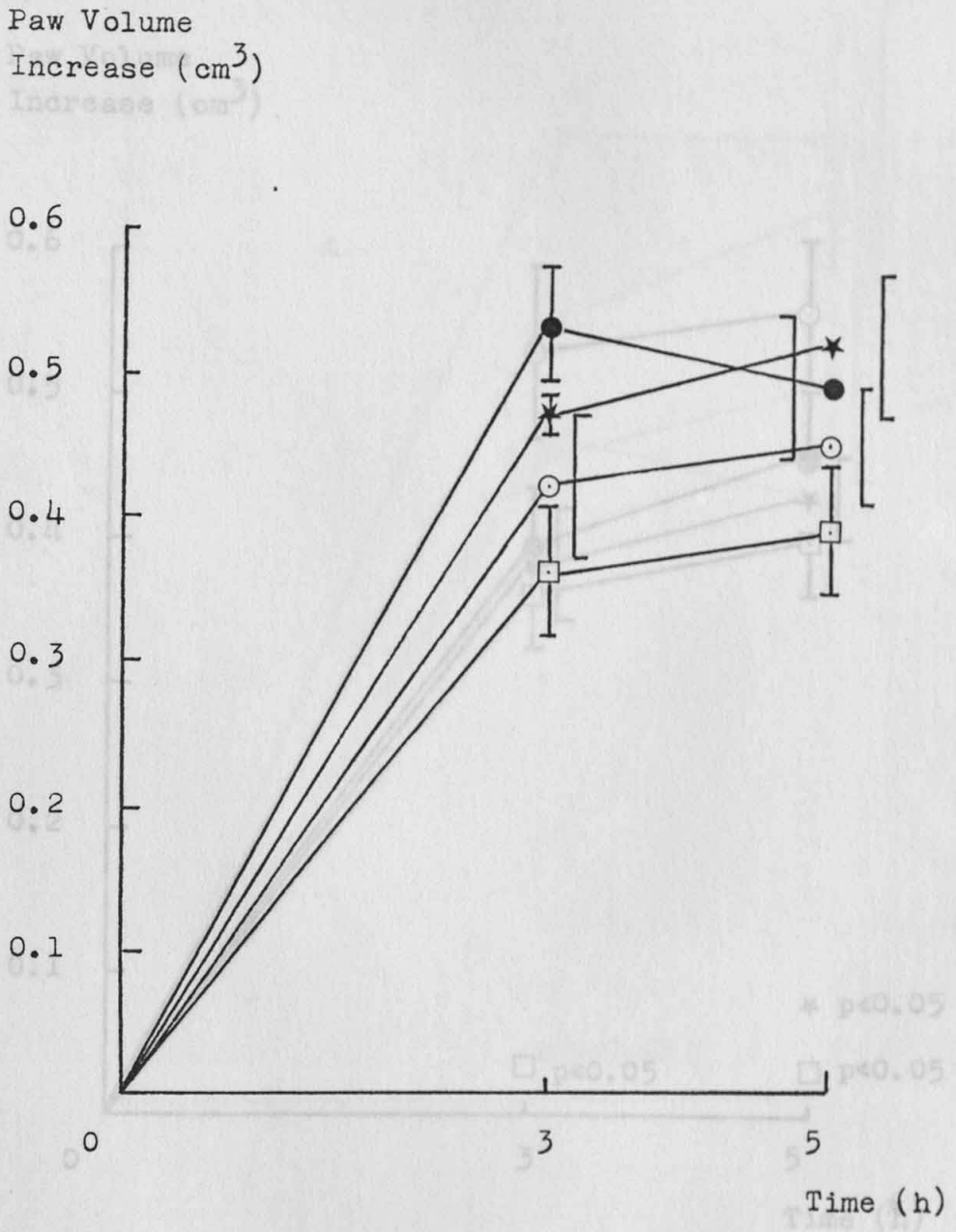


Figure 3.26 The effect on carrageenan induced oedema of spermine given intraperitoneally at doses of 50 µg per Kg ●—●, 500 µg per Kg. ✶—✶ and 5000 µg per Kg □–□. Saline treated controls are also shown ○—○.

Each result represents the mean of five animals ± SEM.

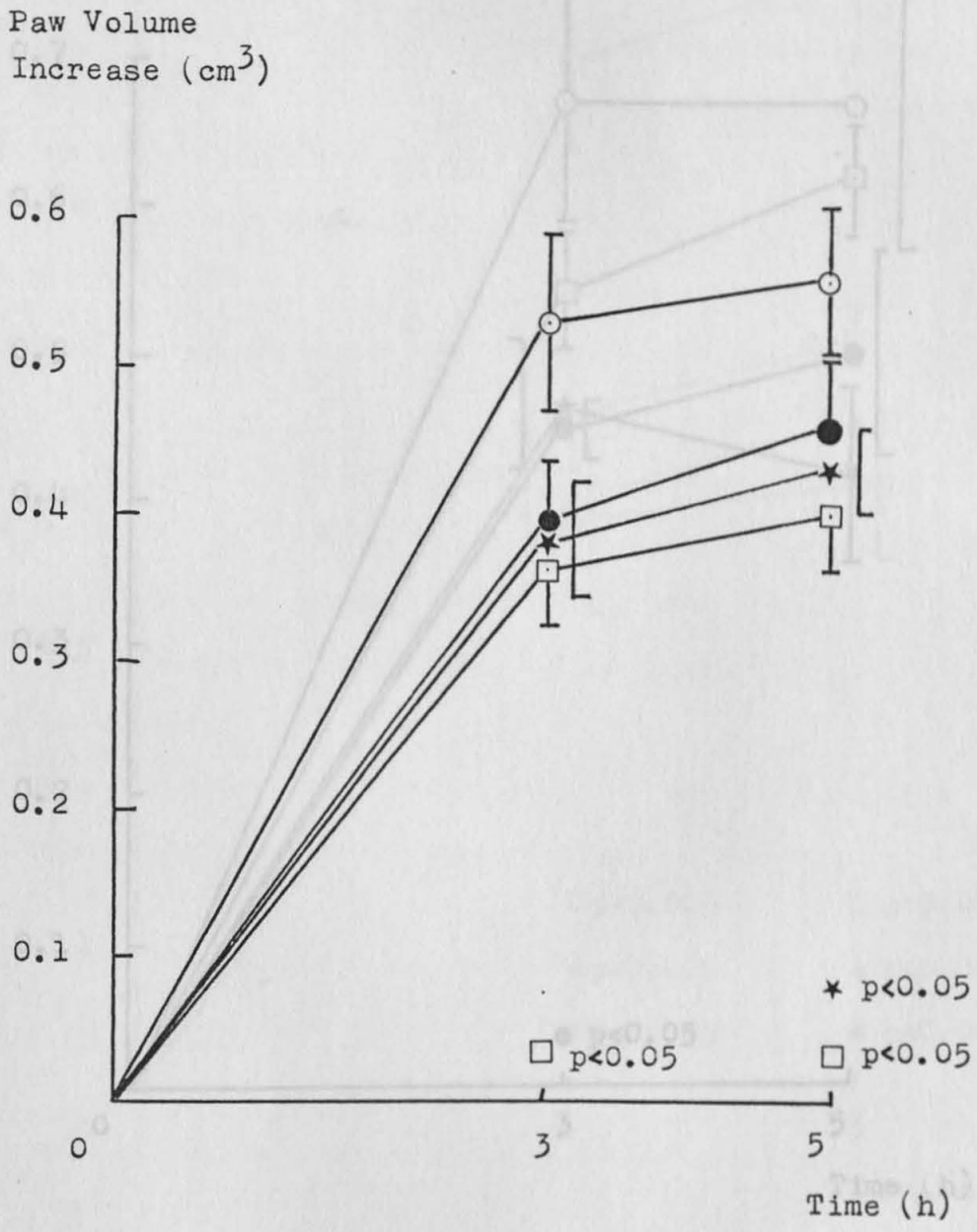


Figure 3.27 The effect on carrageenan induced oedema of spermidine (ip) at doses of 50 μg per Kg •—•, 500 μg per Kg ✶—✶, and 5000 μg per Kg □—□ compared to saline treated controls ○—○.

Each result represents the mean of five animals ± SEM.

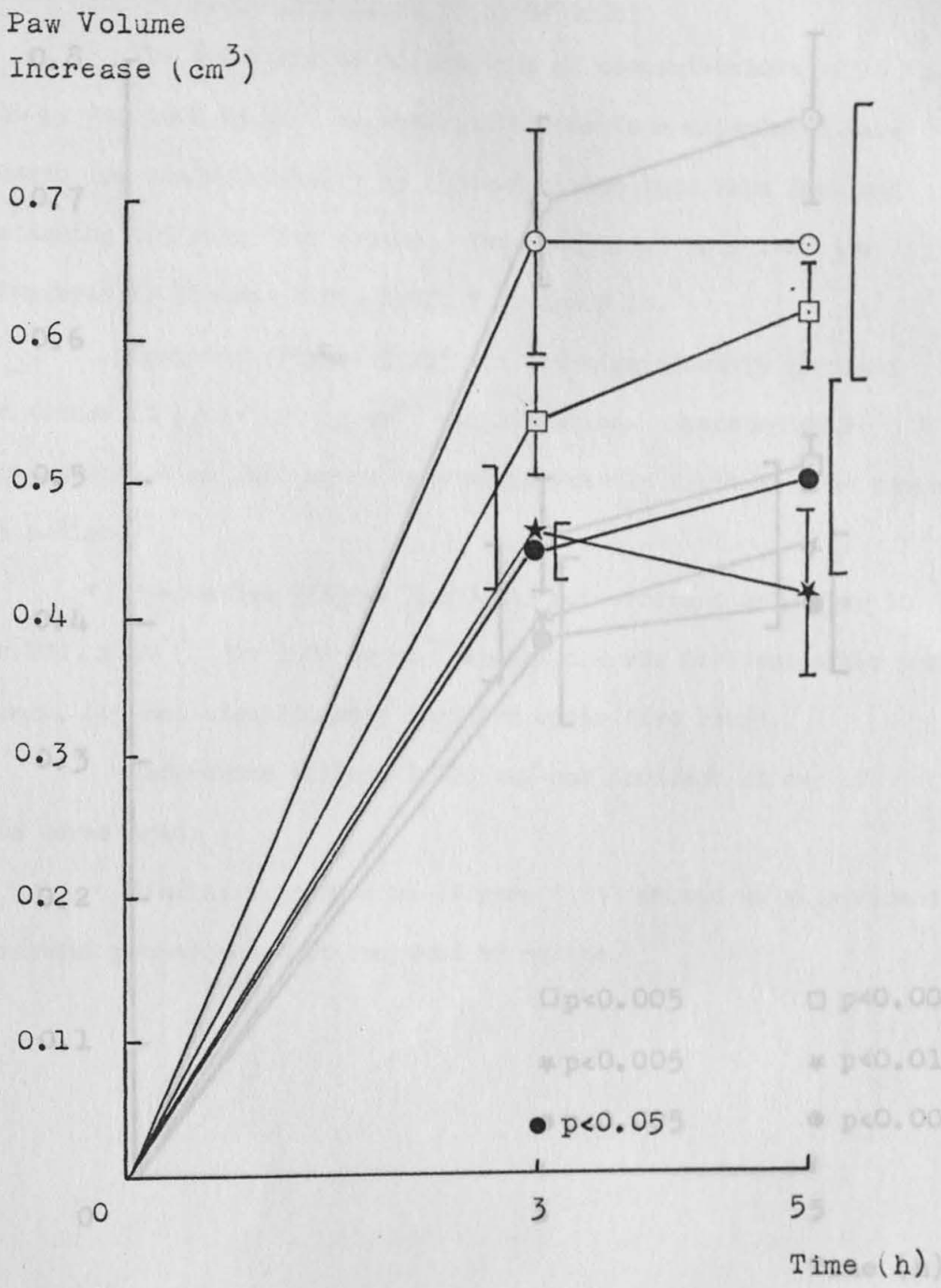


Figure 3.28 The effect on carrageenan induced oedema of cadaverine at doses of 50 µg per Kg ●—●, 500 µg per Kg ✶—✶ and 5000 µg per Kg □——□ compared to saline treated controls

Each result represents the mean of seven animals ± SEM.

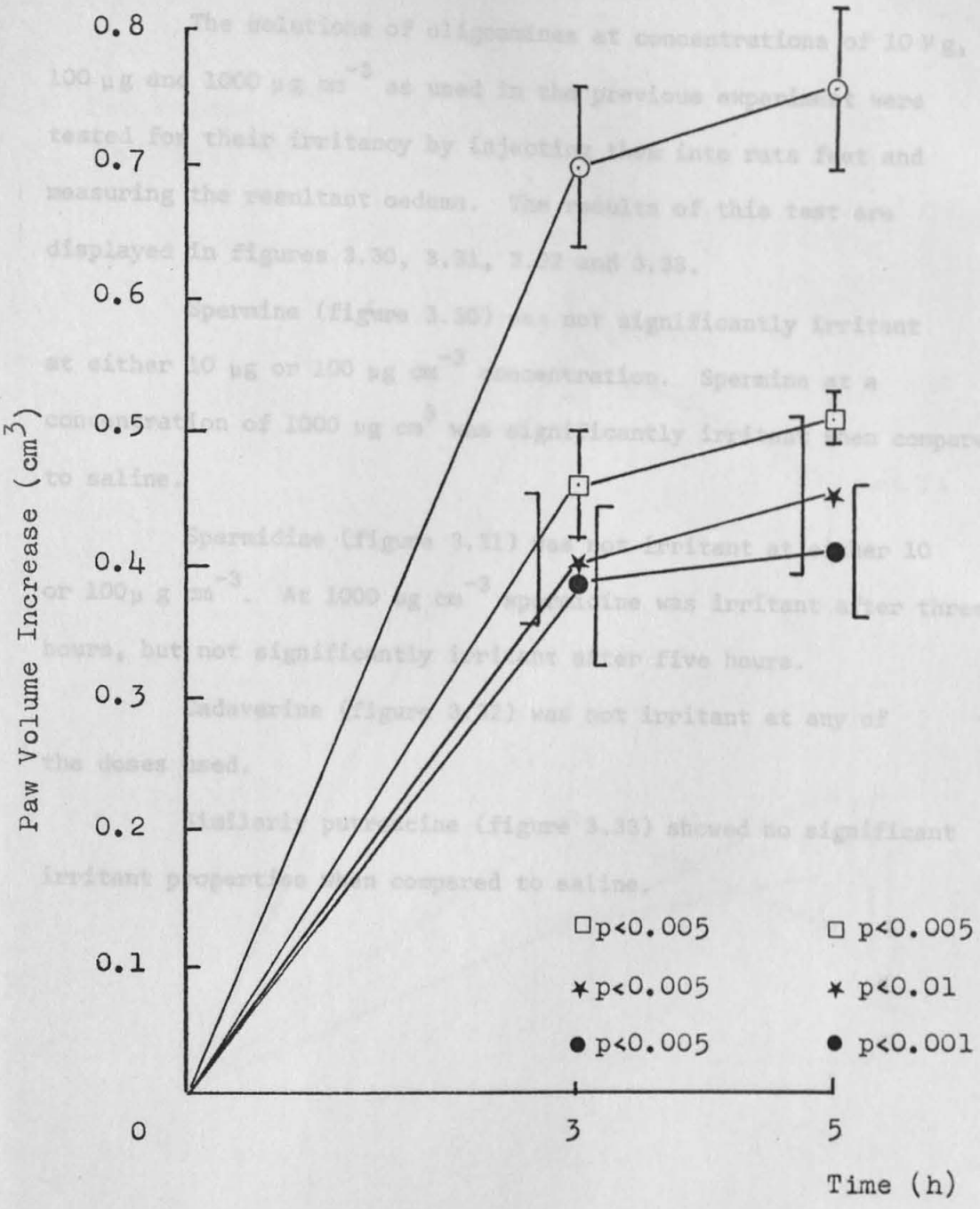


Figure 3.29 The effect on carrageenan induced oedema of putrescine at doses of 50 µg per Kg ●—●, 500 µg per Kg ✱—✱ and 5000 µg per Kg □—□ compared to saline treated controls ○—○.

Each result represents the mean of seven animals ± SEM.

3.6.2 The Irritant Properties of Oligoamines

The solutions of oligoamines at concentrations of 10 µg, 100 µg and 1000 µg cm^{-3} as used in the previous experiment were tested for their irritancy by injecting them into rats feet and measuring the resultant oedema. The results of this test are displayed in figures 3.30, 3.31, 3.32 and 3.33.

Spermine (figure 3.30) was not significantly irritant at either 10 µg or 100 µg cm^{-3} concentration. Spermine at a concentration of 1000 µg cm^{3} was significantly irritant when compared to saline.

Spermidine (figure 3.31) was not irritant at either 10 or 100µ g cm^{-3}. At 1000 µg cm^{-3} spermidine was irritant after three hours, but not significantly irritant after five hours.

Cadaverine (figure 3.32) was not irritant at any of the doses used.

Similarly putrescine (figure 3.33) showed no significant irritant properties when compared to saline.

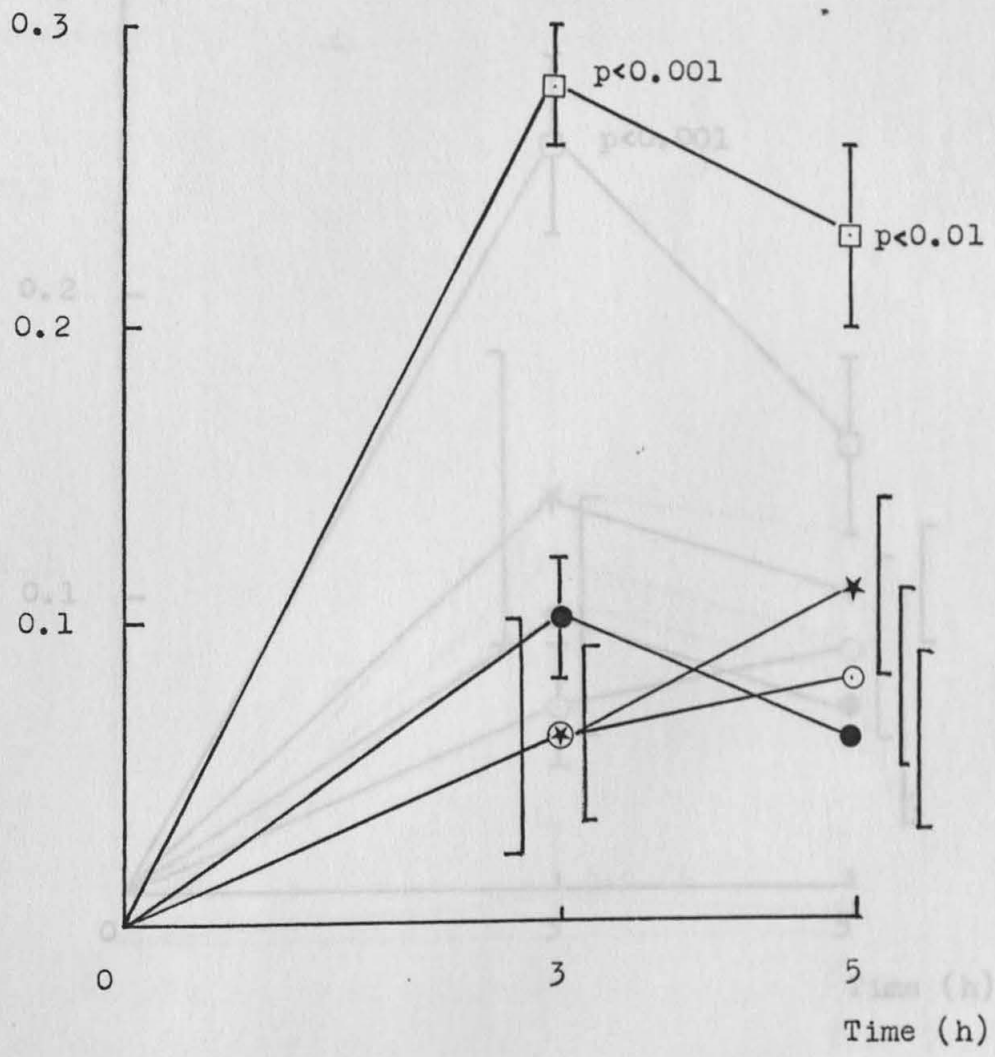


Figure 3.30. Increase in foot volume of rats following injection of spermine at

10 μ g cm^{-3} ●—●

100 μ g cm^{-3} ✶—✶,

and 1000μ g cm^{-3} □—□ compared to saline treated controls ○—○.

Each result represents the mean of five animals ± SEM.

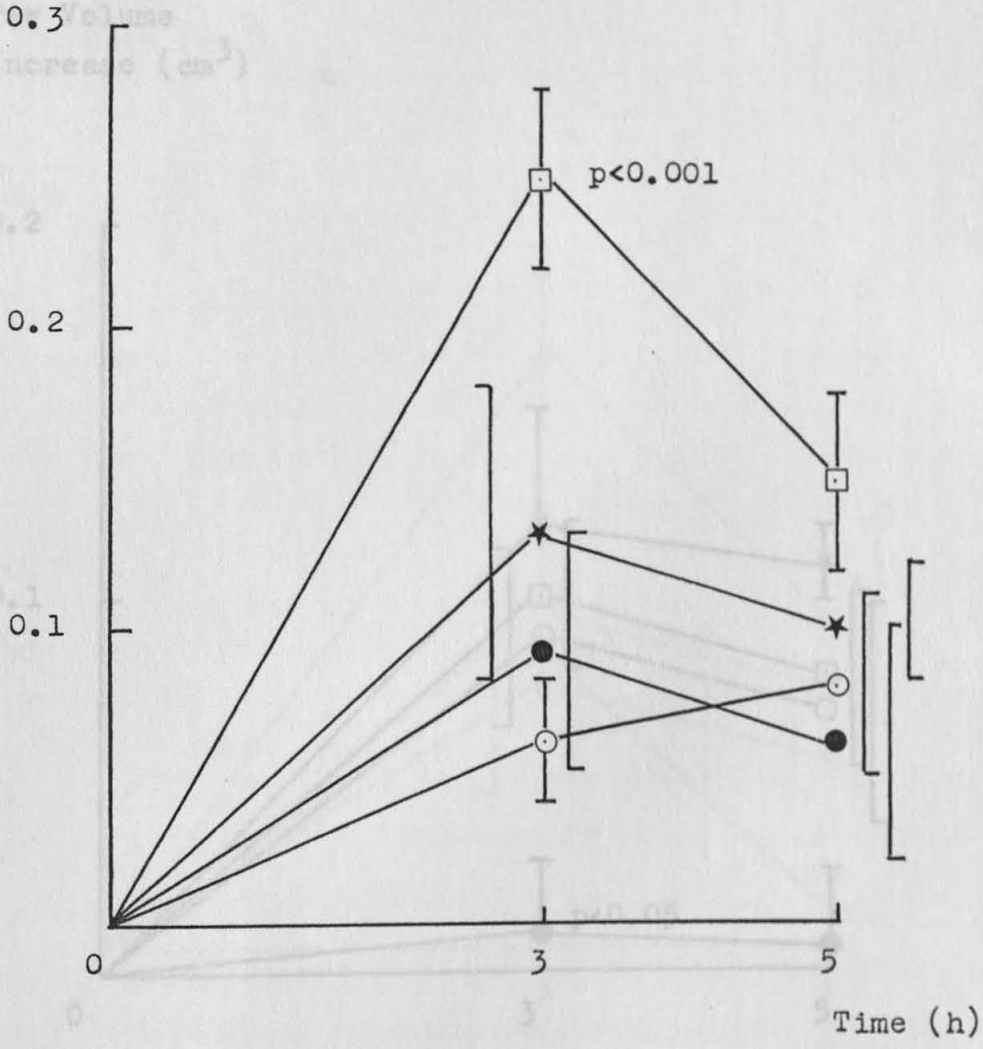


Figure 3.31 Increase in foot volume of rats following injection of spermidine at 10 µg cm^{-3} ●—●, 100 µg cm^{-3} ✱—✱ and 1000µg cm^{-3} □—□ compared to saline treated controls ○—○. Each result represents the mean of five animals ± SEM.

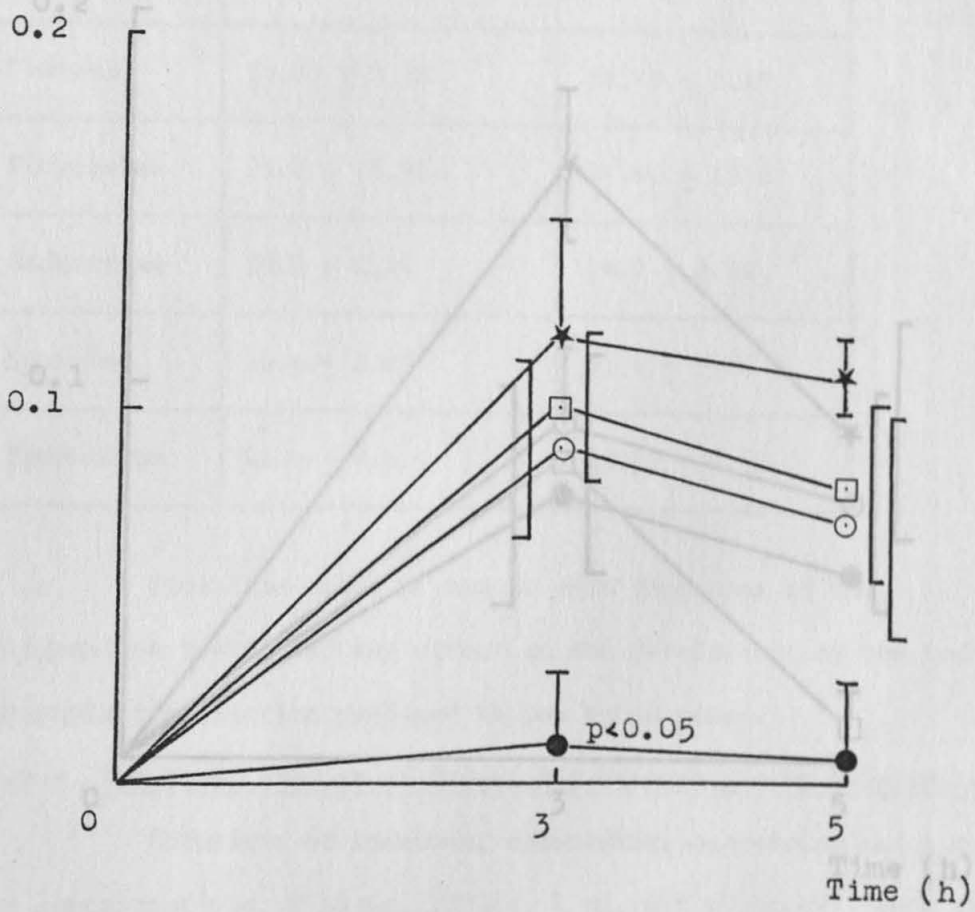


Figure 3.32 Increase in foot volume of rats following injection of cadaverine at 10µgcm^{-3} ●—●, 100 µg cm^{-3} ✱—✱, and 1000 µg cm^{-3} □—□ compared to saline treated controls ○—○.
Each result represents the mean of five animals ± SEM.

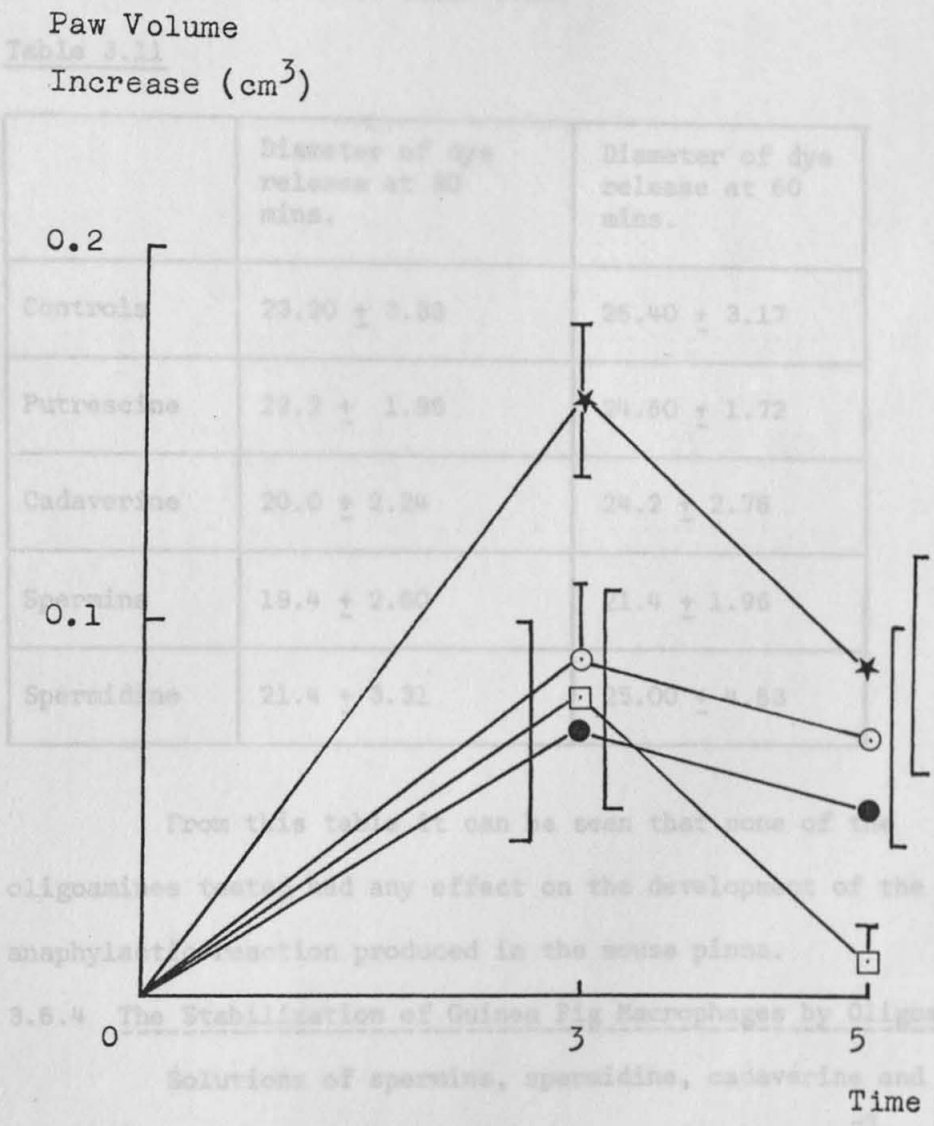


Figure 3.33 Increase in foot volume of rats following injection of putrescine at 10μ g cm^{-3} ●—●, 100μ g cm^{-3} ✶—✶ and 1000μ g cm^{-3} □—□ compared to saline treated controls ○—○. Each result represents the mean of five animals ± SEM.

3.6.3 The Effect of Oligoamines on Pinnal Anaphylaxis in the Mouse

The effect upon the development of a local anaphylactic reaction in the mouse pinna was investigated using doses of 50 µg per Kg of the oligoamines spermine, spermidine, cadaverine and putrescine. The results are shown in table 3.11.

Table 3.11

	Diameter of dye release at 30 mins.	Diameter of dye release at 60 mins.
Controls	23.20 ± 3.83	26.40 ± 3.17
Putrescine	22.2 ± 1.96	24.60 ± 1.72
Cadaverine	20.0 ± 2.24	24.2 ± 2.76
Spermine	19.4 ± 2.60	21.4 ± 1.96
Spermidine	21.4 ± 3.31	25.00 ± 4.63

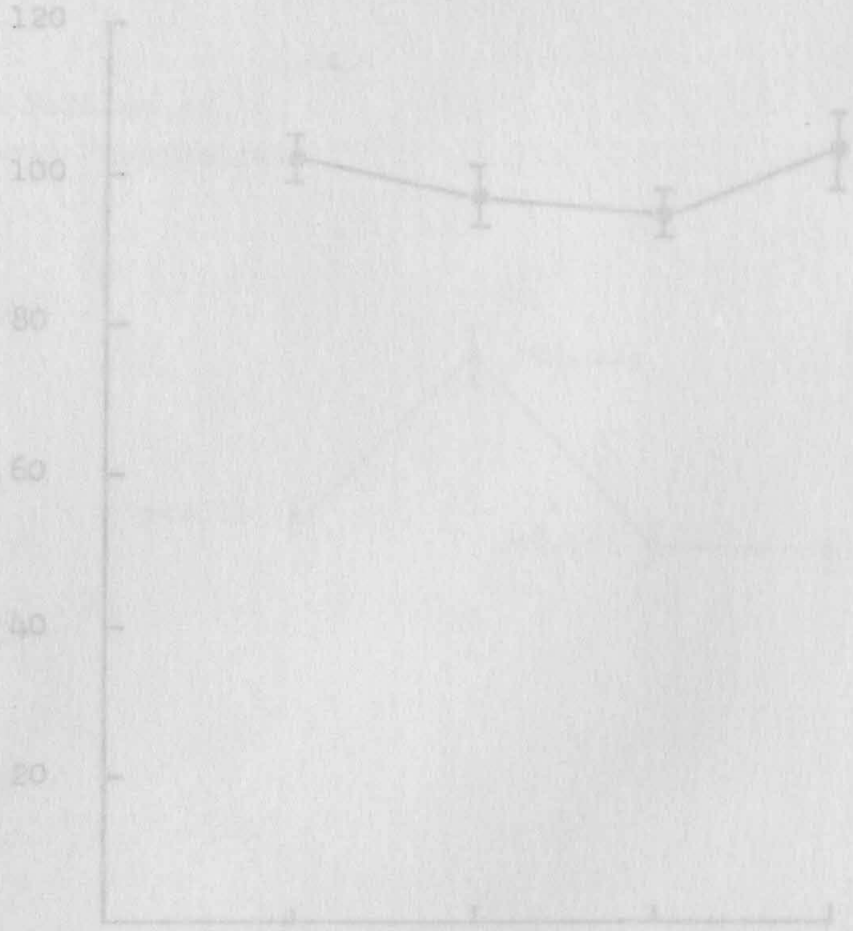
From this table it can be seen that none of the oligoamines tested had any effect on the development of the local anaphylactic reaction produced in the mouse pinna.

3.6.4 The Stabilization of Guinea Pig Macrophages by Oligoamines

Solutions of spermine, spermidine, cadaverine and putrescine at concentrations of 10 µg, 100 µg, 1 mg. and 10 $mg.cm^{-3}$ were tested for their ability to stabilize guinea-pig macrophages.

Figure 3.34 shows the effect of spermine on these cells. In this case spermine had no effect at the concentrations used. Spermidine (figure 3.35) exhibits contrasting effects at different concentrations. At the lower concentrations used it was lytic while at the highest dose spermidine had no significant effect. Cadaverine (figure 3.36) had no effect at the lower concentrations used, but

had a stabilizing effect at the highest concentration used. Putrescine (figure 3.37) behaved in the same fashion, that is it had no effect at the lower concentrations but stabilized at the highest concentration used.



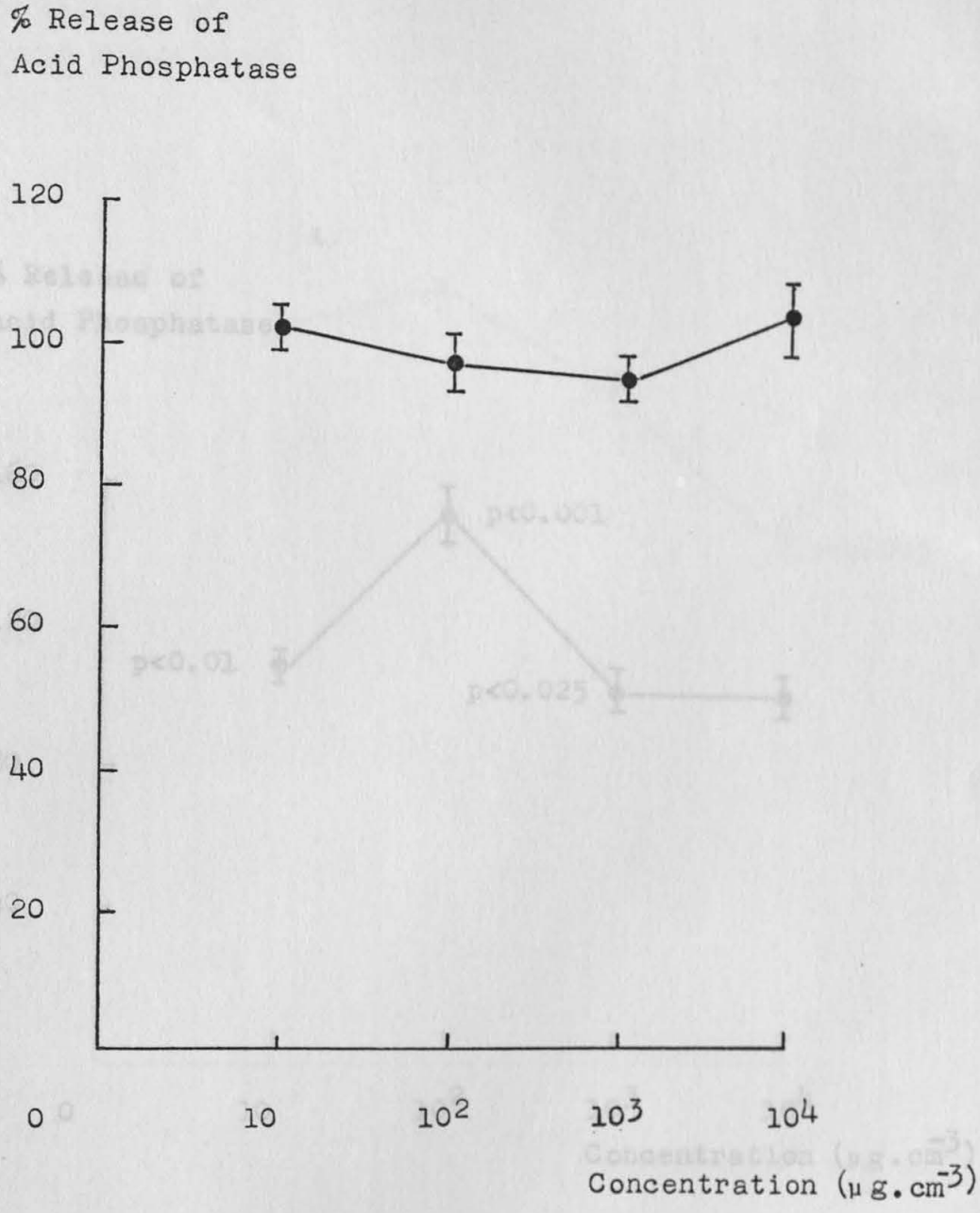


Figure 3.34 The effect of spermine on the release of acid phosphatase from macrophages.

Each result represents the mean of four observations ± SEM.

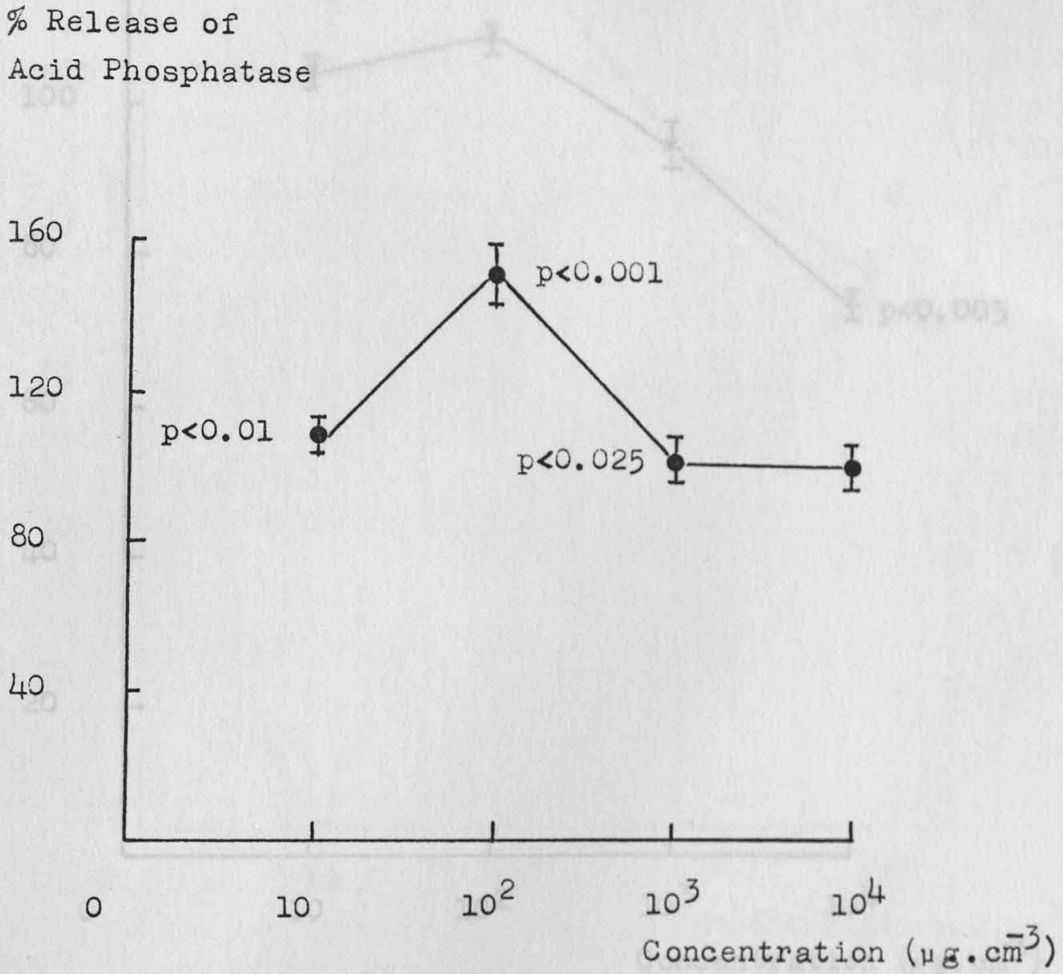


Figure 3.35 The effect of spermidine on the release of acid phosphatase from macrophages.

Each result represents the mean of four observations ± SEM.

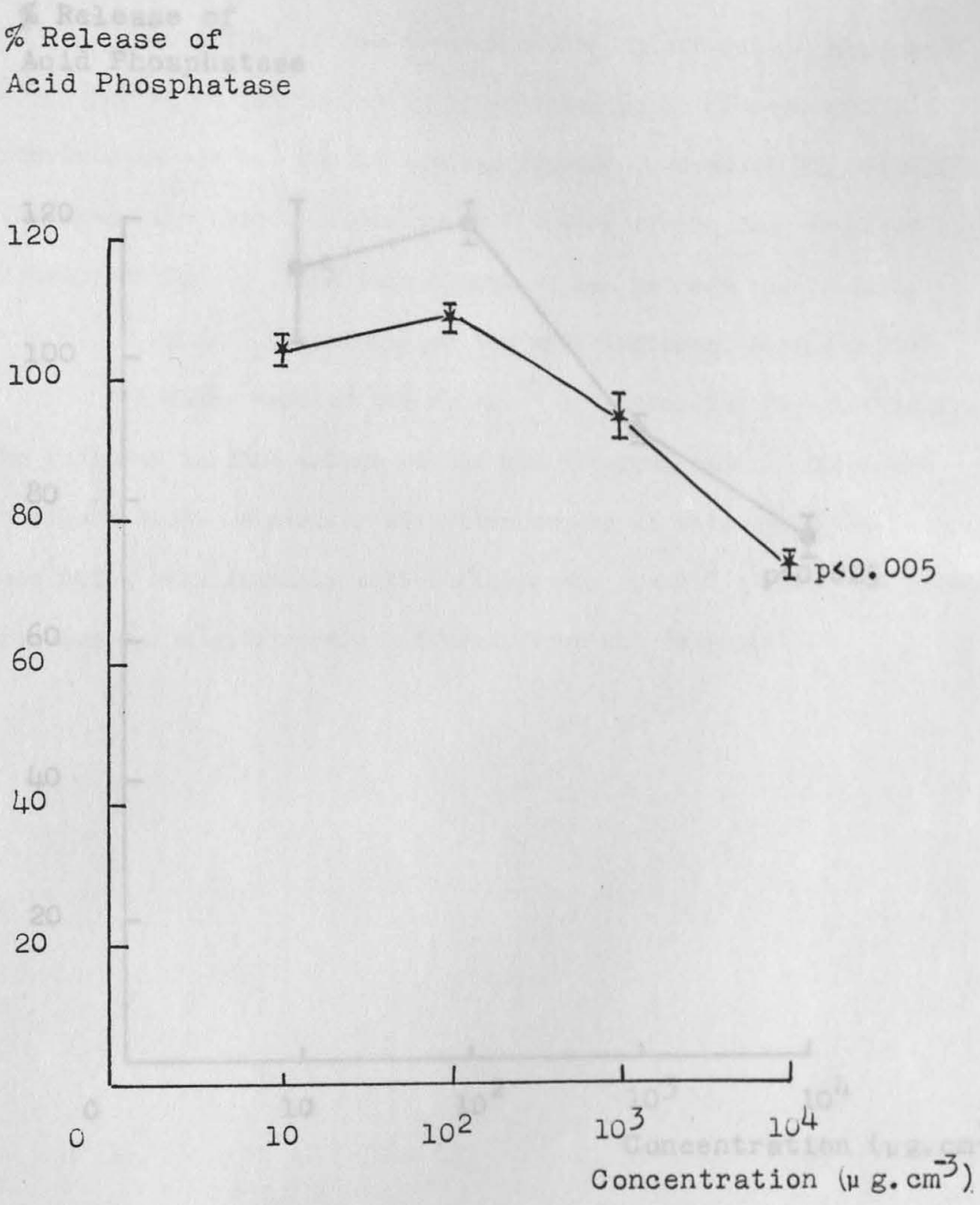


Figure 3.36 The effect of cadaverine on the release of acid phosphatase from macrophages.

Each result represents the mean of four observations ± SEM.

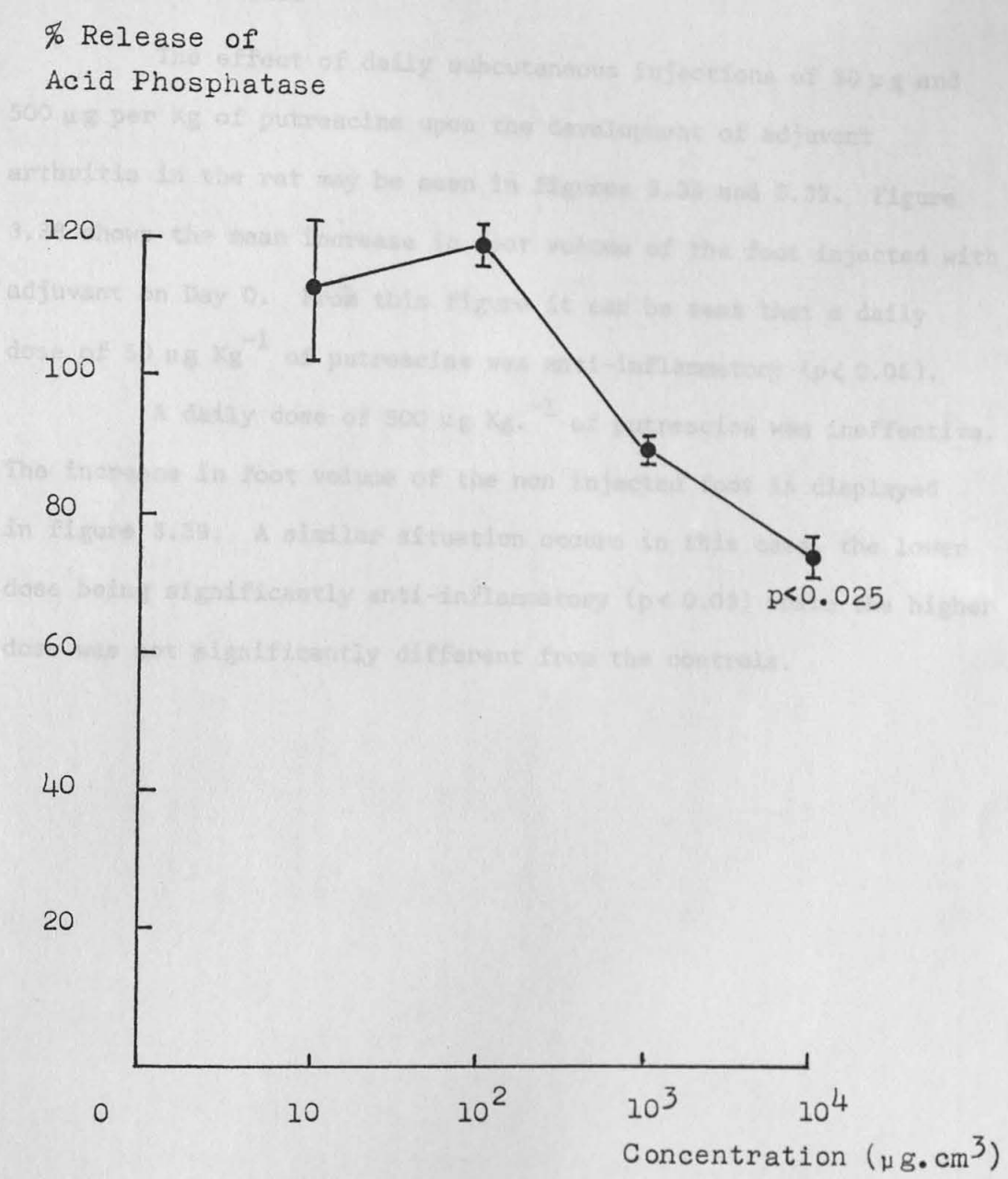


Figure 3.37 The effect of putrescine on the release of acid phosphatase from macrophages.

Each result represents the mean of four observations ± SEM.

3.6.5 The Anti-Inflammatory Action of Putrescine in the Adjuvant Arthritic Rat

The effect of daily subcutaneous injections of 50 μg and 500 μg per Kg of putrescine upon the development of adjuvant arthritis in the rat may be seen in figures 3.38 and 3.39. Figure 3.38 shows the mean increase in foot volume of the foot injected with adjuvant on Day 0. From this figure it can be seen that a daily dose of 50 μg Kg^{-1} of putrescine was anti-inflammatory ($p < 0.05$).

A daily dose of 500 μg $Kg.^{-1}$ of putrescine was ineffective. The increase in foot volume of the non injected foot is displayed in figure 3.39. A similar situation occurs in this case, the lower dose being significantly anti-inflammatory ($p < 0.05$) while the higher dose was not significantly different from the controls.

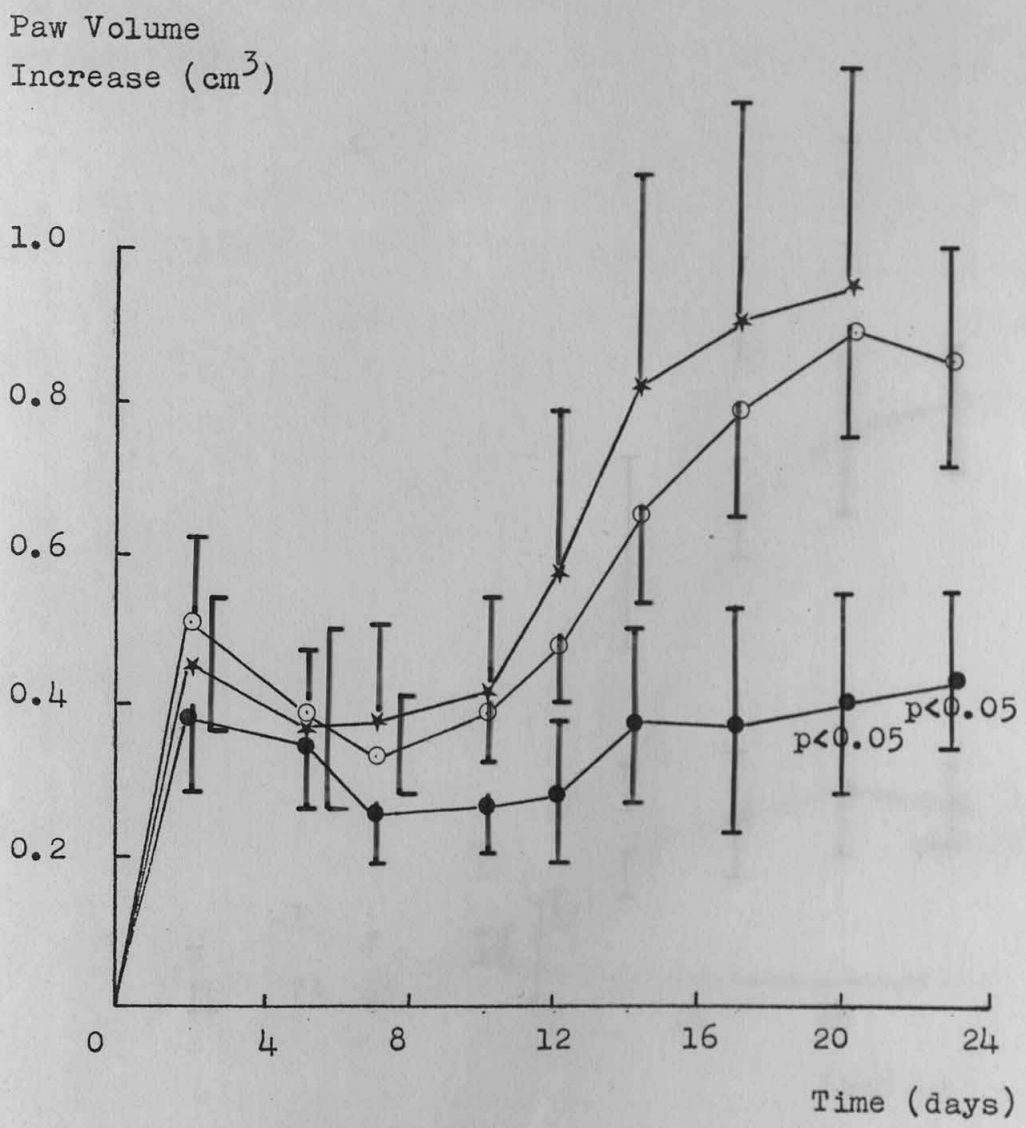


Figure 3.38 The effect on the injected foot volume of adjuvant arthritic rats given daily injections of putrescine at doses of 50 µg Kg^{-1} ●—● and 500 µg Kg^{-1} ✶—✶ compared to saline treated controls ○—○.

Each result represents the mean of six animals ± SEM.

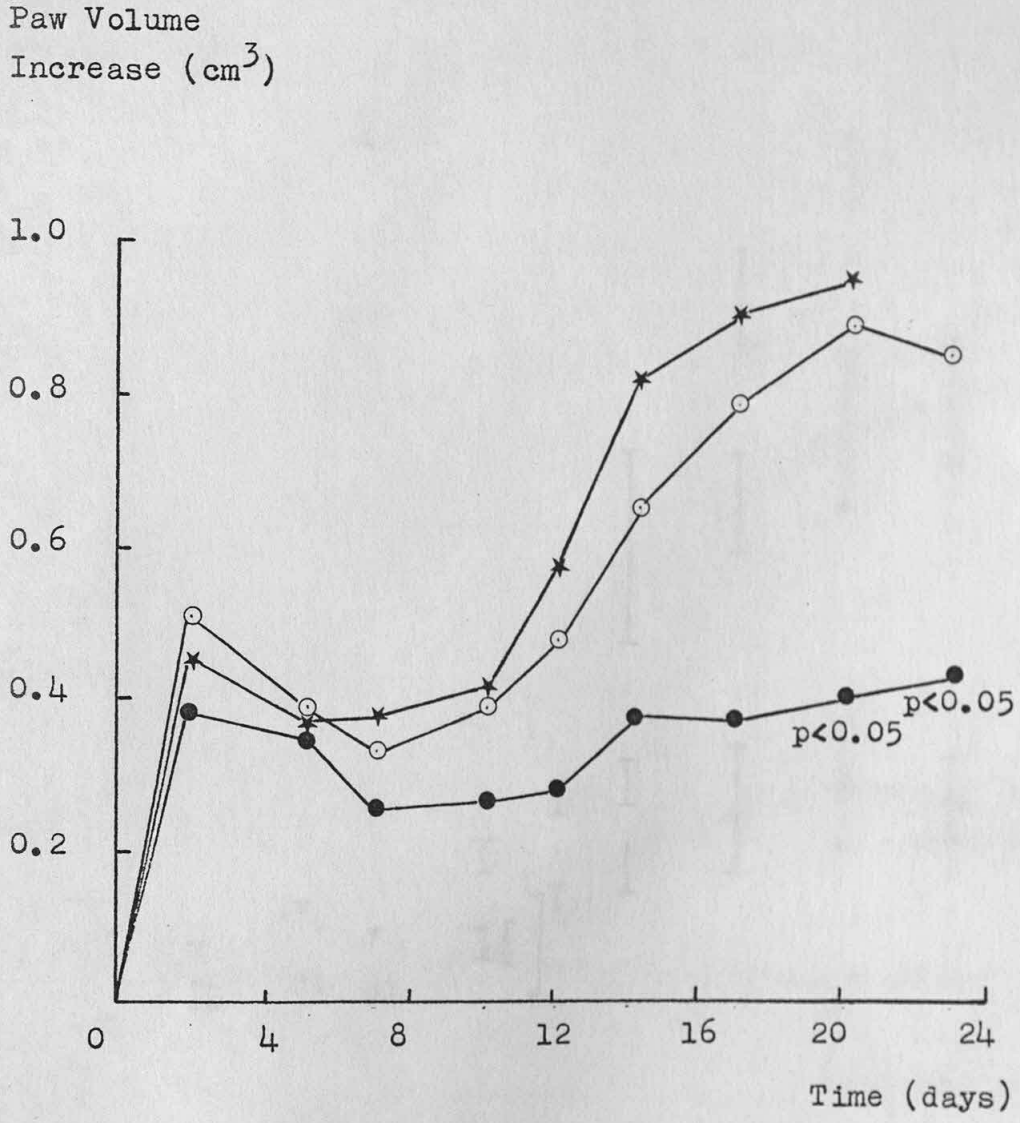


Figure 3.38 The effect on the injected foot volume of adjuvant arthritic rats given daily injections of putrescine at doses of 50 µg Kg^{-1} ●—● and 500 µg Kg^{-1} ✶—✶ compared to saline treated controls ○—○.

Each result represents the mean of six animals ± SEM.

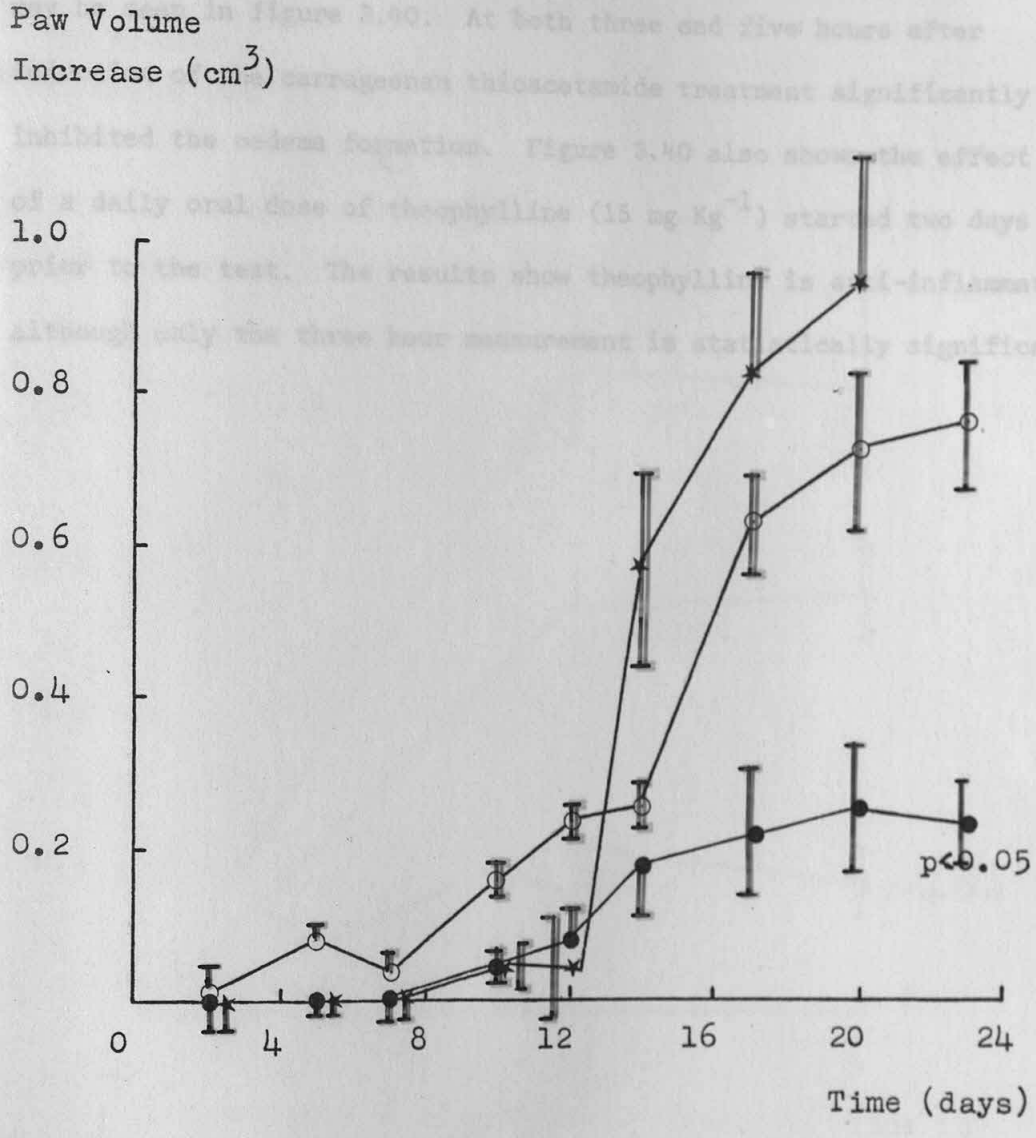


Figure 3.39 The effect on the non-injected foot volume of adjuvant arthritic rats given daily injections of putrescine at doses of 50 µg Kg^{-1} ●—● and 500 µg Kg^{-1} ✶—✶ compared to saline treated controls O—O.

Each result represents the mean of six animals ± SEM.

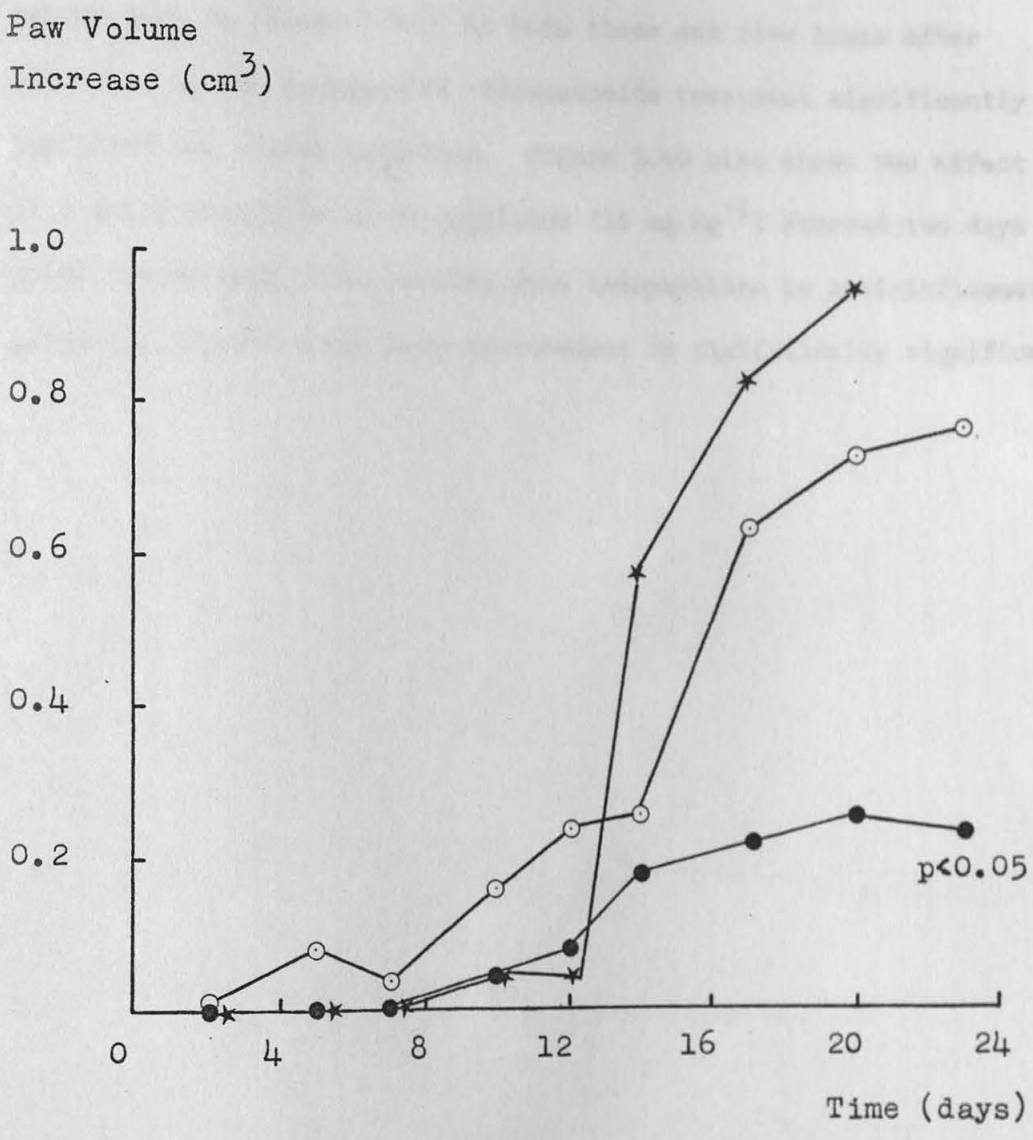


Figure 3.39 The effect on the non-injected foot volume of adjuvant arthritic rats given daily injections of putrescine at doses of 50 µg Kg^{-1} ●—● and 500 µg Kg^{-1} ✱—✱ compared to saline treated controls O—O.

Each result represents the mean of six animals ± SEM.

3.6.6 The Effect of Theophylline and Thioacetamide on Carrageenan Oedema

The effect of a single dose of thioacetamide (150 mg Kg^{-1}) given intraperitoneally 24 hours before the injection of carrageenan may be seen in figure 3.40. At both three and five hours after injection of the carrageenan thioacetamide treatment significantly inhibited the oedema formation. Figure 3.40 also shows the effect of a daily oral dose of theophylline (15 mg Kg^{-1}) started two days prior to the test. The results show theophylline is anti-inflammatory although only the three hour measurement is statistically significant.

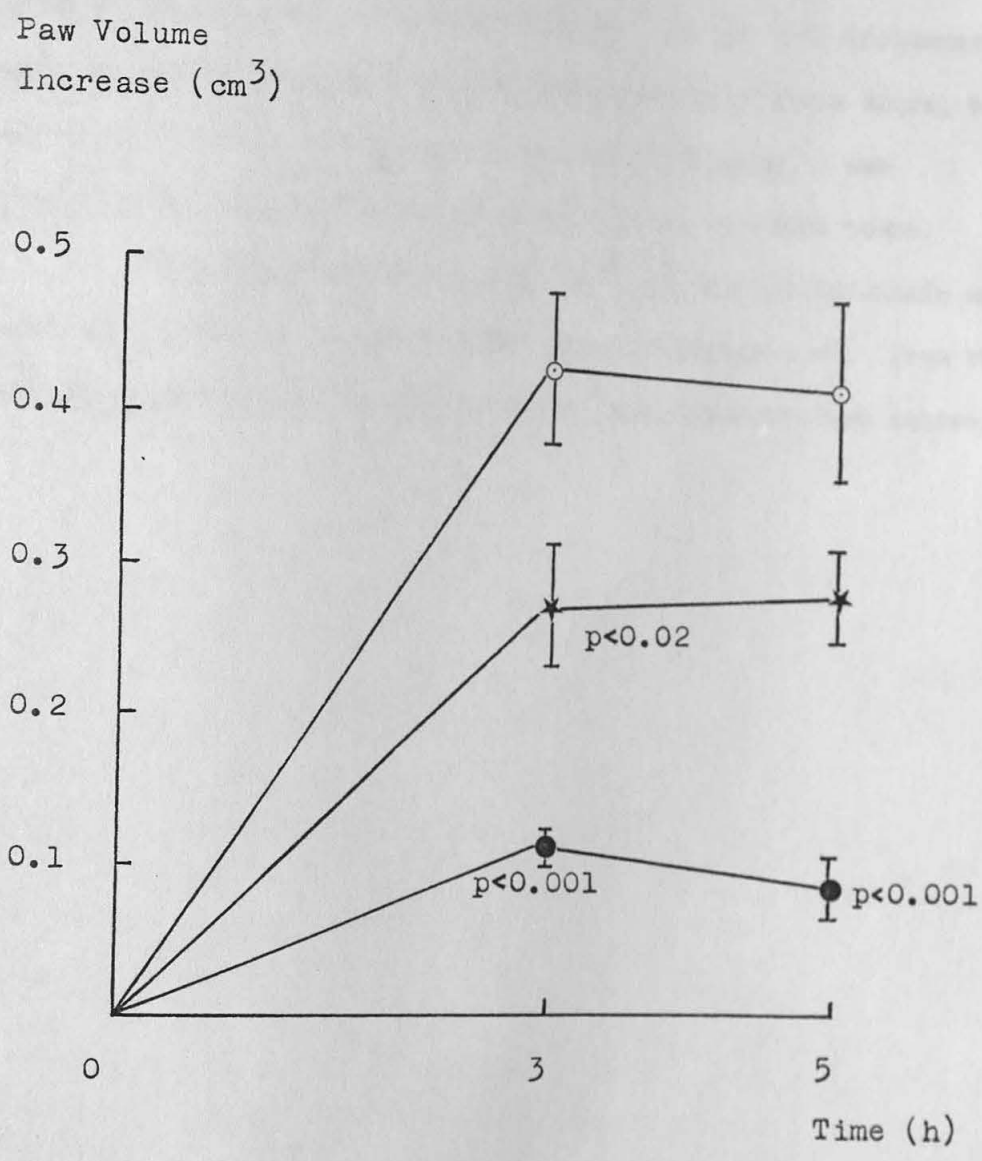


Figure 3.40 The effect on carrageenan induced oedema of thioacetamide ●—● and theophylline ✱—✱ compared to saline controls O—O. Each result represents the mean of seven animals ± SEM.

3.7 The Role of Endotoxin in Inflammation

3.7.1 The Anti-inflammatory Effect of Endotoxin

The anti-inflammatory effect of bacterial endotoxin, given intraperitoneally, on the development of carrageenan oedema is shown in figure 3.41. A dose of 5 μg Kg^{-1} is not anti-inflammatory while a dose of 20 μg Kg^{-1} is anti-inflammatory at three hours, but not at six hours. The highest dose used, 100 μg Kg^{-1}, was significantly anti-inflammatory at both three and five hours.

The irritancy of a 100 μg cm^{-3} solution of endotoxin was also evaluated and the results are shown in figure 3.42. From these results endotoxin can be seen to be no more irritant than saline.

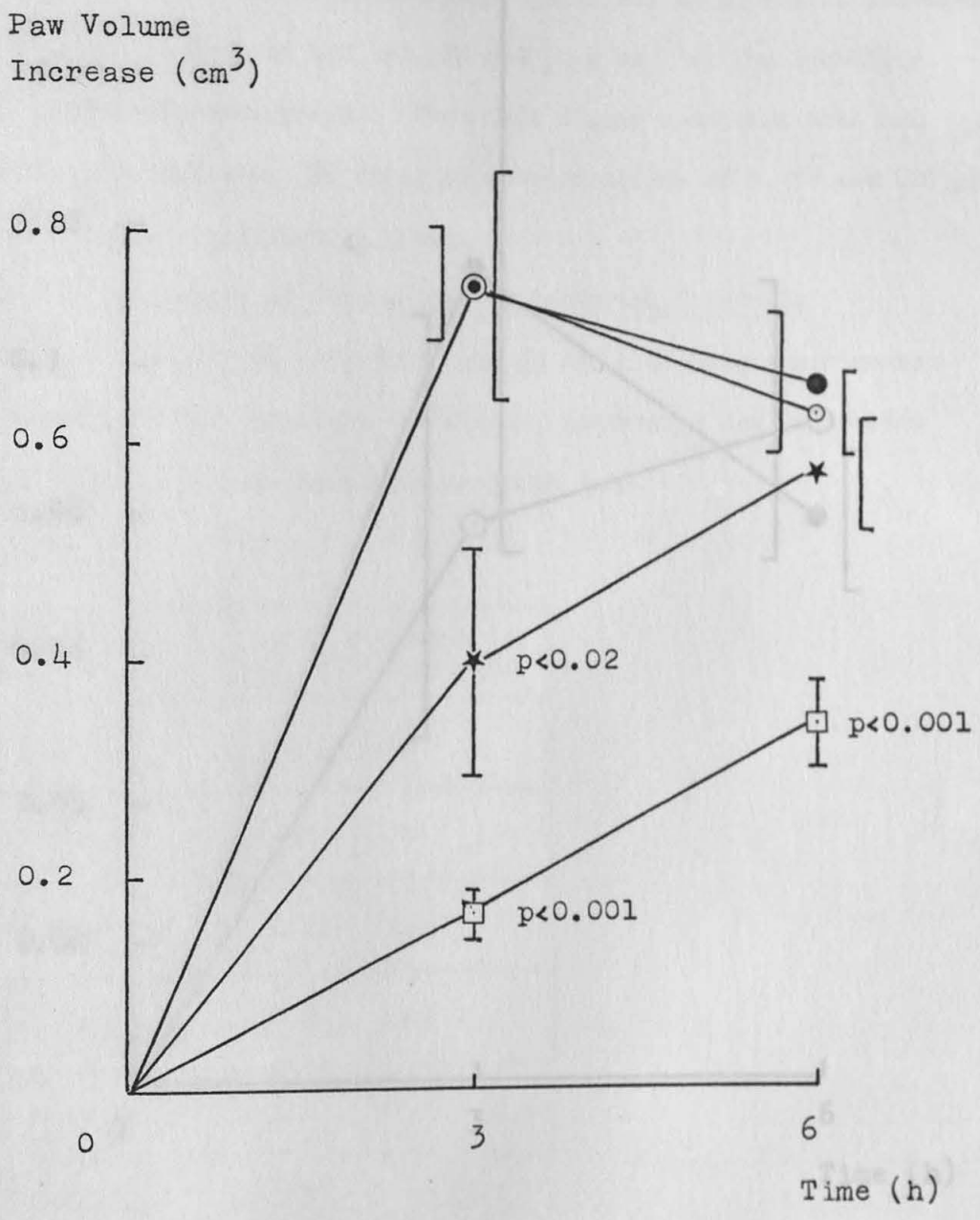


Figure 3.41 The effect of carrageenan induced oedema of an intraperitoneal injection of bacterial endotoxin at doses of 5μg Kg^{-1} ●—●, 20 μg Kg^{-1} ✱—✱ and 100 μg Kg^{-1} □—□ when compared to saline treated controls ○—○.

Each result represents the mean of six animals ± SEM.

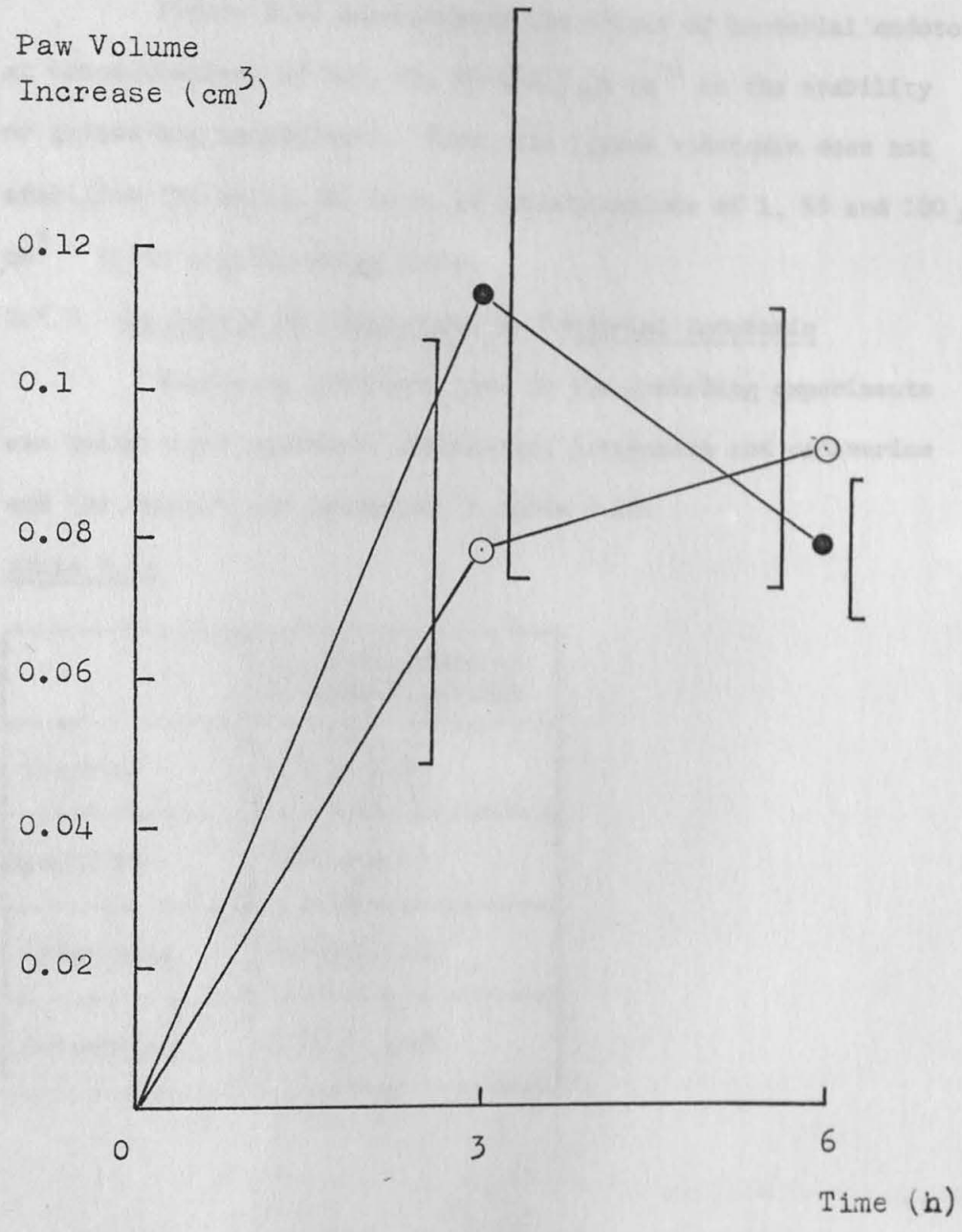


Figure 3.42 The increase in foot volume of rats following injection of a solution of endotoxin (100 µg cm^{-3}) ●—● compared to saline treated controls ○—○.

Each result represents the mean of seven animals ± SEM.

3.7.2 The Degree of Stabilization of Guinea Pig Macrophages by Endotoxin

Figure 3.43 demonstrates the effect of bacterial endotoxin at concentrations of 100, 50, 10 and 1 $\mu g\ cm^{-3}$ on the stability of guinea-pig macrophages. From this figure endotoxin does not stabilize the cells, in fact, at concentrations of 1, 50 and 100 $\mu g\ cm^{3}$ it is significantly lytic.

3.7.3 The Levels of Oligoamines in Bacterial Endotoxin

Bacterial endotoxin used in the preceding experiments was assayed for spermine, spermidine, putrescine and cadaverine and the results are presented in table 3.12.

Table 3.12

	µg of polyamine in 25 mg of Endotoxin
Spermine	1.24 ± 0.35
Spermidine	3.58 ± 0.37
Cadaverine	40.22 ± 2.11
Putrescine	8.78 ± 0.68

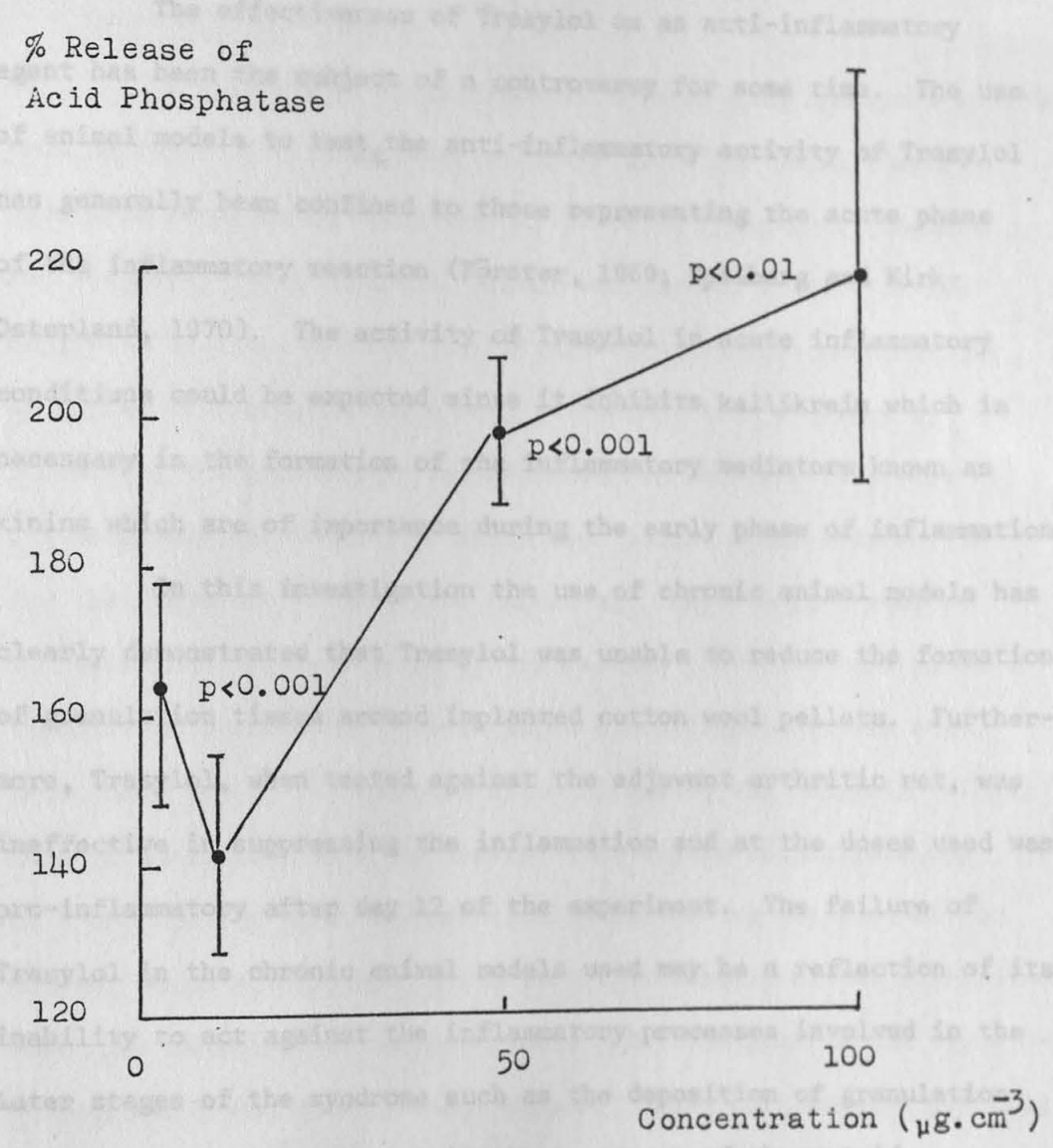


Figure 3.43 The effect of endotoxin on the release of acid phosphatase from macrophages.

Each result represents the mean of eight ± SEM.

4. DISCUSSION

4.1 The Evaluation of the Proteinase Inhibitor Trasylol as a Potential Anti-Inflammatory Agent

The effectiveness of Trasylol as an anti-inflammatory agent has been the subject of a controversy for some time. The use of animal models to test the anti-inflammatory activity of Trasylol has generally been confined to those representing the acute phase of the inflammatory reaction (Förster, 1969; Spilberg and Kirk-Osterland, 1970). The activity of Trasylol in acute inflammatory conditions could be expected since it inhibits kallikrein which is necessary in the formation of the inflammatory mediators known as kinins which are of importance during the early phase of inflammation.

In this investigation the use of chronic animal models has clearly demonstrated that Trasylol was unable to reduce the formation of granulation tissue around implanted cotton wool pellets. Furthermore, Trasylol, when tested against the adjuvant arthritic rat, was ineffective in suppressing the inflammation and at the doses used was pro-inflammatory after day 12 of the experiment. The failure of Trasylol in the chronic animal models used may be a reflection of its inability to act against the inflammatory processes involved in the later stages of the syndrome such as the deposition of granulation tissue. The use of Trasylol in the treatment of rheumatoid arthritis stems from the suggestion that it is capable of inhibiting acid and neutral proteinases released from polymorphonuclear leukocytes during the inflammatory reaction (Spilberg and Kirk-Osterland, 1970). Further support for this theory is offered here since Trasylol can clearly inhibit the proteolytic activity of human rheumatic synovial fluid samples *in vitro* and because of its low molecular size its passage to the site of inflammation would be easier.

The clinical use of Trasylol in rheumatoid arthritis has met with varied results, but its use as an intra-articular preparation may be of benefit (Bruckner and Eisen, 1970; Marcy, Loyau, and Dumas, 1972).

4.2 The Levels of Proteinases and Anti-proteinases in Human Rheumatic Synovial Fluid Samples

The levels of alpha-1-antitrypsin in sera and synovial fluid from patients with rheumatoid arthritis and other joint diseases have been found to be significantly elevated (Brackertz, Hagmann and Kueppers, 1975; Swedlund, Hunder and Gleich, 1974). It was suggested by these workers that higher than normal concentrations of alpha-1-antitrypsin could help to control tissue damage by inhibiting the proteolytic enzymes released from the lysosomes of polymorphonuclear leukocytes which are thought to be responsible for the degenerative processes occurring. Experimental support for this view may be offered by the results presented here since a significant correlation between alpha-1-antitrypsin levels and proteinase levels in rheumatic synovial fluids was obtained. This data is consistent with the concept that alpha-1-antitrypsin may help to regulate joint inflammation by inhibiting the proteolytic enzymes which attack joint tissues. Significant positive correlations were also obtained between proteinase and total protein and alpha-2-macroglobulin and total protein levels. These correlations probably reflect the general increase of protein levels within the joint due to both tissue breakdown and extravasation of proteins from the surrounding tissues into the joint capsule.

4.3 The Role of Endotoxin in Inflammation

Smith, Ford-Hutchinson and Walker (1977) have stated that endotoxin contamination of anti-inflammatory factors isolated from natural sources may cause spurious results.

These workers base their theory on the growth of gram-negative

bacteria in the column packing material (Sephadex) which on application of hypertonic samples are lysed and endotoxin (LPS) is released. The LPS then behaves as a low molecular weight material in the separation rather than its true molecular weight (< 200,000).

This explanation may be possible since hypertonic solutions would cause cell death and destruction, releasing LPS, but actual lysis of the cells is unlikely. The sample used in this fractionation was unusual because it had been derived from human plasma using the techniques of ultrafiltration and concentration which had the effect of concentrating the sample fifty times and thereby greatly increasing the tonicity (Ford-Hutchinson, Insley, Elliot, Sturgess and Smith, 1973). In comparison the materials investigated in this project were either dissolved in or equilibriated with elution buffer prior to application to the column. The samples did not contain an excess of inorganic ions and the contribution of the higher molecular weight molecules to the overall osmotic pressure should be relatively small. The change in tonicity on addition of the sample should, therefore, be minimal. These workers also found that a dose of 2μ gKg^{-1} of LPS was anti-inflammatory when given intravenously to rats used in a carrageenan induced paw oedema test. The results from the present study indicate that when given by the intraperitoneal route (the method utilized for the injection of all test materials) LPS was much less effective in reducing inflammation.

For this investigation the stabilization of monocytes was studied since these cells are considered to be important in the inflammatory process (Lack, 1969). This model was chosen in preference to the stabilization of lysosomes (Hempel, Fernandez and Persellin, 1970; Persellin, Vance and Peery, 1974) since, as Ignarro (1971) pointed out, that model suffers the disadvantage that different experimental conditions can lead to different results.

For comparative purposes LPS was tested in this manner and was shown to have lytic properties when incubated with guinea pig macrophages which has important consequences for some of the work to be discussed later. LPS has always been shown to contain spermine, spermidine, cadaverine and putrescine, some of which are markedly anti-inflammatory (see later) and may account, at least in part, for its anti-inflammatory activity.

The problem of endotoxin contamination still exists and is of importance since Maguire and Wallis (1977) found exclusion of bacteria from the fractionation of anti-lymphocytic serum abolished the anti-inflammatory activity of one of the fractions.

The reasons why LPS should be anti-inflammatory remain unsolved, but its effects are wide ranging and several features may be important. Endotoxin has been shown to cause changes in vascular permeability, neutrophil chemotaxis and release of vasoactive substances, including histamine and kinins. It also causes an increase in serum 11-hydroxycorticosteroid concentration (Munoz, 1961) and activation of the complement system (Bladen, Gewurz and Mergenhagen, 1967).

One of the other problems associated with the study of endogenous anti-inflammatory substances is the counter irritation theory. Although the problem has not been attacked directly in this investigation it has been considered. Thus all the compounds tested for anti-inflammatory activity have also been assayed for their irritant action and an evaluation of both activities should provide an answer to whether the anti-inflammatory activity is due to counter irritation or not.

4.4 The Role of Oligoamines in Inflammation

Although oligoamines possess some properties that may be relevant to inflammation, for example putrescine may be involved in

the production of anti-inflammatory mediators, until recently there has been no evidence of their direct involvement in the inflammatory process. In this investigation the direct anti-inflammatory activity of spermine, spermidine, cadaverine and putrescine has been studied.

Using the carrageenan induced oedema of the rat foot as a test model spermine was not anti-inflammatory, similarly cadaverine exhibited very little activity. Spermidine was anti-inflammatory and an approximate dose dependant relationship was observed. Putrescine was intensely anti-inflammatory at all the doses used, but this effect was not dose related.

The irritancy of the solutions used in the above test was not related to the anti-inflammatory activity. Both cadaverine and putrescine were not irritant. Spermine was irritant at the highest dose used and spermidine was also mildly irritant at the highest dose level.

None of the oligoamines had any effect on the development of a local anaphylactic reaction in the mouse ear.

The in vitro test of anti-inflammatory activity involving the incubation with guinea-pig monocytes and measuring the stabilizing effect of the oligoamines was also used. In this case a distinct difference was noted between the diamines putrescine and cadaverine which have some stabilizing effects and the polyamines spermine, which had no effect and spermidine which had either no effect or was lytic.

Putrescine was also evaluated using the adjuvant rat model. In this case putrescine inhibited the development of the primary and secondary inflammatory lesions, an increase in dose negating this effect.

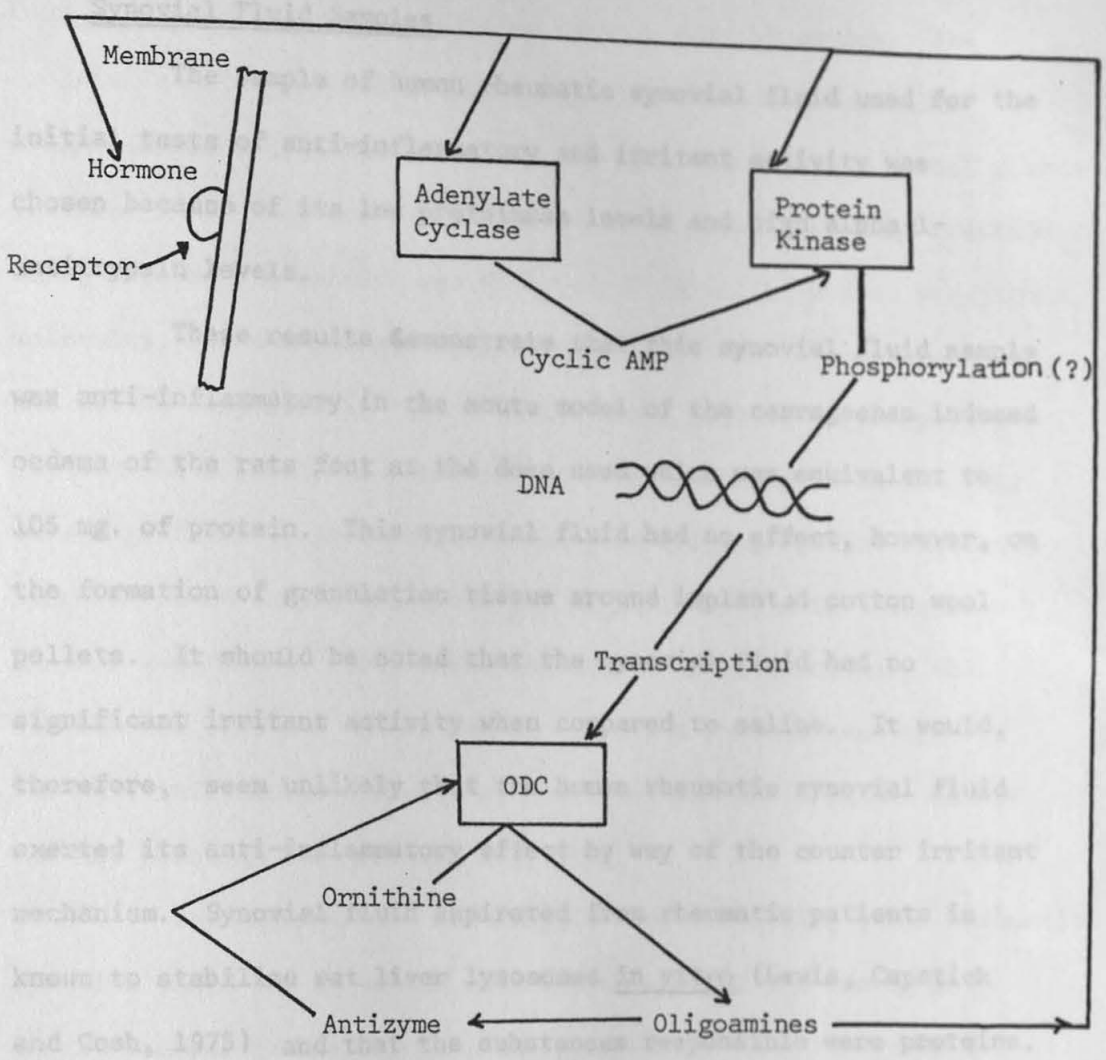
In conclusion cadaverine, spermidine and putrescine all

possess some degree of direct anti-inflammatory activity which does not appear to be related to their irritancy. In addition, putrescine had a marked effect on the chronic inflammatory process. The reason for a negation of this effect by increasing the dose is unknown, but it may well be a reflection of putrescines toxicity since it was noted that granulation tissue formed around the site of injection in the higher dose while no such problem occurred in the lower dosed group. It must be noted, however, that the dose used was far below those which have been found to be non-toxic to rats when administered acutely. A more likely explanation may be that the increase in putrescine triggered the production of ODC antizyme thus synthesis of putrescine within the body is slowed down. Some evidence is available to support this view (Heller and Canellakis, 1980) since N18 neuroblastoma cells were found to increase ODC antizyme production when incubated in a putrescine containing medium. This effect was only observed above a certain threshold level of the diamine.

The mechanism by which putrescine and the other oligoamines operate is unknown, but from these results they have little effect on monocytes or on the anaphylactoid type of reaction. However, it is possible that either putrescine itself is an anti-inflammatory mediator or it may be involved in the production of anti-inflammatory mediators. (Bartholeyns, Fozard and Prakash, 1980).

The effects of thioacetamide and theophylline also confirms the role of ODC and oligoamines in inflammation. These compounds are known to increase the levels of ODC in the liver, thus the general levels of oligoamines and particularly putrescine would be elevated. The use of theophylline, an inhibitor of phosphodiesterase, also raises the possibility of the involvement of cyclic AMP in this process. The involvement of cyclic AMP in putrescine biosynthesis is

well known and is described in the figure below:-



(Bachrach, 1980)

Thus the involvement of cyclic AMP is clear and in addition an excess of putrescine would not only result in production of antizyme, but would ultimately switch off ODC synthesis.

Finally, prostaglandins may play some part in ODC induction (Verma, Rice and Boutwell, 1977), thus these mediators, which are produced during the inflammatory process, may cause an elevation of putrescine biosynthesis with a resultant anti-inflammatory effect.

4.5 The Anti-Inflammatory Activity of Human Rheumatic Synovial Fluid Samples

The sample of human rheumatic synovial fluid used for the initial tests of anti-inflammatory and irritant activity was chosen because of its low proteinase levels and high alpha-1-antitrypsin levels.

These results demonstrate that this synovial fluid sample was anti-inflammatory in the acute model of the carrageenan induced oedema of the rats foot at the dose used which was equivalent to 105 mg. of protein. This synovial fluid had no effect, however, on the formation of granulation tissue around implanted cotton wool pellets. It should be noted that the synovial fluid had no significant irritant activity when compared to saline. It would, therefore, seem unlikely that the human rheumatic synovial fluid exerted its anti-inflammatory effect by way of the counter irritant mechanism. Synovial fluid aspirated from rheumatic patients is known to stabilize rat liver lysosomes *in vitro* (Lewis, Capstick and Cosh, 1975) and that the substances responsible were proteins. Lewis (1977) observed that "although proteins stabilizing lysosomal membranes have properties that suggest that they may be anti-inflammatory, there is no direct evidence that they have an anti-inflammatory action against the usual animal models". The observations from these experiments may offer some additional evidence in support of that statement.

Pooled human rheumatic synovial fluid, when fractionated on Sephadex G-150, produced two pooled samples (F1 and F2) with the split occurring at about 6300 daltons. Both these fractions significantly inhibited the carrageenan induced oedema of the rat paw. The anti-inflammatory effect of the higher molecular weight fraction (F1)

could be due to lysosomal stabilizing proteins (Lewis, Capstick and Cosh, 1975) or to proteins with an unknown mode of action. The anti-inflammatory activity associated with the lower molecular weight fraction (F2) was obviously not due to any of the normal plasma proteins since they are all too large to be present in this fraction. Also, because the extract was dialysed it is unlikely that very small molecules, for example steroid, were present. It is possible that the active component in this fraction was a peptide or polypeptide. Although both fractions were irritant when compared to saline they were significantly less irritant than the original pooled rheumatic synovial fluid sample which was not significantly anti-inflammatory in this experiment. From these results it appears unlikely that the counter irritant theory was in operation in this case since the anti-inflammatory effects of the fractions and original sample did not parallel the irritant activity.

The implantation of dialysis sacs containing human rheumatic synovial fluid had no effect when tested against the carrageenan induced oedema of the rat paw, the possibility that the synovial fluid was capable of releasing dialysable molecules which were anti-inflammatory does not, therefore, arise.

The incubation of pooled human rheumatic synovial fluid with guinea-pig monocytes demonstrated the lytic activity of the unfractionated fluid. Upon fractionation this lytic activity was lost since both fractions (F1 and F2) were capable of stabilizing the membranes of the monocytes. Earlier work using synovial fluids showed that while some fluids stabilized lysosomes others were lytic (Capstick, Lewis and Cosh, 1975). Thus the data presented here may be a reflection of the diverse origin of the pooled rheumatic synovial fluid or the lytic substances present are only effective above a certain threshold level.

4.6 The Anti-Inflammatory Activity of Liver Extracts from Dimethylnitrosamine Treated Rats

Pinals (1973) demonstrated that saline extracts of livers from rats, treated with the hepatotoxin dimethylnitrosamine two to six days earlier, could delay the onset and severity of the inflammation induced by adjuvant in the rat. It has also been shown (Parrott, 1977) that this extract is also effective in supressing the inflammation induced by the injection of carrageenan into the foot of a rat. In both cases extracts of normal livers were ineffective.

In this study the liver extract from dimethylnitrosamine treated rats was fractionated by gel filtration to produce three fractions pooled according to their proteolytic activity. The high molecular weight fraction (> 100,000 daltons) was significantly anti-inflammatory while the other two fractions were ineffective. The irritancy of these fractions paralleled their anti-inflammatory activity, the most irritant fraction being the high molecular weight one. It may be possible, therefore, that this high molecular weight fraction is not truly anti-inflammatory, but operates by causing counter irritation.

Using an *in vitro* method it is clear that the high molecular weight fraction undergoes degradation, which is enhanced by the presence of trypsin. The dialysable molecules released during this process are also demonstrably anti-inflammatory *in vivo*. The nature of these active substances is unknown, but it is possible that oligoamines may play a part in the mechanism of action of DMNO liver extracts.

It is well known that liver damage caused by partial hepatectomy and treatment with carbon tetrachloride produces general tissue regeneration with a concomitant elevation of ODC activity

and oligoamine levels (see Introduction). In this case liver extracts from DMNO treated rats have greatly elevated levels of cadaverine and putrescine, spermine levels are depressed and spermidine levels are unchanged. This liver extract is capable of releasing all four oligoamines when incubated in a dialysis sac although the presence of trypsin does not enhance this release. Previous experiments (section 4.4) have shown that oligoamines possess anti-inflammatory activity and thus the liver extracts may operate via this route and explain the results of Pinals (1973).

4.7 The Anti-Inflammatory Properties of Sponge Induced Inflammatory Exudate

The fractionation of sponge exudate by gel filtration confirms the results of Billingham, Robinson and Robson (1969). A possible mechanism of action for the anti-inflammatory substances found in sponge exudate may be explained by its action on monocytes *in vitro*. The unfractionated exudate was capable of stabilizing the membranes of these cells. On fractionation both fractions (S1 and S2) are capable of stabilizing the cells at the lower concentrations used, but at higher concentrations the fractions were lytic. From this it may be concluded that, during fractionation, either the lytic substances have been concentrated from the original material or they have been introduced by the process. In the case of the latter hypothesis it may be possible the contamination with endotoxin is the reason for the introduction of the lytic compounds since no special precautions were taken during the fractionation procedure.

The study of sponge exudate using antisera yielded few positive results. However, it can be concluded that the major proteins present in the exudate are normally found in rat plasma also the proteins that apparently adhere to lysosomes are normal plasma

proteins. These results do not preclude the possibility of a protein or polypeptide, present in low concentration, having anti-inflammatory capabilities.

From *in vitro* studies of the proteolytic activity of this inflammatory exudate it is possible to conclude that it possesses inherent proteolytic activity. This activity is at a low level when compared to rheumatoid synovial fluid, but is sufficient to account for the self degradation of the exudate and the subsequent release of dialysable molecules.

The *in vivo* study of these dialysable molecules proved that their presence induced a change in the rat such that when the animal was later challenged by an inflammatory stimulus the resultant oedema was significantly smaller than control animals. There is some suggestion from this experiment that trypsin negates this effect. This may be because the active component is destroyed by trypsin, suggesting that it is a protein, or trypsin accelerates its release to leave only low levels when challenged. The use of sponge exudate dialysate collected *in vitro* showed that it was anti-inflammatory, but this was allied to its irritancy. In addition the residue after dialysis was also irritant and anti-inflammatory.

A possible mode of action for these dialysable molecules is suggested by the work of Sarcione and Bogden (1966) who demonstrated the synthesis of alpha-2-glycoprotein in isolated perfused livers from injured rats. They argued that, since injury at sites peripheral to the liver stimulates production of the protein, the injured tissues must release a factor into the blood which prompts its hepatic synthesis. Evidence to support the existence of an extra-hepatic factor (humoral initiator substance or HIS factor) was provided by Bogden, Gray and Fuss (1966) who found that serum from injured rats caused increased synthesis of alpha-2-

glycoprotein in the livers from normal animals. A mechanism similar to this could account for the anti-inflammatory activity of these dialysable molecules and would complement the work of Billingham, Gordon and Robinson (1971) who found that livers from injured rats synthesized an anti-inflammatory protein when perfused with normal blood. These workers established that actinomycin D which prevents protein synthesis, prevented the formation of the anti-inflammatory protein found in sponge exudate.

Sponge induced inflammatory exudate both, contains oligoamines and is capable of releasing them when incubated in dialysis sacs. This fact offers another possible mode of action of sponge exudate. The oligoamines have been shown to be anti-inflammatory and are present in similar amounts in sponge exudate, thus the anti-inflammatory effects of the exudate may be due, at least in part, to the oligoamines. In addition, the release of oligoamines by the exudate offers an explanation of the anti-inflammatory effects of the dialysate.

4.8 Conclusions

The discovery that oligoamines have a direct anti-inflammatory action may be important in several areas of study.

In this work it has been shown that an anti-inflammatory exudate both contains and is capable of releasing oligoamines in amounts which would be pharmacologically active. Although the mode of action of the oligoamines is unknown it is obviously worthy of further investigation and a possible link with the cyclic AMP and prostaglandin systems does not appear unlikely.

The role of liver damage and the resultant tissue regeneration in inflammation has also been clarified since this involves the production of putrescine which has been shown to be anti-inflammatory. It is interesting to note that treatment with

the hepatotoxin carbon tetrachloride causes a conversion of spermidine to putrescine, the more potent anti-inflammatory agent.

Drug action, especially that of the steroidal drugs, may also be explained since steroids are known to cause an increase in ODC activity and thus putrescine levels. A possible reason for this effect may be offered by the work of Pitt and Lewis (1979) who suggested that steroidal drugs caused slight liver damage which would elevate ODC and putrescine levels.

In conclusion oligoamines may be very important to our understanding of the basic mechanisms of inflammation and also the development of new types of drugs may be possible.

5. REFERENCES

ANDERSSON, A.C., HENNINGSSON, S. and ROSENGREN, E. (1978) Increased formation of diamines and polyamines in the pregnant rat. J. Physiol., 285, 311 - 324.

ATKINSON, D.C., BOURA, A.L.A. and HICKS, R. (1969). Observations on the pharmacological properties of inflammatory exudate Eur. J. Pharmac., 8, 348-354.

ATKINSON, D.C. and HICKS, R. (1971). Relationship between the anti-inflammatory and irritant properties of inflammatory exudate. Br.J. Pharmac., 41, 480-487.

ATKINSON, D.C. and HICKS, R. (1975a) The anti-inflammatory activity of Irritants, Agents and Actions, 5/3, 239-249.

ATKINSON, D.C. and HICKS, R. (1975b). The possible occurrence of endogenous anti-inflammatory substances in the blood of injured rats. Br. J. Pharmac., 53, 85-91.

ATKINSON, D.C., WHITTLE, B.A. and HICKS, R. (1971). A further observation on the anti-inflammatory activity of inflammatory exudate. Eur.J. Pharmac., 16, 254-256.

BACHRACH, U. (1975) Cyclic AMP - mediated induction of ornithine decarboxylase of glioma and neuroblastoma cells. Proc. Nat. Acad. Sci. U.S.A., 72, 3087-3091.

BACHRACH, U. (1980). The induction of ornithine decarboxylase in normal and neoplastic cells. IN: Polyamines in biomedical research, (Ed. J.M. Gaugas), pp 81-107, J. Wiley and sons, New York.

BARRETT, A.J. and STARKEY , P.M. (1973). The interaction of alpha-2-macroglobulin with proteinases. Characteristics and specificity of the reaction and a hypothesis concerning its molecular mechanism. Biochem. J., 133, 709-724.

BARTHOLEYNS, J. FOZARD, J.R. and PRAKASH, N.J. (1981). Dexamethasone inhibition of carrageenan paw oedema in the rat requires de novo synthesis of putrescine Br. J. Pharmac., 72, 182P-183P.

BARTHOLEYNS, J. FOZARD, J.R. and PRAKASH, N.J. (1981) Blockade of the anti-inflammatory action of dexamethasone by DL-α-difluoromethylornithine; comparison with actinomycin D and Cycloheximide Br. J. Pharmac. In Press.

BILLINGHAM, M.E.J., GORDON, A.H. and ROBINSON, B.V. (1971). Role of the liver in inflammation. Nature New Biol, 231, 26-27.

BILLINGHAM, M.E.J., MORLEY, J., HANSON, J.M., SHIPOLINI, R.A., and VERNON, C.A. (1973). An anti-inflammatory peptide from bee venom. Nature, 245, 163-164.

BILLINGHAM, M.E.J. and ROBINSON, B.V. (1972). Separation of irritancy from the anti-inflammatory component of inflammation exudate. Br. J. Pharmac., 44, 317-320.

BILLINGHAM, M.E.J., ROBINSON, B.V. and GAUGAS, J.M. (1970). Two anti-inflammatory components in anti-lymphocytic serum. Nature, 227, 276-277.

BILLINGHAM, M.E.J., ROBINSON, B.V. and ROBSON, J.M. (1969a) Partial purification of the anti-inflammatory factor(s) in inflammatory exudate. Br.J. Pharmac., 35, 543-557.

BILLINGHAM, M.E.J., ROBINSON, B.V. and ROBSON, J.M. (1969b). Anti-inflammatory properties of human inflammatory exudate. Br. Med. J., 2, 93-96.

BLADEN, H.A., GEWURZ, H. and MERGENHAGEN, S.E. (1967) Interaction of the complement system with the surface and endotoxic lipopolysaccharide of Veillonella Alcalescens J. Exptl. Med., 125, 767-786.

BOGDEN, A.E., GRAY, J.H. and FUSS, F.R. (1966). Initiation of alpha-2-glycoprotein synthesis by a trauma associated humoral factor. Proc. Am. Ass. Cancer Res., 7, 8.

BRACKERTZ, D., HAGMANN, T. and KUEPPERS, F. (1975). Proteinase inhibitors in rheumatoid arthritis. Ann Rheum. Dis., 34,225-231.

BRACKERTZ, D. and KUEPPERS, F. (1977). Alpha-1-antitrypsin phenotypes in rheumatoid arthritis. Lancet, 1, 934-935.

BRAY, M.A., CUNNINGHAM, F.M., FORD-HUTCHINSON, A.W. and SMITH, M.J.H. (1981) Leukotriene B_4: a mediator of vascular permeability. Br.J. Pharmac. IN PRESS.

BRISEID, K., ARNTZEN, F.C. and DYRUD, O.K. (1971). Inhibition of carrageenan-induced rat paw oedema by substances causing a reduction of kininogen and prekallikrein in plasma. Acta. Pharmac, Toxicol., 29, 265-274.

BRUCKNER, F.E. and EISEN, V. (1970). In: New Aspects of Trasylol Therapy Vol. 4. (Eds. Haberland, G.L., Huber, P. and Matis, P.) Schatlauer Verlag page 17.

BRUNKHORST, W.K. (1958) Ph.D. thesis, University of Wisconsin, Madison.

BUISSERET, P.D., PEMBREY, M.E. and LESSOF, M.H. (1977). Alpha-1-antitrypsin phenotypes in rheumatoid arthritis and ankylosing spondylitis. Lancet, 1, 1358-1359.

BURLEIGH, M.C. (1973) Action on Collagen of Cathepsins B1 and D Biochem. Soc. Trans., 1, 380-381.

BURLEIGH, M.C., BARRETT, A.J. and LAZARUS, G.S. (1974). Cathepsin B1. A lysosomal enzyme that degrades native collagen. Biochem.J., 137, 387-398.

CANELLAKIS, E.S., HELLER, J.S., KYRIAKIDIS, D. and CHEN, K.Y. (1978). Intracellular levels of ornithine decarboxylase its half-life, and a hypothesis relating polyamine sensitive membrane receptors to growth. In. Advances in Polyamine Research, Vol. 1 (Eds. R.A. Cambell, D.R. Morris, D. Bartos, G.D. Daves and F. Bartos), pp 17-30, Raven Press, New York.

CHANG, WEN-CHANG, MUROTA, S. and TSURUFUJI, S. (1976). Role of prostaglandin E in carrageenan induced inflammation in rats. Biochem. Pharmac., 25, 2045-2050.

CHANG, Y. and BLIVEN, M.L. (1979). Anti-arthritic effect of bee venom. Agents Actions, 9, 205-211.

CHURCH, M.K., JAMES, G.W.L. and MILLER, P. (1974). The inhibitory effect of insulin on pinnal anaphylaxis in the mouse. Br.J. Pharmac., 54, 454P.

CONTI, C.R., CLUFF, L.E. and SCHEDER, E.P., (1961). Studies on the pathogenesis of staphylococcal infection IV. The effect of bacterial endotoxin. J. exp. Med., 113, 845-859.

COTRAN, R.S. (1967). Delayed and prolonged vascular leakage in inflammation. III. Immediate and delayed reactions in skeletal muscle. Exp. Molec. Path, 6, 143-155.

COX, D.W. and HUBER, O. (1976). Rheumatoid arthritis and alpha-1-antitrypsin. Lancet, 1, 1216-1217.

CRAWFORD, I.P. (1973). Purification and properties of normal human alpha-1-antitrypsin. Arch. Biochem. Biophys., 156, 215-222.

CYGIELMAN, S. and ROBSON, J.M. (1963). The effect of irritant substances on the deposition of granulation tissue in the cotton pellet test. J. Pharm. Pharmac. 15, 794-797.

DIETZ, A.A., RUBINSTEIN, H.M. and HODGES, L. (1974). Measurement of alpha-1-antitrypsin in serum by immunodiffusion and by enzymatic assay. Clin. chem., 20, 396-399.

DIPASQUALE, G. and GIRED, R.J. (1961) Anti-inflammatory properties of lyophilized inflammatory exudates. Am.J.Physiol., 201, 1155-1158.

DIPASQUALE, G., GIRED, R.J., BEACH, V.L. and STEINETZ, B.G. (1963) Antiphlogistic action of granuloma pouch exudates in intact or adrenalectomised rats. Am.J. Physiol., 205, 1080-1082.

DI ROSA,M., GIROUD, J.P. and WILLOUGHBY, D.A. (1971). Studies on the mediators of the acute inflammatory response induced in rats in different sites by carrageenan and turpentine. J. Pathol., 104, 15-29.

DI ROSA,M. and SORRENTINO, L. (1968). The mechanism of the inflammatory effect of carrageenan. Eur.J.Pharmac., 4, 340-342.

DI ROSA,M. and WILLOUGHBY, D.A. (1971) Screens for anti-inflammatory drugs. J. Pharm. Pharmac., 23, 297-298.

DOHERTY, N.S. and ROBINSON, B.V. (1976a). Lysosomal stabilization as the possible mechanism of action of an endogenous anti-inflammatory protein. Biochem. Pharmac., 25, 2039-2044.

DOHERTY, N.S. and ROBINSON, B.V. (1976b). Some biological and pharmacological properties of inflammatory exudates. J. Pharm. Pharmac., 28, 859-864.

ELLIOT, P.N.C., FORD-HUTCHINSON, A.W., HARFORD, D.J., INSLEY, M.Y., SMITH, M.J.H. and STURGESS, E.A. (1973) Anti-inflammatory activity in human plasma. J. Pharm. Pharmac.,25, 408.

FAGERHOL, M.K., and LAURELL, C.B. (1970). The Pi system inherited variants of serum alpha-1-antitrypsin. Progr. Med. Genet., 7, 96-111.

FERREIRA, S.H. and VANE, J.R. (1974). New Aspects of the mode of action of nonsteroidal anti-inflammatory drugs. Ann. Rev. Pharmac., 14, 57-73.

FLOWER, R.J. and BLACKWELL, G.J., (1979). Anti-inflammatory steroids induce biosynthesis of a phospholiphase A_2 inhibitor which prevents prostaglandin generation. Nature, 278, 456-459.

FORD-HUTCHINSON, A.W., INSLEY, M.Y., ELLIOTT, P.N.C., STURGESS, E.A. and SMITH, M.J.H. (1973). Anti-inflammatory activity in human plasma. J.Pharm.Pharmac., 25, 881-886

FÖRSTER, O., (1969). Proteases in inflammation: on the possible mode of action of the protease inhibitor Trasylol. In: Inflammation Biochemistry and Drug Interaction (Eds. A. Bertelli and J. C. Houck). Excerpta. Medica, Amsterdam. Pages 53-65.

FRANCHIMONT, P., HEYREN, G. and HAVWAERT, C.H. (1978) Adverse reactions to the principal drugs used in rheumatoid arthritis. J. Rheumatol (suppl). 5(4), 85-92.

FRENOY, J.P., RAZAFIMAHALEO, E. and BOURILLON, R. (1972). Etudes sur la structure de l'alpha-2-macroglobuline humaine. III Isolement et caracterisation d'une sous-unite. Biochim. Biophys. Acta, 257, 111-121.

GANROT, K. (1973). Rat alpha-2- acute phase globulin, a human alpha-2-macroglobulin homologue. Interaction with plasmin and trypsin. Biochim. Biophys. Acta., 322, 62-67.

GANROT, P.O. (1966). Determination of alpha-2-macroglobulin as trypsin-protein esterase. Clin.Chim.Acta., 14, 495-501.

GANROT, P.O. (1972). Variation of the concentration of some plasma proteins in normal adults, in pregnant women and in newborns. Scand.J.Lab. Invest., 29, Suppl. 124, 83-88.

GANROT, P.O. and SCHERSTEN, B. (1967). Serum alpha-2-macroglobulin concentration and its variation with age and sex. Clin. Chim. Acta., 15, 113-120.

GAUGAS, J.M., BILLINGHAM, M.E.J., and REES, R.J.W. (1970). Suppressive effect of homologous and heterologous inflammatory exudate on tuberculin sensitivity in the guinea pig. Am.Rev. Resp.Dis., 101, 432-434.

GLENN, E.M., GRAY, J. and KOOYERS, W. (1965). Chemical changes in adjuvant polyarthritis of rats. Am.J.Vet. Res., 26, 1195-1203.

GLENN, E.M. and KOOYERS, W. (1966). Plasma inflammation units: an objective method for investigating effects of drugs on experimental inflammation. Life. Sci., 5, 619-628.

GOODMAN, M.L., WAY, B.A. and IRWIN, J.W. (1979). The inflammatory response to endotoxin. J. Pathol. 128, 7-14.

GORNALL, A.G., BARDAWILL, C.J. and DAVID, M.M. (1949). Determination of serum proteins by means of the biuret reactions. J. Biol. Chem., 177, 751-766.

HABERMAN, E. (1968) Inhibitors of kallikrein and other proteinases from animal blood. Ann.N.Y. Acad. Sci., 146, 479-490.

HANNONEN, P., RAINA, A., and JÄNNE, J. (1972). Polyamine synthesis in the regenerating rat liver: stimulation of S-adenosylmethionine decarboxylase, and spermidine and spermine synthases after partial hepatectomy. Biochim.Biophys.Acta, 273, 84-90.

HANSON, J.M., MORLEY, J. and SORIA-HERRERA, C. (1974). Anti-inflammatory property of 401 (MCD-Peptide), a peptide from the venom of the bee Apis mellifera. Br.J. Pharmac., 50, 383-392.

HARRIS, E.D., DI BONA, D.R. and KRANE, S.M. (1969). Collagenases in human synovial fluid. J. Clin. Invest., 48, 2104-2113.

HARRIS, J.M. and SPENCER, P.S.J. (1962). A modified plethysmographic apparatus for recording volume changes in the rat paw. J. Pharm. Pharmac., 14, 464-466.

HEIDE, I. HEIMBURGER, N. and HAUPT, H. (1965). An inter-alpha trypsin inhibitor of human serum. Clin.Chim.Acta., 11, 82-85.

HEIM, W.G. (1968). Relation between rat slow alpha-2-globulin and alpha-2-macroglobulin of other mammals. Nature 217, 1057-1059.

HEIMBURGER, N. and HAUPT, H. (1965). Charakterisie rung von alpha-1-X-glykoprotein als chymotrypsin-inhibitor des human plasmas. Clin.Chim.Acta.,12, 116-118.

HELLER, J.S. and CANELLAKIS, E.S. (1980). Minimal requirements for the induction of the antizyme to ornithine decarboxylase. In:Polyamines in biomedical research, (Ed.J.M. Gaugas), pp 135-145, J. Wiley and sons, New York.

HELLER, J.S. KYRIAKIDIS, D., FONG, W.F. and CANELLAKIS, E.S. (1977) Ornithine decarboxylase antizyme is a normal component of uninduced H-35 cells and rat liver. Eur. J. Biochem. 81, 545-550.

HEMPEL, K.H., FERNANDEZ, L.A. and PERSELLIN, R.H. (1970). Effect of pregnancy sera on isolated lysosomes. Nature, 225, 955-956.

HENCH, P.S. (1933). Analgesia accompanying hepatitis and jaundice in cases of chronic arthritis, fibrositis and sciatic pains. Proc. Mayo. Clinic, 8, 430-436.

HICKS, R. (1975). Inflammation and repair in damaged tissue. Pharmaceut. J., 214, 220-222.

HILL, S.R. and HOLLEY, H.L. (1966). The ameliorating effect of pregnancy and jaundice on certain rheumatic diseases. In: Arthritis and Allied Disorders, Ed.J.L. Hollander, Lea and Febiger, Philadelphia, pp 612.

HINSHAW, L.B. BRAKE, C.M. and EMERSON, T.E. (1965). IN: Shock and hypertension: pathogenesis and treatment, Grune and Stratton, New York, pp 431-441.

HOGAN, B.L.M., and MURDEN, S. (1974). Effect of growth conditions on the activity of ornithine decarboxylase in cultured hepatoma cells. J. Cell. Physiol., 83, 345-352.

HÖLTTÄ, E, SINERVIRTA, R. and JÄNNE, J. (1973). Synthesis and accumulation of polyamines in rat liver regenerating after treatment with carbon tetrachloride. Biochim. Biophys. Res. Comm., 54, 350-357.

HURLEY, J.V., RYAN, G.B. and FRIEDMAN, A. (1966). The mononuclear response to intrapleural injection in the rat. J. Path. Bact., 91, 575-587.

HURLEY, J.V. and SPECTOR, W.G. (1965). A topographical study of increased vascular permeability in acute turpentine induced pleurisy. J. Path. Bact., 89, 245-254.

HUTTUNEN,R. and KORHONEN, L.K. (1973). New trypsin and chymotrypsin binding factor in rat plasma. Ann. Med. Expt. Biol.

IGNARRO, L.J. (1971) Dissimilar effects of anti-inflammatory drugs on the stability of lysosomes from peritoneal and circulating leukocytes and liver. Biochem. Pharmac., 20, 2861-2870.

JÄNNE, J., PÖSÖ, H. and RAINA, A. (1978). Polyamines in rapid growth and cancer. Biochim. Biophys. Acta., 473, 241-293.

JÄNNE, J. and RAINA, A. (1968). Stimulation of spermidine synthesis in the regenerating rat liver: relation to increased ornithine decarboxylase activity. Acta. Chem. Scand., 22, 1349-1351.

JONES, J.M., CREETH, J.M. and KEKWICK, R.A. (1972). Thio reduction of human alpha-2-macroglobulin. The subunit structure. Biochem.J., 127, 187-197.

JORI, A. and BERNARDI, D. (1966). Presence of a general irritation and inhibition of a local inflammation. Med. Pharmac. exp., 14, 500-506.

KANE, M.A., MAY, J.E. and FRANK, M.M. (1973). Interactions of the classical and alternate complement pathway with endotoxin lipopolysaccharide. J.Clin.Invest., 52, 370-376.

KAPLAN, P.D., KUHN, C. and PIERCE, J.A. (1973). The induction of emphysema with elastase. I. The evolution of the lesion and the influence of serum. J. Lab. Clin. Med., 82, 349-356.

KINDMARK, C.O. and LAURELL, C.B. (1972). Sequential changes of the plasma protein pattern in inoculation hepatitis. Scand. J.Clin.Lab.Invest., 29, Suppl. 124, 105-115.

KOPITAR, M. and LEBEZ, D. (1975). Intracellular distribution of neutral proteinases and inhibitors in pig leucocytes. Isolation of two inhibitors of neutral proteinases. Eur. J. Biochem., 56, 571-581.

LACK, C.H. (1969) IN: Lyosomes in Biology and Pathology (Ed. J.T. Dingle and H.B. Fell). North-Holland, Amsterdam. p493-508.

LADEN, C., BLACKWELL, R.Q. and FOSDICK, L.S. (1958). Anti-inflammatory effects of counterirritants. Am.J.Physiol., 195, 712-718.

LAURELL, C.B. (1966) Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Anal. Biochem., 15, 45-52.

LEVINE, J.H., LEAMING, A.B. and RASKIN, P. (1978). On the mechanism of activation of hepatic ornithine decarboxylase by cyclic AMP. In R.A. Campbell, D.R. Morris, D. Bartos, G.D.Daves and F. Bartos (Eds), Advances in Polyamine Research, Vol. 1, Raven Press, New York, pp51-58.

LEWIS, D.A., (1977). Endogenous anti-inflammatory Proteins. Biochem. Pharmac., 26, 693-698.

LEWIS, D.A., BEST, R., and BIRD, J. (1977). Anti-inflammatory action of azapropazone. J.Pharm.Pharmac., 29, 113-114.

LEWIS, D.A., BEST, R., BIRD, J. and PARROTT, D.P. (1978). Formation of anti-inflammatory proteins following liver damage in rats. In: Proceedings of the 7th Internation congres of pharmacology. Paris. p335.

LEWIS, D.A. and CAPSTICK, R.B. (1977). Pharmacological and biochemical properties of azapropazone and other non-steroidal anti-inflammatory agents. Drugs Exptl. Clin.Res., 2(1), 71-77.

LEWIS, D.A., CAPSTICK, R.B. and BEST, R. (1976). The control of rheumatic disease by endogenous protein. Biochem. Pharmac., 25, 1435-1436.

LEWIS, D.A., CAPSTICK, R.B. and COSH, J.A. (1975). Naturally occurring anti-inflammatory factors in the synovial fluids of patients with rheumatic disease and their possible mode of action. Ann.Rheum.Dis., 34, 213-218.

LIYANAGE, S.P., CURREY, H.L.F. and VERNON-ROBERTS, B. (1975). Influence of tubercle aggregate size on severity of adjuvant arthritis in the rat. Ann.Rheum.Dis., 34, 49-53.

LOGAN, G. and WILHELM, D.A. (1966). Vascular permeability changes in inflammation. I. The role of endogenous permeability factors in ultraviolet injury. Brit.J.Exp.Pathol., 47, 300-313.

MAGUIRE, E.D. and WALLIS, R.B. (1977). The role of bacterial contamination in the isolation of apparent anti-inflammatory factors from rabbit anti-lymphocytic serum. Br.J.Pharmac. 59, 261-268.

MARCY, R., LOYAU, G. and DUMAS, M. (1972). Etude des effets de l'aproteinine administreé par voie para et intra-articulaire II. Etude clinique. Therapie, 27, 1031-1041.

MARMION, B.P. (1976) A microbiologists view of investigative rheumatology. IN: Infection and Immunology in the rheumatic diseases. Blackwell Scientific, Oxford. pp 245-258.

MARTIN, G.V. and SOUTHWELL, W.J. (1965). Enzymes in inflammation. Exp.Med.Surg. Suppl., Issue 23, p 150-164.

MATHIES, H. (1979). The mechanism of action of antirheumatic agents. Triangle, 18, 63-73.

McARTHUR, J.N., DAWKINS, P.D., SMITH, M.J.H. and HAMILTON, E.B.D. (1971). Mode of Action of antirheumatic drugs. Brit. Med. J., 2, 677-679.

McARTHUR, J.N., SMITH, M.J.H. and FREEMAN, P.C. (1972). Anti-inflammatory substance in human serum. J.Pharm. Pharmac., 24, 669-670.

MEIR, R., SCHULER , W. and DESAULLES, P. (1950). Zur Frage des Mechanismus der Hemmung des Bindegeivebswachstums durch Cortisone. Experientia (Basel), 6, 469-471.

MERGENHAGEN, S.E., SNYDERMAN, R., GEWURZ, H. and SHIN, H.S. (1969). Significance of complement to the mechanism of action of endotoxin. Curr. Top. Microbiol. Immun., 50, 37-77.

MIELENS, Z.E., DROBECK, H.P., ROZITIS, J. and SANSOME,S. (1969). Inhibition of experimental inflammation by oral toxic agents. Toxic Appl. Pharmacol., 14, 293-300.

MIZUTANI, A., INOUE, H. and TAKEDA, Y. (1974) Changes in polyamine metabolism during wound healing in rat skin. Biochim. Biophys.Acta., 338, 183-190.

MUNOZ, J. (1961). Permeability changes produced in mice by Bordetella pertussis J. Immun., 86, 618-626.

MUSIANI, P. and TOMASI, T.B. (1976). Isolation, chemical and physical properties of alpha-1-antitrypsin. Biochemistry, 15, 798-804.

NEWBOULD, B.B. (1963). Chemotherapy of arthritis in rats by myco-bacterial adjuvant. Brit.J.Pharmac., 21, 1-10.

OBENRADER, M.F. and PROUTY, W.F. (1977). Production of monospecific antibodies to rat liver ornithine decarboxylase and their use in turnover studies.
J. Biol.Chem., 252, 2866-2872.

OKA,T., KANO, K. and PERRY, J.W. (1978). Studies on SAMD of Mouse Mammary Gland. IN. Advances in polyamine research, Vol.I (Eds. R.A. Cambell, D.R. Morris, D. Bartos, G.D. Daves and F.Bartos). pp 59-69, Raven Press, New York.

OHLSSON, K. (1971). Interaction between human or dog leucocyte proteases and plasma protease inhibitors. Scand.J.Clin. Lab Invest., 28, 225-230.

ONO, M., INOUE, H., SUZUKI, F. and TAKEDA, Y. (1972). Studies on ornithine decarboxylase from the liver of thioacetamide treated rats. Purification and some properties. Biochim. Biophys. Acta, 284, 285-297.

PARROTT, D.P. (1977). An investigation into the possible anti-inflammatory proteins in arthritis. Phd. thesis, University of Aston.

PARROTT, D.P. and LEWIS, D.A. (1977) Protease and anti-protease levels in blood of arthritic rats. Ann.Rheum.Dis., 36, 166-169.

PARTSCH, G., DESSER, H. and TAUSCH, C. (1978). Aliphatic diamines and histamine in the serum of patient with chronic polyarthritis. Z.Rheumatol., 37, 329-334.

PAULUA, H.E. and WHITEHOUSE, M.W. (1973) Nonsteriod anti-inflammatory agents. Ann.Rev.Pharmac., 13, 107-125.

PEARSON, C.M. (1956). Development of arthritis, periarthritis and periostitis in rats given adjuvants. Proc. Soc. Expt. Biol. Med., 91, 95-101.

PEARSON, C.M. (1963). Experimental joint disease. Observations on adjuvant-induced arthritis. J. Chronic Dis., 16, 863-874.

PEARSON, C.M. and WOOD, F.D. (1959). Studies on polyarthritis and other lesions induced in rats by injection of myco-bacterial adjuvant. I. General clinical and pathological characteristics and some modifying factors. Arth.Rheumat., 2, 440-459.

PEARSON, C.M. and WOOD, F.D. (1963). Studies of arthritis and other lesions induced in rats by the injection of mycobacterial adjuvant. VII . Pathological details of the arthritis and spondylitis. Am.J.Path., 42, 73-86.

PERSELLIN, R.H. (1972). Lysosome stabilization by adjuvant arthritis serum. Arth. Rheumat., 15, 144-152.

PERSELLIN, R.H., VANCE, S.E. and PEERY, A. (1974). Effect of pregnancy serum on experimental inflammation. Br.J. Exp.Path., 55, 26-32.

PINALS, R.S. (1973) Effect of hepatic injury on adjuvant arthritis. Ann.Rheum.Dis., 32, 471-474.

PITT, E. and LEWIS, D.A. (1979). Stimulatory action of steroidal anti-inflammatory drugs on plasma alpha-1-antitrypsin levels in the rat. Br.J.Pharmac., 66, 454-455P.

RAINA, A., JÄNNE,J., HANNONEN, P., HÖLTTÄ, E. and AHONEN, J. (1973) Polyamine synthesizing enzymes in regenerating liver and in experimental granuloma. IN: Polyamines in normal and neoplastic growth, Ed.D.H. Russell, Raven Press, New York, pp 167-180.

RICHMAN, R.A., UNDERWOOD, L.E., VAN WYK, J.J. and VOINA, S.J. (1971). Synergistic effect of cortisol and growth hormone on hepatic ornithine decarboxylase activity. Proc.Soc.Exp. Biol.Med., 138, 880-884.

RIMON, A., SHAMASH, Y. and SHAPIRO, B. (1966). The plasmin inhibitor of human plasma VI. Its action on plasmin, trypsin, chymotrypsin and thrombin. J.Biol.Chem., 241, 5102-5107.

RINDANI, T.H. (1956). Recovery of an anti-inflammatory fraction from inflammatory exudate. Indian.J.Med.Res., 44, 673-676.

RINDERKNECHT, H., GEOKAS, M.C., SILVERMAN, P. and HAVERBACK, J.B. (1968). A new ultra sensitive method for the determination of proteolytic activity. Clin.Chim.Acta., 21, 197-203.

ROBERTS, R.G., RIESEN, W.A. and HALL, P.K. (1974). In: Bayer-Symposium V: Proteinase Inhibitors. (Eds.H. Fritz, H.Tschesche, L.J. Greene and E. Truscheit). Springer-Verlag, Berlin. p.63.

ROBINSON, B.V. and ROBSON, J.M. (1964). Production of an anti-inflammatory substance at a site of inflammation. Br. J.Pharmac., 23, 420-432.

ROBINSON, B.V. and ROBSON, J.M. (1966). Further studies on the anti-inflammatory factor found at site of inflammation. Br.J.Pharmac., 26, 372-384.

ROSENTHALE, M.E. (1974) IN: Anti-inflammatory agents II, Academic Press, London, pp 123-192.

RUSSELL, D.H., and SNYDER, D.H. (1969). Amine synthesis in regenerating rat liver: Extremely rapid turnover of ornithine decarboxylase. Mol.Pharmacol., 5, 253-262.

RUSSELL, D.H., BYUS, C.V. and MANEN, C.A. (1976). Proposed model of major sequential biochemical events of a trophic response. Life Sci., 19, 1297-1306.

RUSSELL, D.H., GILES, H.R., CHRISTIAN, C.D. and CAMPBELL, J.L. (1978). Polyamines in amniotic fluid, plasma and urine during normal pregnancy. Am.J. Obstet.Gynaecol., 132, 649-652.

RYAN, G.B. (1967). The origin and sequence of the cells found in the acute inflammatory response. Aust.J.Exp.Biol. Med.Sci., 45, 149-162.

SARCIONE, E.J. and BOGDEN, A.E. (1966). Hepatic synthesis of alpha-2-acute phase globulin of rat plasma. Science, 153, 547-548.

SCHÖNENBERGER, M.,SCHMIDTBERGER, M. and SCHULTZ, H.E. (1958). Alpha-2-macroglobulin. Z. Naturforsch B., 13, 761-772.

SCHROCK T.R.,OAKMAN, N.J. and BUCHER, N.L.R. (1970) Ornithine decarboxylase activity in relation to the growth of rat liver. Biochim. Biophys. Acta, 204, 564-577.

SCHULTZE, H.E., HEIDE, K.and HAUPT, H. (1962). Alpha-1-antitrypsin aus human serum. Klin. Wochenschr., 40, 427-429.

SCHUMACHER, V.G. and SCHLUMBERGER, H.D. (1963). Uber veranderungen der alpha-2-globuline aus serums. Deut. Mech. Wschr., 88, 645-651.

SEILER, N. and ASKAR, A. (1971). A micro method for the quantitative estimation of putrescine in tissues. J. Chromatogr., 62, 121-127.

SIELER, N.V. and WIECHMANN, M. (1967). Die Mikrobestimmung von Spermin und Spermidin als 1-Dimethylaminonapthalin -5-sulfonsäure - Derivate. Hoppe-Seylers. Z. Physiol. Chem., 48, 1285-1290.

SHELDON, W.H. and BAUER, H. (1960) Tissue mast cells and acute inflammation in experimental cutaneous mucomycosis of normal, 48/80 treated and diabetic rats. J.Exp.Med., 112, 1069-1083.

SJÖBLOM, K.G. and WOLLHEIM, F.A. (1977). Alpha-1-antitrypsin phenotypes and rheumatic diseases. Lancet, 1, 41-42.

SMITH, D.B., O'NEILL, A.N. and PERLIN, A.S. (1955). Studies on the heterogeneity of carrageenan. Can.J.Chem., 33, 1352-1360.

SMITH, M.J.H. and FORD-HUTCHINSON, A.W. (1975). An anti-inflammatory substance in normal human plasma. Agents and Actions, 5/4, 318-321.

SMITH, M.J.H., FORD-HUTCHINSON, A.W. and WALKER, J.R. (1977). Anti-inflammatory activity of bacterial endotoxin. J.Pharm.Pharmac., 29, 702-704.

SPECTOR, W.G. and COOTE, E. (1965). Differentially labelled blood cells in the reaction to paraffin oil. J.Path. Bact., 90, 589-598.

SPECTOR, W.G., WALTERS, M.N.H and WILLOUGHBY, D.A. (1965). The origin of the mononuclear cells in inflammatory exudates induced by fibrinogen. J.Path.Bact., 90, 181-192.

SPECTOR, W.G. and WILLOUGHBY, D.A. (1957). Histamine and 5-hydroxytryptamine in acute experimental pleurisy. J. Pathol. Bacteriol. 74, 57-65.

SPECTOR, W.G. and WILLOUGHBY, D.A. (1968). The Pharmacology of Inflammation. English Universities Press, London.

SPILBERG, I. and KIRK-OSTERLAND, C. (1970). Anti-inflammatory effect of the trypsin-kallikrein inhibitor in acute arthritis induced by urate crystals in rabbits. J.Lab. Clin. Med., 76, 472-479.

SWEDLUND, H.A., HUNDER, G.G. and GLEICH, G.J. (1974). Alpha-1-antitrypsin in serum and synovial fluid in rheumatoid arthritis. Ann.Rheum. Dis., 33, 162-164.

SWINGLE, K.F. (1974). Evaluation for anti-inflammatory activity. In: Anti-inflammatory agents, chemistry and pharmacology Vol.II (Eds. R.A. Scherrer and M.W. Whitehouse), Academic Press, p 33-122.

SWINGLE, K.F. and SHIDEMAN, F.E. (1967). Phases of inflammatory response to subcutaneous implantation in the rat. Pharmacologist, 9, 243.

SYMONS, A.M., LEWIS, D.A. and ANCILL, R.J. (1969). Stabilizing action of anti-inflammatory steroids on lysosomes. Biochem.Pharmac., 18, 2581-2582.

TSURUFUJI, S., SUGIO, K. and TAKEMASA, F. (1979). The role of glucocorticoid receptor and gene expression in the anti-inflammatory action of dexamethasone. Nature (Lond.) 280, 408-410.

VAN ARMAN, C.G., BEGANY, A.J., MILLER, L.M. and PLESS, H.H. (1965) Some details of the inflammation caused by yeast and carrageenan (with appendix on kinetics of the reaction). J. Pharmac., 150, 328-334.

VANE, J.R. (1971) Inhibition of prostaglandin synthesis as a mechanism of action for asprin-like drugs. Nature New Biol. 231, 232-235.

VERMA, A.K., RICE, H.M. and BOUTWELL, R.K. (1977). Inhibition of tumour promoter -induced ornithine decarboxylase activity in epidermis by inhibitors of prostaglandin synthesis. Biochem. Biophys. Res. Commun., 79, 1160-1166.

VERMA, A.K. and BOUTWELL, R.K. (1980) . Inhibition of tumour promoter induced mouse epidermal ornithine decarboxylase activity and prevention of skin carcinogenesis by Vitamin A acid and its analolgues (Retinoids). In: Polyamines in biomedical research, (Ed. J.M. Gaugas), pp 185-202, J. Wiley and sons, New York.

VERNON, C.A., HANSON, J.M. and BRIMBLECOMBE, R.W. (1969). Peptides, British Patent No. 1324823.

VERNON-ROBERTS, B., LIYANAGE, S.P. and CURREY, H.L.F. (1976) Adjuvant arthritis in the rat. Distribution of fluorescent material after footpad injection of rhodamine-labelled tubercle bacilli. Ann.Rheum.Dis., 35, 389-397.

VINEGAR, R., MACKLIN, A.W., TRUAX, J.F. and SELPH, J.L. (1971). Histopathalogical and pharmacological study of carrageenan inflammation in the rat. Pharmacologist, 13, 284.

VINEGAR, R., SCHREIBER, W. and HUGO, R (1969). Biphasic development of carrageenan oedema in rats. J. Pharmac. Exp.Ther., 166, 96-103.

VON BUCH, O. and WAGNER-JAUREGG, T. (1960). Zur Problematik antiphlogistischer Teste. Arzneimettel Forsch, 10, 834-836.

VON BUCH, O. and WAGNER-JAUREGG, T. (1962). Hemmung des polyvinylpyrrolidon -Ö dems der Rattenpfote durch intraperitoneale Injektion ödemerzeugender Substanzen. Arzneimittel-Forsch, 12, 639-640.

WALZ, D.T., DiMARTINO, M.J. and MISHER, A. (1971). Adjuvant-induced arthritis in rats. II. Drug effects on physiologic, biochemical and immunologic parameters. J. Pharmac. Exp. Ther., 178, 223-231.

WEISSMANN, G., SMOLEN, J.E. and HOFFSTEIN, S. (1978). Polymorphonuclear leukocytes as secretory organs of inflammation. J.Invest. Derm., 71, 95-99.

WILLIAMS-ASHMAN, H.G., COPPOC, G.L. and WEBER, G. (1972). Imbalance in Ornithine metabolism in hepatomas of different growth rates as expressed in formation of putrescine, spermidine and spermine. Cancer Res., 32, 1924-1932.

WILLIS, A.L. (1969). Parallel assay of prostaglandin-like activity in rat inflammatory exudate by means of cascade superfusion. J.Pharm.Pharmac., 21, 126-128.

WINTER, C.A., RISLEY, E.A. and NUSS, G.W. (1962). Carrageenan induced oedema in hind paw of the rat as an assay for anti-inflammatory drugs. Proc. Soc. Expt.Biol.Med., 111, 544-547.

WOOLLEY, D.E., ROBERTS, D.R. and EVANSON, J.M. (1976). Small molecular weight Bl serum protein which specifically inhibits human collagenases. Nature, 261, 325-327.