THE INHIBITORY EFFECT OF CYCLIC 3',5' ADENOSINE MONOPHOSPHATE AND PUTRESCINE IN INFLAMMATION

A thesis submitted by

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SUMMARY

1. The adenylate cyclase activators (salbutamol, isoprenaline and sodium phenobarbitone) were effective in inhibiting the carrageenan-induced oedema in rats, with isoprenaline being the most anti-inflammatory.

2. Isoprenaline was short-acting and significantly inhibited the carrageenan oedema only at 3 hours after the injection of carrageenan. Salbutamol significantly inhibited the carrageenan oedema at both 3 and 5 hours after carrageenan injection.

3. The anti-inflammatory effect of the β-adrenergic agonists, isoprenaline and salbutamol were inhibited by the β-adrenergic antagonist, propranolol.

4. The anti-inflammatory effect of the β-agonists coincided with the increase in plasma cyclic AMP level in rats measured at 3½ hours and 5½ hours after being given the drugs.

5. The phosphodiesterase inhibitor, 3-isobutyl-1-methyl xanthine was also anti-inflammatory in the carrageenan oedema.

6. DL-α-difluoromethyl ornithine, an irreversible inhibitor of ornithine decarboxylase, prevented the isoprenaline inhibition of carrageenan oedema by about 11%, suggesting the involvement of putrescine in the anti-inflammatory effect of isoprenaline.

7. Theophylline and salbutamol both inhibited the development of adjuvant arthritis in the rats.

8. The putrescine level in the theophylline-treated adjuvant rats killed on day 26 was twice that of the controls.

9. Putrescine inhibited the carrageenan oedema when injected together with carrageenan into the hind paw of the rats.

10. Putrescine also inhibited serotonin-induced oedema in the rats.

11. Putrescine was also shown to scavenge superoxide anions generated from the stimulation of guinea-pig polymorphonuclear leucocytes by N-formyl-methionyl-leucyl-phenylalaine (FMLP).

12. Putrescine failed to inhibit the development of granuloma in the cotton pellet test.

13. 1,3 diaminopropane was shown to be anti-inflammatory in the carrageenan oedema but not 1,2 diaminoethane.

14. Anti-inflammatory effect of drugs that raise cyclic AMP level may be due to cyclic AMP and polyamines.

KEY WORDS : Isoprenaline, salbutamol, cyclic AMP, putrescine, Anti-inflammatory.

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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 when the inflammation is chronic it can be serious. In man, the most prevalent inflammatory conditions are known collectively as the rheumatic diseases. This is the overall name given to diseases characterized primarily by pain and stiffness in joints, bones, muscles, tendons or ligaments. These diseases include a large number of disorders such as rheumatoid arthritis, osteoarthrites, ankylosing spondylitis, gout etc.

Rheumatoid arthritis is a common destructive joint disease. Rheumatoid arthritis, though not life threatening can be a serious condition since severe forms of the disease can quickly lead to loss of mobility. The chronic inflammatory lesions occurring in the joints can cause pain, swelling and deformity in the affected parts and may lead to considerable destruction of tissues and cause life-long crippling. The systemic effects of rheumatoid arthritis include cardiovascular, haematological, neurological and pulmonary abnormalities.

The severity usually fluctuates, giving some remissions followed by exacarbation. The disease is of world-wide distribution. Women are generally more susceptible than men.

1.1 The Inflammatory Process

When living tissues are injured, a characteristic series of changes follows in the small blood vessels and related tissues within the damaged area. This process may be considered as a protective function designed to minimize or eliminate the cause of damage and subsequently to remove the damaged tissue and lead to its repair.

The changes that occur in the first few hours after injury involve three processes:

(i) brief arteriolar vasoconstriction (a few seconds) followed by prolonged dilatation of arterioles, capillaries and venules which is caused by the liberation of vasoactive compounds from the injured tissue itself. The dilatation of the blood vessels causes increased blood flow to the damaged area (Spector and Willoughby, 1968).

(ii) increased vascular permeability of the vessels due to the process of vasodilatation. This causes loss of fluid into the surrounding tissue which leads to the formation of local swelling or oedema (Cotran, 1967). The permeability changes are due to the openings of the endothelial cell junctions (Bohm, 1977). The accumulation of fluid itself has a function in diluting toxic materials such as irritants or bacterial toxins thereby reducing the intensity of tissue damage.

(iii) emigration of leucocytes from circulating blood into the extravascular tissues. The changes in the vascular channels together with the altered permeability cause an increase in the viscosity

of the blood which is responsible for pushing the leucocytes against the vascular endothelium to which they adhere and eventually migrate into the extravascular tissues.

These processes give rise to the four characteristic signs of acute inflammation : redness, heat, swelling and pain.

There are many mediators involved in the development of oedema in inflammation.

Histamine is the major mediator in the initial phase of the response. Capillary and arteriolar responses and permeability changes are almost entirely mediated by histamine released from mast cells (present in almost all tissues). A variety of evidence supports this view. 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). Tissue depletion of histamine has been shown to delay the onset of inflammation produced by injection of bacteria (Shelden and Bauer, 1960). Vascular permeability in the initial phase of inflammation can be inhibited by anti-histamine treatment (Hurley and Spector, 1965). In rats and mice, 5-hydroxytryptamine is released at the same time as histamine in this phase.

In the later phase, many mediators may be involved in the response of which kinin, prostaglandin and complement systems seem most important. In carrageenan-induced oedema in the rat, kinins are released during the second phase (Di Rosa, Giroud and Willoughby,

1971). The kinins are a family of polypeptides and include bradykinin, kallidin and leucokinin. Like histamine, kinins can produce vasodilatation, increase in vascular permeability and pain (Elliot et al. 1960; Elliot et al. 1963). Kinins are formed by enzymatic degradation of a precursor (a kininogen) such as plasma α-2-globulin by a proteolytic lysosomal enzyme called kallikrein.

Prostaglandins are long chain fatty acids which are released whenever tissues are mechanically or chemically stimulated (Ferreira and Vane, 1967). In inflammation, enzyme phospholipase-A_2 released initially from damaged cells and later on from phagocytozing polymorphonuclear leucocytes (Anderson, Brocklehurst and Willis, 1971) converts membrane phospholipids to arachidonic acid. Arachidonic acid can then be metabolized via the cyclo-oxygenase pathway giving rise to various types of metabolites, like the prostaglandins, prostacyclin and thromboxanes, or via the lipoxygenase pathway, giving rise to the leukotrienes and various types of polyunsaturated hydroxy fatty acids (Higgs, Moncada and Vane, 1980).

Prostaglandins, mainly from the E series and predominantly prostaglandin E_2 are found in numerous types of inflammatory response in experimental animals and man (Ferreira and Vane, 1979). Thromboxanes and prostacyclin are also generated in inflammation. Comparable concentrations of thromboxane B_2, 6-oxo-PGF_1 and prostaglandin E_2 have been detected in carrageenan-induced inflammatory exudates in the rat (Higgs and Salmon, 1979) and thromboxane B_2 has also been found in joint fluids aspirated from patients with rheumatoid arthritis (Trang et al. 1977).

E-type prostaglandins exert pro-inflammatory actions, such as vasodilatation (Crunkhorn and Willis, 1971), increase vascular permeability (Arora et al, 1970) and cause pain (Ferreira, 1972). They also potentiate the action of other inflammatory mediators (Vane, 1976) and prostaglandin E_2 has also been shown to be chemotactic for rabbit polymorphonuclear leucocytes (Higgs, McCall and Youlten, 1975). It has been suggested that prostacyclin, which is a potent vasodilator, contributes to the exudate formation in inflammation but the role played by thromboxanes is less clear (Higgs and Salmon, 1979; Capasso et al, 1975). Thromboxane B_2 is chemotactic for mouse polymorphonuclear leucocytes (Kitchen et al, 1978) but not for human leucocytes (Goetzl and Gorman, 1978).

The lipoxygenase products of arachidonic acid metabolism which are also formed in inflammation (Bragt and Bonta, 1979; Siegel et al, 1981) are mainly considered as chemotactic agents for polymorphonuclear leucocytes (Goetzl and Sun, 1979; Ford-Hutchinson, Bray and Smith, 1980; Palmer et al, 1980). Leukotriene B_4 is one of the most potent endogenous chemotactic factor discovered (Ford-Hutchinson et al, 1980). It has been reported that leukotriene B_4 is equipotent with prostaglandin E_2 and prostacyclin in enhancing bradykinin-induced plasma exudation in rabbit skin (Eakins et al, 1980c, 1981; Higgs et al, 1981; Bray et al, 1981) and it has also been shown that vasodilator prostaglandins synergize with leukotriene B_4 to produce plasma exudation (Bray et al, 1981; Wedmore and Williams, 1981).

Many of the non-steroidal anti-inflammatory drugs are known to inhibit prostaglandin synthesis which is thought to be their mode

of action (Ferreira and Vane, 1979) and indeed, the ability of these compounds to inhibit prostaglandin synthesis seems to parallel their therapeutic efficacy.

The complement system (C') is a special group of normal serum proteins (C'1 to C'9) which interact sequentially to affect a variety of inflammatory events. Activation of the complement cascade leads to the generation of biologically active proteins which may act as humoral inflammatory mediators. These active proteins can mediate changes in vascular permeability (Lepow, 1971). They also attract both polymorphonuclear and mononuclear leucocytes, influence the release of lysosomal enzymes, enhance phagocytosis and damage cell membranes, ultimately causing lysis of target cells.

For the cellular event in inflammation, the main cellular constituents of the inflammatory reaction are the polymorphonuclear leucocytes and the mononuclear cells (monocytes, macrophages). In most acute inflammations, the polymorphonuclear leucocytes are the first cells to enter the injured area, followed in several hours to days by mononuclear cells (Ryan, 1967). All leucocytes behave similarly in their entry to the inflammation site. They adhere briefly to the inner surface of the vascular endothelium (margination) and then migrate into the tissues by amoeboid motion. Passage occurs mainly through the walls of venules and takes place through the endothelial cell junctions. The gap closes up behind the leucocyte and the basement membrane is penetrated with similar ease and is easily reconstituted. Polymorphs usually predominate for 24 - 72 hours. The emigration of leucocytes appears to be divorced from the leakage

of protein and indeed emigration can take place with little or no permeability increase (Hurley, 1963).

The migration of the cells is usually assisted by the process of chemotaxis, the chemical attraction of cells by mediators. The most important mediator of leucocyte chemotaxis is the fifth component of complement which can be generated by activation of the complement pathway. Damaged tissues, bacteria, bacterial constituents and immune complexes all activate complement and lead to leucocyte chemotaxis.

Once in the tissues the leucocytes start to phagocytoze and digest foreign materials and to demolish the severely damaged tissue prior to repair. Polymorphonuclear 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^-, $\dot{OH}$ and H_2O_2) which have a bactericidal action and inflammatory mediators such as prostaglandins and thromboxanes are released. These polymorphonuclear leucocytes also secrete lysosomal proteinases which aid tissue demolition.

When the foreign material has been removed, the repair process begins by the development of granulation tissue. Fibroblast cells migrate into the area from surrounding connective tissue and deposit a layer of collagen fibres around the lesion.

After the granulation phase, the tissue to some extent reverts to a normal condition, with the addition of considerable deposited fibres, forming scar tissue.

If the stimulus is persistent, a lesion develops in which acute inflammation, demolition, repair and (sometimes) regeneration co-exist in the inflamed area. This persisting process, during which destruction and inflammation are proceeding at the same time as attempts at repair, is chronic inflammation.

1.2 The Pathogenesis of Rheumatoid Arthritis

1.2.1 The Normal Joint

In a normal joint, the ends of the bones are covered with a smooth elastic cartilage, which consists of collagen fibres (giving strength) and proteoglycans (binding the fibres together and giving elasticity) (Anderson et al, 1964; Muir et al, 1970).

The joint itself is enclosed in a capsule made of tough fibrous tissue. The inner layer of this capsule consists of specialized connective tissue cells called the synovium. Within the joint space is found the synovial fluid which consists of a dialysate of plasma with the addition of hyaluronic acid and hyaluronate protein (Sandson and Hamerman, 1962). The hyaluronate protein and hyaluronic acid are synthesized by the synovial lining cells themselves. The synovial fluid has two functions, firstly to provide nutrition to the avascular cartilage and secondly to act as a lubricant between cartilage surfaces. The schematic diagram of a normal joint is shown in Figure 1.

The synovial lining cells were found to be of 2 types (Barland, Novikoff and Hamerman, 1962). Type A are phagocytic and the cells are rich with lysosomes. It was suggested that they have a dual function, both secretory and phagocytic.

The type B cell is structurally adapted for a synthetic role, producing hyaluronic acid and hyaluronate protein. Coulter (1962)

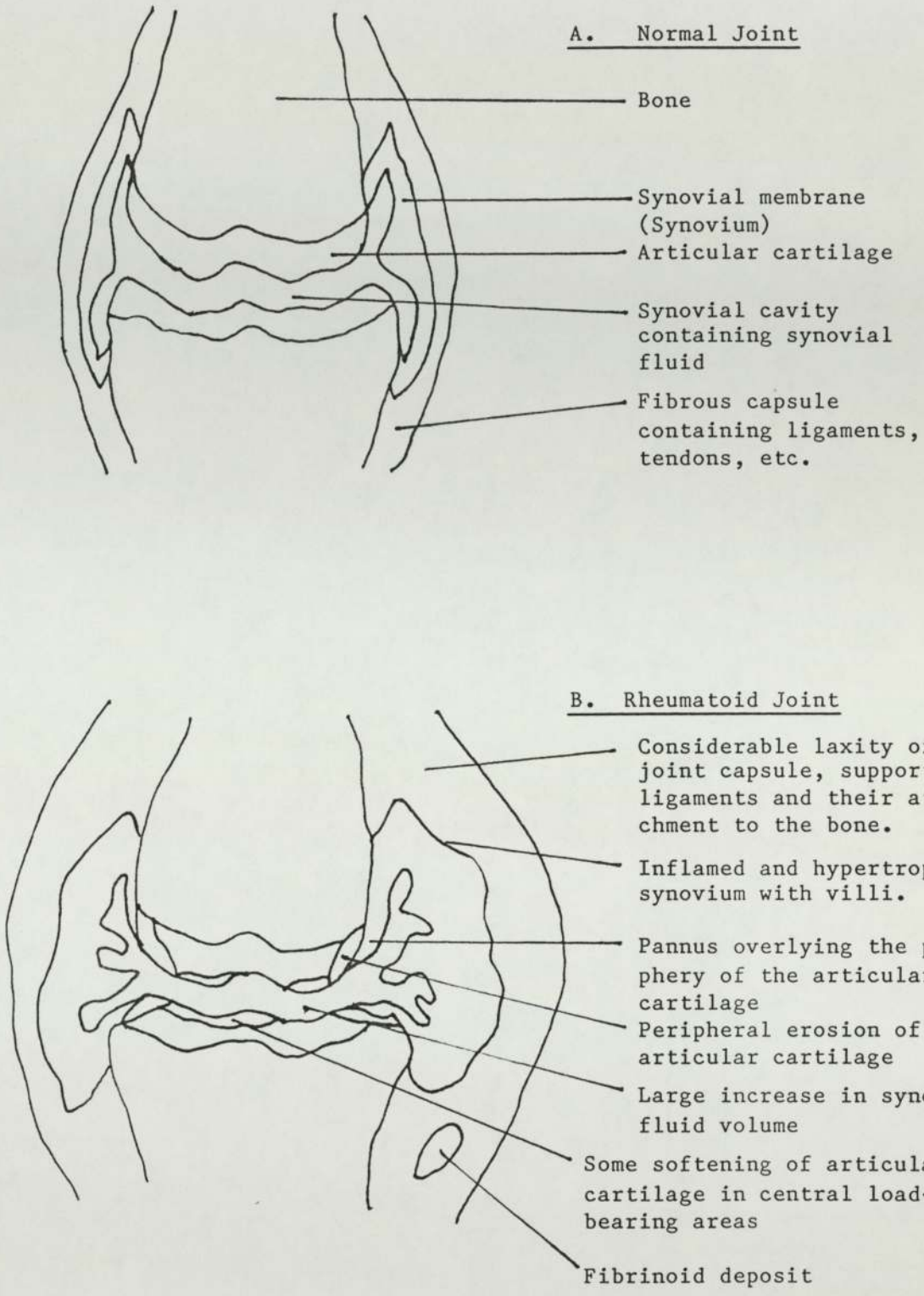

Figure 1 The Schematic Diagram of a Normal and a Rheumatoid Joint

noted a well-developed endoplasmic reticulum characteristic of protein producing cells.

Hyaluronic acid is an important constituent of synovial fluid, making it viscous so that it can function as a lubricating agent for the relatively rough cartilage surfaces (Walker et al, 1970).

1.2.2 The Rheumatoid Joint

Human rheumatoid joints are generally swollen and inflamed. The lesions occur mainly in the synovial membranes of the smaller joints. The condition starts as an acute inflammation of the joint. The earliest changes are capillary and arteriolar vasodilatation followed by vascular congestion and oedema with subsequent development of a cell-rich infiltrate. The normal one or two-cell layer thick synovial membrane and loose connective tissue which cover the joint capsule becomes thickened in rheumatoid arthritis and in the active phases of this chronic disease shows marked hyperplasia and hypertrophy (Hamerman et al, 1969) with multilayered cell proliferation to the extent that displaced tissue forms folds or villi on the surface.

The changes in the synovium are accompanied by the formation of excessive amounts of synovial fluid which is made up of local secretions and blood transudate with a high protein content, a large number of polymorphonuclear leucocytes but lesser number of lymphocytes, mononuclear cells consisting of shed synovial cells as well as monocytes and secretory products of cells (for example, immunoglobulins, lymphokines, kinins, enzymes etc).

The synthesis of hyaluronic acid is increased in the rheumatoid synovial tissue but it is not polymerized to the normal extent and has a lower molecular weight (Castor et al, 1966). This causes a fall in the viscosity of the synovial fluid and can lead to increased stress and friction on the cartilage surfaces.

In the region of the capsular attachment to the margins of bone where the synovium is in close approximation to the cartilaginous bone ends, characteristic erosions of cartilage and bone are found, covered with tissue, termed the pannus, which is an extension of the vascular synovial connective tissue and which is believed to be the source of degradative enzymes which destroy cartilage and bone. The schematic diagram of a rheumatoid joint is shown in Figure 1.

In rheumatoid arthritis, the degradation of cartilage, bone and other connective tissue of the joint occurs predominantly in regions contiguous with the hyperplastic pannus although proteoglycans may be degraded from regions of the articular cartilage remote from the pannus (Janis and Hammerman, 1969). Electron microscopy shows that there is loss of collagen fibrils in the cartilage immediately ahead of the advancing pannus, suggesting that enzymatic degradation of fibres is the first step in joint structural damage (Krane, 1974). Pannus formation can lead to distortion of the joint resulting in permanent deformity and eventual solidification of the joint (ankylosing).

Pannus contains a heterogenous population of cells including synovial cells, fibroblasts, lymphocytes (T lymphocytes probably more

than B lymphocytes), monocytes and endothelial cells (Kobayashi and Ziff, 1975). In contrast to the synovial fluid, pannus usually contains relatively few polymorphonuclear leucocytes.

Coagulated fibrin can form a layer on the synovial surface which impairs the free movement of the opposed articular surfaces, causing further damage to the membrane.

The destruction of joint structures in arthritis may result from the failure of normal mechanisms controlling the interactions among cells of the various tissues of the joint. The destruction of extracellular matrices of joint tissues during arthritis may be mediated in part by proteinases synthesized by the tissues themselves. It has been shown that cultured monocytes aided by T lymphocytes produce a monokine-like material, designated mononuclear cell factor (MCF) which stimulates the biosynthesis and release of neutral proteinases such as collagenase and proteoglycanase, and prostaglandin E_2 from the macrophage type synovial cells (Murphy et al, 1981).

These proteinases could degrade connective tissue macromolecules such as collagen and proteoglycan at neutral pH. Cultures of rheumatoid synovium has long been known to produce large amounts of collagenase and prostaglandin E_2 (Dayer et al, 1977). A synovial factor (SF) obtained from cultures of either normal or rheumatoid synovial fragments can also stimulate the production of prostaglandin E_2 and neutral proteinases by cells derived from human synovium, cartilage and bone (McGuire et al, 1982).

Normal human synovium and cartilage in cultures produce collagenase in a latent form and the cultures also produce a neutral proteinase inhibitor called TIMP (tissue inhibitor of metallo-proteinases) (McGuire et al, 1981; Murphy et al, 1981). TIMP is produced in a considerable amount by normal synovium but it cannot be detected in cultures of rheumatoid synovium (McGuire et al, 1981). The result suggests that TIMP may be important in determining the amount of active enzyme present in human synovial tissue. Latent collagenase can be activated by proteinases such as plasmin, produced by the action of plasminogen activator on plasminogen. Plasminogen activator can be synthesized by macrophages and synovial cells (Werb et al, 1977).

The stimulation of synovial cells as well as chondrocytes and bone cells by materials such as MCF and SF to produce prostaglandins may contribute to the high levels of prostaglandin E_2 found in synovial fluids from patients with inflammatory joint diseases (Robinson and Levine, 1974). Enhanced production of prostaglandin by cells of joint tissues could alter vascular permeability and could also stimulate bone resorption (Robinson et al, 1975a; Robinson et al, 1975b). Prostaglandins could also regulate the proliferation of the synovial pannus and be involved in the mobility and activity of the synovial cells (Dayer et al, 1979). Dayer and Krane (1978) showed that production of collagenase is dependent on the presence of small amounts of prostaglandin E_2 and suggested that prostaglandin may modulate production of collagenase by synovial cells.

1.2.3 The Immunopathology of Rheumatoid Arthritis

The exact cause of rheumatoid arthritis remains unknown. Infectious organisms, hereditary, hormonal abnormality, metabolic defects, diet and climate are among the number of factors proposed as the cause of the disease. Marmion (1976) concluded that there was no direct evidence that infectious organisms such as viruses and mycoplasmas were the cause of rheumatoid arthritis.

For the present, it is widely believed that the inflammation in rheumatoid arthritis is triggered by immune processes. An abnormal immune response is thought to form an integral part of the pathogenesis of inflammation in rheumatoid arthritis. The pathogenesis of rheumatoid arthritis appears to involve both humoral and cell mediated immunity (Yu and Peter, 1974).

Approximately 80% of patients with rheumatoid arthritis are seropositive. This means that rheumatoid factor (antibody) is present in the serum. Rheumatoid factors are immunoglobulins with antibody specificity for antigenic site on the Fc part of the heavy chain of immunoglobulin G(lgG). Rheumatoid factors are mostly of lgM class of immunoglobulins but they can be of lgG, lgD or lgA class. Diagnosis of rheumatoid arthritis frequently involves the detection of rheumatoid factor in the serum or the joint fluid (only lgM rheumatoid factor is recorded in routine testing).

Some patients with sustained rheumatoid arthritis and deformities may remain seronegative but there is a high degree of clinical

correllation between the presence of rheumatoid factor and subcutaneous nodules and sustained, symmetrical, deforming hands and wrists arthritis and visceral manifestations (Christian and Paget, 1978).

lgG rheumatoid factor is found in high concentrations in the joint fluid. The resulting lgG/anti-lgG immune complex found in both seropositive and seronegative rheumatoid arthritis may play a major pathogenic role in synovitis (Paget and Gibofsky, 1979).

Circulating antibody alone is not responsible for the joint lesions seen in rheumatoid arthritis as seropositivity may be present in other diseases such as scleroderma and tuberculosis where a destructive arthritis is not a characteristic feature (Christian and Paget, 1978).

The source of the rheumatoid factor and other immunoglobulins in rheumatoid arthritis is thought to be the synovial membrane. It has been suggested that the rheumatoid membrane acts like an ectopic lymph node populated by B lymphocytes and plasma cells, synthesizing immunoglobulins which then diffuse into the blood and synovial fluid (Smiley et al, 1968).

The pathogenic role of the rheumatoid factor in rheumatoid arthritis has not been clearly defined, although most investigators beleived that it is intimately involved in the inflammatory reaction leading to tissue damage in rheumatoid arthritis. Many studies have shown that immune complexes consisting of rheumatoid factor bound to its antigen (immunoglobulin) and binding complement are commonly found

in the synovial membrane and synovial fluid of rheumatoid arthritis patients (Zvaifler, 1973; Winchester et al, 1970).

Hollander et al (1965) suggested that rheumatoid factor was formed against IgG which had been denatured by some unknown pathological influence, leading to the formation of high molecular weight antigen-antibody complexes of IgG - IgM.

Cell mediated immunity also appears to be involved in the pathogenesis of rheumatoid arthritis. Recent work suggests that antibody production results from the failure of T lymphocytes to control and coordinate the immune response to an unknown stimulating antigen (Paget and Gibofsky, 1979). This leads to a continuation of antibody production due to a lack of T lymphocyte suppression - the perpetuation of what should have been a transient process. There is also evidence to suggest that lymphokines (a product of stimulated T cells) are able to greatly increase the production of collagenase and prostaglandin E_2 from rheumatoid synovial tissue (Dayer et al, 1977). Thus the products of T lymphocytes could activate rheumatoid synovial cells to produce an enzyme which destroys cartilage and subsequently leads to bone dissolution. This provides an important link between the immune response and joint destruction.

A hypothesis concerning the pathogenesis of inflammation of rheumatoid arthritis is shown in Figure 2. It postulates that an unknown antigenic stimulus induces an immune response resulting in antibody production and subsequent immune complex formation (for example, IgG - anti-IgG or an unknown antigen x - anti-x). Such

immune complexes activate the cascade of complement-generating factors that promote chemotactic migration of polymorphonuclear leucocytes and macrophages to the joint. Deposits of immunoglobulin and complement have been demonstrated in the acutely inflamed vessel walls of rheumatoid arthritis patients as well as in the synovial membrane, synoviocytes and synovial exudate cells (McDuffie, 1978). Levels of complement components are reduced in the synovial fluid in a majority of patients with rheumatoid arthritis (Pekin and Zvaifler, 1964). This implies that complement is being consumed by active immune processes taking place in the joint itself.

The polymorphonuclear leucocytes and macrophages phagocytose the complexes and release lysosomal enzymes causing articular damage. T lymphocytes become sensitized by the antigenic stimulus and produce lymphokines which induce macrophages and synovial cells to release prostaglandins and collagenase which cause cartilage degradation.

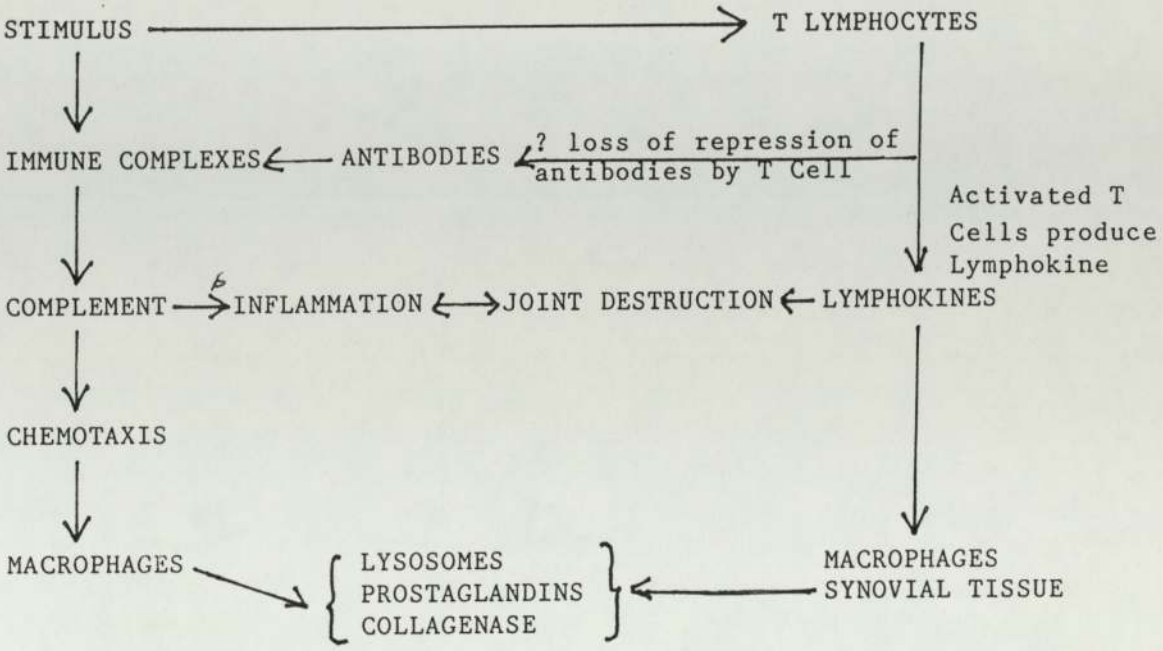

Figure 2 : Pathogenesis of Rheumatoid Arthritis

1.3 Lysosomes in Inflammation

It has been shown that lysosomal enzymes are selectively released when polymorphonuclear leucocytes or macrophages phagocytoze particles such as immune complexes, micro-organisms or zymosan fragments (Weissmann et al, 1971; Wright and Malwista, 1973; Ringrose, Parr and McLaren, 1975). Several mechanisms have been proposed to explain this selective release of enzymes.

When a polymorphonuclear leucocyte encounters a suitable ingestible particle, the surface membrane of the cell invaginates and surrounds it. The vesicle containing the particle, now called a phagosome or phagocytic vesicle, pinches off from the surface and is introduced into the cytoplasm of the cell (Mudd et al, 1934; Zucker-Franklin and Hirsch, 1964; Korn and Weissman, 1967). Digestion of the engulfed particle is initiated by the fusion of the lysosomal membranes with the phagosome membrane to form a phagolysosome. The lysosomes subsequently discharge their contents into the phagosome, a process called degranulation (Hirsch and Cohn, 1960; Cohn and Hirsch, 1960).

It has been suggested that the lysosomal enzymes are not entirely kept to the phagolysosome but some are discharged into the surrounding tissue. This might happen if there is premature merging of a lysosome with a phagosome before the phagosome is entirely sealed off. This is termed 'regurgitation during feeding'. There is thus a route out of the cell for loss of enzymes (Weissmann, Zurier and Hoffstein, 1972). Such a process has been observed by electron microscopy (Zucker-Franklin and Hirsch, 1964).

Another mechanism which has been proposed considers that the membranes of the phagolysosomes might also merge with the outer cell membrane during the process of digestion thus releasing the lysosomal constituents. This is termed "reverse endocytosis" (Weissmann, Zurier and Hoffstein, 1972) or "frustrated phagocytosis" (Henson, 1971; Becker and Henson, 1973; Henson, 1974). This selective release of lysosomal constituents might happen when the cells encounter immune complexes or aggregated immunoglobulins deposited on solid surfaces (for example, collagen membranes) (Hawkins, 1971). They adhere to the surface of the immunoglobulins and selectively release their lysosomal enzymes.

The large quantities of destructive enzymes present in polymorphonuclear leucocyte lysosomes include at least 20 different substances such as acid phosphatase, lipase, β-glucuronidase, sulfatases, esterases and proteinases (Weissmann, 1974). These enable the polymorphs to destroy a wide variety of foreign matter. Evidence that lysosomal enzymes are probably important mediators of both tissue destruction and inflammation was presented by Weissmann, Spilberg and Krakauer (1969). They found that they were able to induce chronic inflammation and cartilage degradation by injecting lysates of purified lysosomes into the joints of experimental animals. With respect to the tissue destruction observed in inflammatory reactions, the most important lysosomal enzymes are the proteinases (Weissmann, 1977). These proteinases such as collagenase and elastase are capable of degrading two major components of extra-cellular matrix : collagen and proteoglycan. These proteinases may also contribute to inflammatory reactions by generating chemotactic factors from

complement C5 (Ward and Hill, 1970) and releasing kinin-like materials from kininogens (Movat et al, 1973).

Phospholipase A_2 released by lysosomes can interact with cell membrane phospholipids with a resultant increase in arachidonic acid (Kunze and Vogt, 1971; Anderson, Brocklehust and Willis, 1971). This leads to an increase in the production of pro-inflammatory arachidonic acid metabolites, for example, prostaglandins and leukotrienes.

Polymorphonuclear leucocyte lysosomes also contain a cationic protein capable of increasing vascular permeability as a result of mast cell disruption (Janoff et al, 1965).

Large quantities of lysosomal enzymes are found in rheumatoid synovial fluid compared with normal synovial fluid and also compared with other inflamed tissues (Luscombe, 1963; Coppi and Borardi, 1968; Andersson, 1970).

1.4 The Role of Oxygen Radicals in Inflammation

When a polymorphonuclear leucocyte or a macrophage encounters a phagocytable particle or when their membranes are stimulated by the appropriate stimuli, for example, phorbol myristate acetate and concanavalin A, both these cells undergo a 'respiratory burst' which is characterised by an increase in oxygen consumption, activation of the hexose monophosphate shunt and generation of oxygen derived free radicals and their metabolic products. The active species of oxygen include superoxide anion (O_2^-), hydroxyl radical ($OH^\bullet$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$).

The enzyme system responsible for the increase in oxygen consumption and superoxide anion generation has been identified as a membrane-associated nicotinamide adenine nucleotide oxidase (Patriarca et al, 1971; Babior et al, 1976; McPhail et al, 1976; Nakamura et al, 1981).

Detail reviews of the mechanism of the production of oxygen radicals by phagocytosing leucocytes have been given by Badwey and Kanovsky (1980) and Fantone and Ward (1982).

Hydrogen peroxide is formed by the interaction of two superoxide dismutase (SOD).

$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

In the presence of traces of free iron salts as catalyst,

superoxide anion and hydrogen peroxide can react together to produce the highly toxic hydroxyl radical ($OH^{\cdot}$) (Halliwell, 1981). This reaction can be expressed as follows:

$$Fe^{2+} + H_2O_2 \longrightarrow OH^{\cdot} + Fe^{3+} + OH^{-} \quad (a)$$

Reaction (q) is referred to as the Fenton reaction. The iron II required for the reaction is thought to be derived from the more stable iron III form by a reductive process involving superoxide:

$$Fe^{3+} \text{ complex} + O_2^{-} \longrightarrow Fe^{2+} \text{ complex} + O_2 \quad (b)$$

with a net result :

$$H_2O_2 + O_2^{-} \longrightarrow O_2 + OH^{\cdot} + OH^{-} \quad (c)$$

Reaction (c) is referred to as the iron catalysed Haber-Weiss reaction.

The hydroxyl radical is considered the most toxic of the univalent reduction products derived form oxygen. It is able to attack or destroy cell membranes, deoxyribonucleic acid and most other cell constituents (Wilson, 1978).

The oxygen radicals are implicated in the generation and development of inflammation. In vitro studies have shown that hydroxyl

radicals produced in the presence of iron degrades hyaluronic acid responsible for the lubricating properties of normal synovial fluid, thus increasing its susceptibility to degradation by the lysosomal enzyme β-N-acetylglucosaminidase A (McCord, 1974; Greenwald and May, 1980; Wong et al, 1981).

Fong et al (1973) reported the peroxidation of lysosomal membranes by hydroxyl radical resulting in the rupture of the membranes and release of the lysosomal enzymes. Lysosomal membrane contains large amounts of polyunsaturated fatty acids which can undergo lipid peroxidation. Lipid peroxidation is an autocatalytic process which can be induced by hydroxyl radicals (Tappel, 1975; Gutteridge and Stock, 1976; Gutteridge, 1977). In this process, the subsequent generation of organic oxygen radicals is followed by the formation of lipid peroxides (Pryor, 1976) which can further fragment to produce gaseous hydrocarbons and reactive aldehydes, which may damage structural membranes (Riley et al, 1974; Gutteridge, 1980). Such damage to lysosomes can amplify destructive processes by the release of hydrolytic enzymes.

In cells, superoxide anions can be scavenged by superoxide dismutase, and hydrogen peroxide can be catalysed by catalase to water and molecular oxygen (Fridovich, 1975; Halliwell, 1978). However, superoxide dismutase and catalase are either found to be absent (Blake et al, 1981) or their levels low in synovial fluid (Igari, 1982; Biemond, 1984).

In vitro studies have shown that superoxide anions have the capacity to generate chemotactic factors. When human plasma is incubated with a hypoxanthine-xanthine oxidase system which can produce superoxide anion and hydroxyl radical, a potent chemotactic agent is generated (Petrone et al, 1980). Further studies have demonstrated the production of a chemotactic lipid from arachidonic acid follwoing incubation with an O^{2-} and H_2O_2 generating system (Perez and Goldstein, 1980). Thus it appears that oxygen metabolites from phagocytic cells may function as a positive feedback mechanism to potentiate the inflammatory response through the generation of chematactic factors.

An additional mechanism by which phagocyte-derived free radicals may augment the inflammatory response is by the inactivation of normal serum inhibitors of leucocyte proteinases, thereby causing an increased destruction of the structural components of tissue such as elastin (Carp and Jonoff, 1979; Matheson et al, 1979). In vitro studies have shown that hydroxyl radicals generated from human neutrophils that are phagocytosing immune complexes, inhibit α-1-proteinase inhibitor activity, the primary inhibitor of leucocyte elastase.

Superoxide anion and hydroxyl radical have been shown to be directly involved in the induction of inflammation (Ohmori et al, 1978). The injection of a hypoxanthine-xanthine oxidase system into the hind foot paw of the rats produced an acute oedema which

was maximum at about 20 minutes after the injection. The foot oedema was inhibited slightly when superoxide dismutase or catalase was injected together with the hypoxanthine-xanthine oxidase system but when both enzymes were used in combination, the inhibition of foot oedema was enhanced. Hydroxyl radical scavengers inhibited the foot oedema markedly suggesting that hydroxyl radical plays a major role in the hypoxanthine-xanthine oxidase induced foot oedema. Hydrogen peroxide has been shown to induce histamine release from rat mast cells. (Ohmori et al, 1979) and superoxide anion has been demonstrated to induce the release of serotonin from human platelets (Handini et al, 1977). Hydroxyl radical has also been suggested to increase vascular permeability, by degrading or inactivating a natural anti-inflammatory protein which is synthesized in vivo to regulate endothelial cell contraction (Oyanagui, 1981).

1.5 Animal Models Used in the Investigation of the Inflammatory Process

There have been many attempts to produce experimental arthritis. In 1960, Gardner published a comprehensive review and classified these experiments according to the method of induction : chemical, physical infective, endocrinological and immunological.

Experimental models of inflammation are used to investigate the mechanism of the inflammatory process and screening of potentially useful anti-inflammatory drugs, but none of the models employed in the laboratory provide adequate simulation of the pathophysiological events underlying the clinical disorder of rheumatoid arthritis. Man appears to be unique in his ability to develop rheumatic diseases.

Therefore, the anti-inflammatory activity of a drug should be tested on several models in vivo which together can mimic a broad spectrum of acute and chronic inflammatory events. The acute models of inflammation are concerned mainly with the manifestation of one of the cardinal signs of inflammation (such as swelling, reddening) without the involvement of joint pathology. In the chronic animal models, the major emphasis is on the articular disease where destructive changes occur in the articular cartilage and bone. Swingle (1974) has reviewed many of the methods of evaluating anti-inflammatory activity.

A commonly used acute inflammatory reaction for screening anti-inflammatory drugs is the carrageenan-induced rat paw oedema

(Winter, Risley and Nuss, 1962). The method is based on the inhibition of an induced swelling of the rat's paw caused by an injection of a small amount of carrageenan solution into the plantar foot-pad of the rat's hind paw. Carrageenan is not known to be antigenic and therefore the inflammatory response is entirely due to the local irritative stimulus. The carrageenan-induced oedema is highly reproducible and can be inhibited by both steroidal and non-steroidal anti-inflammatory drugs (Winter, Risley and Nuss, 1962).

Carrageenan is a mixture of polysaccharides composed of sulphated galactose units and is derived from Irish sea moss (Chondrus Crispus) (Smith et al, 1955). The structural integrity of the polysaccharide is essential for its oedemogenic activity, shown by the loss of some activity by heat denaturation (Vinegar, Schreiber and Hugo, 1969).

From the experiments on the mediators of the carrageenan oedema it seems that there are three distinct phases in the development of the odema, namely an initial release of histamine and 5-hydroxytryptamine, a second phase mediated by kinins and finally a third phase, the mediator of which is suspected to be prostaglandins (Di Rosa, Giroud and Willoughby, 1971).

In the earliest phase of the carrageenan oedema (0-90 minutes) histamine and 5-hydroxytryptamine are released at the same time and in such a concentration that each amine is exerting a maximal effect on vascular permeability. This simultaneous release of two mediators explains the failure of anti-histamine or 5-hydroxytryptamine antagonist

given singly to modify the early foot-oedema. This first phase begins immediately after the injection of carrageenan and diminishes within an hour.

The second phase (90-150 minutes) can be inhibited by cellulose phosphate, a kininogen depleting agent. It seems likely that kinins are responsible for this phase.

The third phase (150-360 minutes) is brought about by prostaglandins and is responsible for the maintenance of the enhanced vascular permeability brought about by the previous mediators. Willis (1969a, 1969b) demonstrated the presence of prostaglandins in carrageenan-induced exudate. This 'prostaglandin phase' seems to be closely associated with the emigration of leucocytes into the inflamed area (Di Rosa, Papadimitriou and Willoughby, 1971). The cellular emigration starts at about 2 hours after sub-plantar injection of carrageenan into the rat hind paw and the cells are predominantly polymorphonuclear leucocytes. The maximum number of cells are found at about 6 hours after carrageenan injection, with the number of polymorphonuclear leucocytes about twice that of monocytes. The anti-inflammatory activity of non-steroidal anti-inflammatory drugs such as indomethacin and phenylbutazone has been found to be most effective on the 'prostaglandin phase' of the carrageenan oedema suggesting that their anti-inflammatory activity is associated with the ability of these agents to suppress cellular emigration (Di Rosa, Papadimitriou and Willoughby, 1971). It has also been shown that there is a good correlation between the ability of non-steroidal anti-inflammatory drugs to suppress mononuclear cells emigration

and the ability of these drugs to suppress the later phase of the carrageenan oedema. However, no such correlation exists between the ability of these drugs to suppress the polymorphonuclear leucocytes emigration and their ability to suppress the oedema. So it is suggested that the mode of action of the non-steroidal anti-inflammatory drugs in carrageenan-induced oedema is primarily to inhibit the migration of mononuclear cells into the injection site (Di Rosa, Papadimitriou and Willoughby, 1971).

It has been shown that oxygen radicals participate in the swelling of the prostaglandin phase of the carrageenan-induced oedema (Oyanagui, 1976a), as has been mentioned in section 1.4 of this thesis. The first phase of the carrageenan oedema which was sustained by histamine and serotonin was not inhibited by superoxide dismutase or catalase. The carrageenan foot-oedema of agranulocyte rats, induced by methotrexate injections, was more susceptible than that of normal rats to superoxide dismutase inhibition, suggesting the importance of macrophages in the prostaglandin phase in this model of inflammation.

The same sequential release of the mediators, histamine and 5-hydroxytryptamine, kinins and prostaglandins occurs in other types of inflammatory response in rats, for example, in carrageenan-induced pleurisy, turpentine-induced foot oedema and turpentine-induced pleurisy (Spector and Willoughby, 1968; Di Rosa, Giroud and Willoughby, 1971).

Rat paw oedema can also be induced by sub-plantar injection in the foot of other irritants such as formalin, mustard, kaolin, dextran, ovalbumin or one of the chemical mediators of inflammation such as histamine, serotonin, bradykinin and prostaglandin E_1 and E_2.

The various irritants can also produce an acute inflammatory reaction when injected into a natural cavity (the pleural and peritoneal cavities, knee joints and anterior chamber of the eye) (Swingle, 1974).

The pleural cavity of rats and guinea-pigs has been the most successfully utilized to study the inflammatory reaction and to investigate the effectiveness of anti-inflammatory drugs (Di Rosa, Giroud and Willoughby, 1971). In this model it is easy to measure the volume of exudate or to determine the amount of proteins, mediators and leucocytes in the exudate.

Ultraviolet-induced erythema (Winder et al, 1958) in guinea-pigs is sometimes used to test the anti-inflammatory activity of a substances. Erythema (redness) is the earliest sign of an inflammatory reaction in the skin and is due to the vasodilatation of blood vellels.

One of the most commonly used models for chronic inflammation is the adjuvant-induced arthritis in rats. The rat adjuvant-induced arthritis model has been used extensively for the screening of drugs of potential use in the treatment of rheumatoid arthritis.

The arthritis in the rat is produced by the sub-plantar injection of Freund's complete adjuvant (a dispersion of heat-killed mycobacterium tuberculosis in mineral oil or paraffin) into the rat's hind foot pad (Pearson, 1956). The sub-plantar injection of the adjuvant results in a primary, non-immune, localized inflammatory response in the paw followed by the secondary immune systemic disease.

The local swelling begins in the injected paw within 24 hours, reaches a peak on day 4 or 5 and becomes stabilized on day 6 to 11. The systemic disease usually starts on day 7 and is characterized by the swelling of the opposite non-injected foot and the appearance of nodules on the ears and tail. The severity of the generalized immune response reaches a peak on day 26 to 28. The polyarthritis formed affects many of the peripheral joints and also some of the joints in the tail and at the base of the spine. The animals develop coarse ruffled fur, loss weight and generally appear to be unwell.

About 50 per cent of the animals recover in 2 to 4 months with the inflammation subsiding, leaving pale granulomatous swellings around the joint. In the remainder, arthritis persists and progresses to destruction of the involved joints with ankylosis (Pearson and Wood, 1959; 1963).

The development of the arthritic syndrome in rats is followed by measuring the changes in thickness or volume of both hind feet (Newbould, 1963).

The secondary systemic inflammatory response is believed by most authorities to be due to a delayed hypersensitivity response (cellular immune mechanism) to mycobacteria antigen(s) (Waksman, Pearson and Sharp, 1960; Pearson, 1963; Pearson and Wood, 1964).

The arthritic activity of Freund's adjuvant seems to reside in the wax D fraction of the mycobacteria (Waksman, Pearson and Sharp,

1960). Wax D is a complex peptidoglycan which can be extracted from the bacterial cell wall.

The histopathology of the synovium in rat adjuvant arthritis is indistinguishable from inflammatory synovitis in man by light and electron microscopy (Muirden and Peace, 1969). Oedema, an early sign of inflammation can be identified at the site of injection before cellular infiltration occurs (Movat, 1966). Mast cells are the first to appear in large numbers (Gryfe et al, 1971) in the inflamed tissues. More than the normal number of mast cells can be seen in the non-injected feet of rats 5 days after the injection of adjuvant. There are also large numbers of lymphocytes and histiocytes appearing in the synovial tissues. Although the number of polymorphonuclear leucocytes greatly increase on the 11th or 12th day after adjuvant injection, they are usually fewer in number than the lymphocytes. The mononuclear cells predominate in the tissues while polymorphonuclear leucocytes predominate in the inflammatory joint exudate (Gryfe et al, 1971). As in rheumatoid arthritis, cartilage destruction is due to an invasive pannus which is made up of proliferating fibroblasts and synoviocytes (Pearson and Wood, 1963).

The incidence of adjuvant arthritis in the rat varies according to the age, breed of the rat and the site of injection of the adjuvant.

The most popular sites for injection of adjuvant are the hind paw and the tail of the rat. The most successful route for producing the disease has been intradermal (Glenn and Gray, 1965). The intradermal route gives easy access to the lymphatic system.

The regional lymph nodes play a leading part in the development of a hypersensitivity to the adjuvant. Newbould (1964) by using coloured adjuvant demonstrated the presence of injected materials in draining lymph nodes, so that absorption from the injected site is through lymph channels. Injection of adjuvant directly into the lymph node has been used and may be the most reliable method of induction of arthritis (Whitehome et al, 1972). It is also possible to transfer the disease to normal rats in a highly inbred strain by the transfer of large numbers of viable lymph nodes or spleen cells collected from several animals from the 8th to 12th day, though the transferred polyarthritis is usually less severe than the original (Pearson and Wood, 1964).

Although the amount of adjuvant injected (ranging from 0.1 to 10 mg/rat) was not found to cause any difference in the incidence or severity of the resulting arthritis (Glenn and Gray, 1965), the size of the tubercle particles in the injection, however, does influence the severity of the disease (Fujihara et al, 1970). Liyanage et al (1975) found that tubercle aggregates smaller than 90 μm were essential to produce arthritis in Sprague-Dawley rats but found no evidence that aggregates in the range of 4.5-63 μm produced a more severe form of arthritis than aggregates in the range 63-90 μm. However, Best et al (1984) showed that a particle size of less than 19 μm was required to produce arthritis in Wistar strain rats and that much smaller particles (below 10 μm) were required to produce secondary lesions. Since it is generally believed that adjuvant arthritis represents a cell-mediated reaction to tuberculous material which has reached the joints from the site of injection, then aggregate size might modify the migration of antigen to the joints.

Adjuvant arthritis can only be induced in rats. Attempts to induce the disease in other species such as rabbit, guinea-pig and mouse have been unsuccessful (Glenn and Gray, 1965).

Various strains of rat differ in their response to the induction of adjuvant arthritis. In Sprague-Dawley rats the incidence of arthritis approaches 90% following adjuvant injection while the Buffalo rats are particularly resistant to the disease (Swingle et al, 1969). This could be due to the relatively poor lymphatic drainage of the site of injection, since full scale polyarthritis could be produced by injecting the adjuvant directly into a lymph node of these rats.

There is no sex differences in the incidence or severity of adjuvant-induced arthritis but the age of the animal does influence the disease. Both very young rats and old rats are relatively resistant to induction of the disease, which seems to be related to the immunological state of the animals (Glenn and Gray, 1965).

The severity of the disease may be modified by steroidal and non-steroidal anti-inflammatory drugs (Pearson and Wood, 1959; Newbould, 1963). It may also be prevented by immunosuppressive drugs such as cyclophosphamide (Brown et al, 1970) and methotrexate (Ward et al, 1964).

The similarities of adjuvant arthritis to the human disease (Pearson 1963; Katz and Piliero, 1969) and the sensitivity of this model to anti-arthritic agents (Newbould, 1963; Graeme et al, 1966; Ward and Cloud, 1966; Winter and Nuss, 1966) support the view that

adjuvant-induced arthritis is the best available model of rheumatoid arthritis.

There are however, distinct dissimilarities between adjuvant and rheumatoid arthritis. The serum counterpart to the 'rheumatoid factor' has so far not been detected in the rat serum (Lowe, 1964). Also, unlike the human disease, there are few polymorphonuclear leucocytes present at the affected joints in adjuvant arthritis.

The growth of granulation tissue around an implanted sterile cotton pellet in rat was first shown by Meier et al (1950) to be reduced by cortisone. The model has been widely used to examine the effects of steroidal and non-steroidal anti-inflammatory drugs. 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 where there is a rapid increase in the wet weight of the pellet. The large amount of fluid available for soaking the pellet escape from the capillaries due to an increase in vascular permeability at the site of implantation. The second phase of the response occur between 3 and 72 hours after implantation and was designated the exudative phase which was measured by assessing the amount of Evans Blue dye passing from the circulation to the implant region. There is an accumulation of exudate around the pellet at this stage. The

third component of the response occurs on the fourth day after implantation where collagen is first detected in the granuloma. Also the greatest increase in the number of fibroblasts takes place between the third and fifth day.

The pattern of early cellular infiltration in this model can be divided into an acute phase lasting for about 2 days in which the polymorphonuclear leucocytes predominate, followed by a further period of several days during which the population of mononuclear cells, mainly macrophages, increases and cell proliferation commences (Freeman et al, 1979).

1.6 Cyclic Nucleotides and Inflammation

The naturally occurring cyclic nucleotides, cyclic 3',5' - adenosine and guanosine monophosphate (cyclic AMP and cyclic GMP respectively) are key regulatory substances that mediate the intracellular action of diverse agents that act at the cell surface.

Several hormones, neurotransmitters, regulators of cell division and other agents interact with receptors at the cell surface and induce activation of one or the other cyclic nucleotide cyclases (enzyme adenylate cyclase or guanylate cyclase) which in turn induce the intracellular production of the cyclic nucleotides from their appropriate precursors, adenosine triphosphate (precursor for cyclic AMP) and guanosine triphosphate (precursor for cyclic GMP). Cyclic nucleotides are present in every cell of the mammalian organ except the erythrocytes and the function they express depends on the particular cell or tissue.

Cyclic AMP acts by stimulating a cyclic AMP-dependent protein kinase which then influences the activity of various enzymes. Cyclic GMP actions are thought to be mediated by a specific protein kinase different from that acted upon by cyclic AMP.

The cyclic nucleotides are broken down by their specific phosphodiesterases. Cyclic AMP and cyclic GMP in general produce events that are opposite to each other (Goldberg et al, 1974).

Numerous in vitro investigations have implicated cyclic AMP as an important intracellular modulator of immediate hypersensitivity reaction. In 1968, Lichtenstein and Margolis presented the evidence implicating cyclic AMP in control of leucocyte function. Adrenaline and theophylline, agents that respectively stimulate the synthesis or decrease the degradation of cyclic AMP in many tissues prevented the antigen-induced release of histamine from leucocytes of patients with hay fever. In this in vitro model of the immediate hypersensitivity reaction, histamine secretion is triggered by the combination of antigen with specific immunoglobulin E (lgE) antibody bound to membranes of basophilic leucocytes (Lichtenstein and Osler, 1964; Lichtenstein and Osler, 1966).

Isoprenaline (a β-adrenergic agonist), prostaglandin E and cholera enterotoxin stimulate the accumulation of cyclic AMP in leucocytes by activating adenylate cyclase and prevent the antigen-induced release of leucocyte histamine (Bourne, Melmon and Lichtenstein, 1971; Bourne, Lichtenstein and Melmon, 1972; Lichtenstein, Gillespie, Bourne and Henney, 1972; Pierce et al, 1971). Methylxanthines, such as theophylline, inhibit enzyme phosphodiesterase and potentiate the effect of isoprenaline and the other agents on both cyclic AMP, an analogue of cyclic AMP also inhibits the histamine release (Bourne, Lehrer, Cline and Melmon, 1971). Dibutyryl cyclic AMP, an analogue of cyclic AMP also inhibits the histamine release from stimulated mast cells.

Increase in the cellular level of cyclic AMP also inhibits histamine release from sensitized human and guinea-pig lung tissue (Orange et al, 1971; Schmutzler et al, 1973).

On the other hand, stimulation of cholinergic receptors by acetylocholine analogues leads to an increase in cyclic GMP level and causes an enhcncement of histamine release from human lung (Kaliner and Austen, 1974).

The inhibition of the release of histamine from immediate hypersensitivity reaction by increasing the intracellular level of cyclic AMP has also been demonstrated *in vivo* using an animal model (Deporter, Capasso and Willoughby, 1976).

In vitro studies have also implicated cyclic AMP and cyclic GMP in the control of lysosomal enzyme release from polymorphonuclear leucocytes. It has been demonstrated that the discharge of the potentially destructive lysosomal enzymes from isolated polymorphonuclear leucocytes in response to a variety of stimuli, including antigen-antibody complexes can be inhibited by elevating polymorphonuclear leucocytes cyclic AMP levels or enhanced by increasing their cyclic GMP levels (Weissmann, Dukor and Zurier, 1971; Weissmann, Zurier, Spieler and Goldstein, 1971; Ignarro, 1975; Zurier et al, 1974).

In vitro experiments on the effects of cyclic AMP on phagocytosis by polymorphonuclear leucocytes have produced conflicting results. Thus whereas Cox and Karnovsky (1973) and Ignarro, Lint and George (1974) claimed that elevated level of cyclic AMP inhibited phagocytosis by polymorphonuclear leucocytes, other workers concluded that the cyclic AMP system was not important in the control of phagocytosis by polymorphonuclear leucocytes (Mangianello et al, 1971; Schell-Frederick and Van Sande, 1974).

Deporter, Dieppe, Glatt and Willoughby (1977) studied the effect of altering endogenous leucocyte cyclic AMP levels on phagocytosis and lysosomal enzyme release in pyrophosphate crystal-induced pleurisy model of acute inflammation in rats. Despite marked increases in leucocyte cyclic AMP concentrations produced by injections of dibutyryl cyclic AMP and theophylline there was no reduction in crystal phagocytosis or the lysosomal enzyme release. In the *in vivo* model of immediate hypersensitivity reaction in rats (Arthus reaction), the administration of dibutyryl cyclic AMP with or without theophylline into the pleural cavity of rats at the time of initiation of an immediate hypersensitivity reaction in the same site increased leucocyte cyclic AMP but had no significant effect on secretion of the lysosomal hydrolase enzyme, β-glucuronidase (Deporter, 1977a). These authors concluded that cyclic AMP did not monitor lysosomal enzyme release *in vivo* which is in direct opposition to the *in vitro* work of Weissmann, Dukor and Zurier (1971). The reason for the lack of agreement between the *in vitro* studies and the *in vivo* studies is not clear.

Cyclic AMP has been demonstrated to inhibit the mobility and migration of leucocytes *in vitro* (Johnson et al, 1972; Pick, 1972). Enstensen et al (1973) showed that chemotaxis of polymorphonuclear leucocytes was inhibited by cyclic AMP and enhanced by cyclic GMP. In vivo, elevation of leucocyte cyclic AMP had been shown to produce a marked reduction in leucocyte migration into a pleural reaction induced by immediate hypersensitivity (passive Arthus reaction) but had no effect on the cell numbers in pleurisy induced either by pyrophosphate or urate crystals (Deporter, 1977b). He suggested that

the inhibitory effect of cyclic AMP on cell migration in Arthus immediate hypersensitivity reaction might be due to the inhibition of chemotactic factors released from the activation of complement. The two types of crystal-induced inflammation are not complement-dependent (Willoughby, Dunn, Yamamoto, Capasso, Deporter and Giround, 1975; McCarthy and Kozin, 1975).

Cyclic AMP has also been demonstrated to inhibit several types of experimental inflammation. Cyclic AMP could inhibit the increase of capillary permeability induced by histamine, serotonin or bradykinin (Bertelli et al, 1966). Cyclic AMP was effective in suppresing carrageenan-induced oedema in the hind paws of rats by oral, subcutaneous and intraperitoneal injections (Ichikawa et al, 1972a). Cyclic AMP also showed a dose-dependent inhibitory action in the formation of granuloma induced by carrageenan, a more chronic type of inflammation, when administered intraperitoneally or directly into granuloma (Ichikawa et al, 1972b). Inhibitory effects of cyclic AMP of the experimental inflammation were augmented by the presence of theophylline. Cyclic AMP also inhibits the rat paw oedema induced by the injections of prostaglandin E and F (Bertelli et al, 1974). Cyclic GMP administration is not only unable to inhibit the oedema induced by prostaglandins but enhances the oedema.

Prostaglandin E_1 and theophylline when given together inhibit the inflammatory response in adjuvant-induced arthritis in rats (Bonta, Parnham and Van Vliet, 1978). They proposed that the anti-inflammatory and anti-arthritic effect of the combined drugs treatment involve cyclic AMP changes in phagocytic cells at the site of tissue

injury and in systemic lymphocytes.

It has also been suggested that one or more of the anti-inflammatory effects of indomethacin and dexamethasone is mediated through an elevation of leucocyte cyclic AMP content (Porter, Dunn and Willoughby, 1979). This hypothesis is corroborated by the finding of Thomas and Whittly (1976) that indomethacin inhibited histamine release from isolated challenged mast cells of the rat, probably through inhibition of mast cell phosphodiesterase activity.

1.7 Anti-Inflammatory Drugs

The two important anti-inflammatory drugs used in the treatment of rheumatoid arthritis are the steroidal and the non-steroidal anti-inflammatory drugs. Certain synthetic steroids derived from the glucocorticoid hormone hydrocortisone (cortisol, 11 , 17 , 21-trihydroxy-4-pregene-3, 20-dione) are potent anti-inflammatory drugs with a wider pharmacological spectrum than their non-steroidal counterparts. They inhibit not only the vascular and cellular changes of acute inflammation but also the self-prepetuating, proliferative and destructive aspects of chronic inflammation. A recent review on the anti-inflammation action of steroids has been given by Skidmore (1981).

Steroid mediated effects can be divided into two types, specific and non-specific, on the basis of mechanism of action. Specific effects are induced as a result of the interaction of the steroid with a specific receptor which differs for each class of steroid. Non-specific effects do not depend on the interaction of steroid with specific receptor but may be exerted by insertion of the steroid into the lipids of the cell membrane with consequent alterations in the structure and properties of these membranes.

The principles of the mechanism of specific steroid action appear to apply to all steroid classes (Schrader and O'Malley, 1978; Mainwaring, 1977; Gorski and Gannon, 1976; Baxter and Funder, 1979). The steroid is thought to enter the cell by passive diffusion and combine with a specific, soluble cytoplasmic receptor protein, altering

its conformation. The receptor-steroid complex is transported to the nucleus where it binds specifically and with high affinity to the chromatin. A new and specific messenger ribonucleic acid (mRNA) is then synthesized which leaves the nucleus, associates with the polysomal apparatus and initiates protein synthesis, and it is this new protein through which the effect of the steroid is expressed. Alternatively, the synthesis of an existing mRNA may be enhanced, thus increasing the synthesis of a protein already being made by the cell.

Steroids exert their anti-inflammatory effects by a number of mechanisms. Anti-inflammatory steroids are vasoconstrictor (Zweifach et al, 1953; McKenzie, 1962) and counteract the vasoconstrictor of acute inflammation. Steroids also prevent the increase in vascular permeability of simple acute inflammations (Tsurufuji, Sugio and Takemasa, 1979; Tsurufuji et al, 1980).

Anti-inflammatory steroids inhibit the accumulation of both polymorphs and mococytes at the inflammatory sites. The steroids suppress the adherence of polymorphs to the vascular endothelium, thus preventing their emigration and accumulation at the site of injury (Mishler, 1977; Skidmore, 1981). The accelerated release of polymorphs from the bone marrow and decreased entry to the tissues account for the neutrophilic leucocytosis seen 4-6 hours after the steroids are given.

Steroids suppress the abundance of both the mononuclear phagocytes (monocytes and macrophages) (Thompson and Van Furth, 1970; Dale et al, 1974) and the lymphocytes (Fauci, 1979). The anti-inflammatory

steroids also inhibit many of the synthetic and seretory functions of macrophages, for example, synthesis of prostaglandins (Robinson et al, 1978, Bray and Gordon, 1978, Bonney et al, 1978) and synthesis and release of neutral proteinases (Coote et al, 1980; Vasalli et al, 1976; Werb, 1978).

Steroids have multiple sites of potential action on joint tissues. They inhibit the production of mononuclear cell factor (MCF) and synovial factor (SF) which stimulate synovial and cartilage cells. The inhibition of these factors will inhibit the production of collagenase and other neutral proteinases, and prostaglandin E_2 from the synovial and cartilage cells. Steroids also inhibit the production of plasminogen activator by these cells while they increase the amount of free TIMP (tissue inhibitor of metalloproteinases) (McGuire et al, 1981b). These actions of steroids will decrease the amount of active proteinases present in the joint and contribute to their potent anti-inflammatory activity.

During inflammation, the plasma levels of anti-proteinases are increased and it has been shown that these plasma anti-proteinases are anti-inflammatory since they inhibit a number of proteinases that are involved in inflammation (Lewis, 1977). The two most abundant human plasma anti-proteinases are α_1-proteinase inhibitor and α_2-macroglobulin. Cortisol has been shown to stimulate α_1-proteinase inhibitor level in the blood of both normal and arthritic rats (parrot and Lewis, 1976).

Steroids have been reported to inhibit phagocytosis by the

polymorphonuclear leucocytes and the macrophages (Mishler, 1977; Smith, 1977; Ralph et al, 1978; Norton and Munck, 1980) and to stabilize the lysosomal membranes thus preventing the release of lysosomal enzymes (Weissmann, 1973; Wright and Malawista, 1973; Ingarro, 1974b; Smith, 1977). Both these effects may reflect the non-specific action of steroids on membrane function and these effects are unlikely to be of significance in vivo (Skidmore, 1981).

It has been shown that anti-inflammatory steroids such as dexamethasone and prednisolone, prevent the release of arachidonic acid from membrane phospholipids and consequently the generation of the pro-inflammatory cyclo-oxygenase and lipoxygenase metabolites of arachidonic acid metabolism, by inhibiting phospholipase A_2 (Blackwell et al, 1978; Flower and Blackwell, 1979; Gryglewski et al, 1975; Gryglewski, 1976; Hong and Levine, 1976; Nijkamp et al, 1976; Tam et al, 1977). The steroids induce the synthesis and release of protein inhibitors of phospholipase A_2 by neutrophils, monocytes and guinea-pig lungs (Hirata et al, 1980; Carnuccio et al, 1980; Oyanagui, 1984; Flower and Blackwell, 1979).

In vivo administration of steroids has also been shown not only to inhibit phospholipase A_2 activity but also alters the activity of enzymes responsible in the synthesis of breakdown of prostaglandins (Hoult and Moore, 1980).

Steroids have many unwanted side-effects. They increase the susceptibility of infection by altering the body's defence against infections (for example, decreased lymphocyte population etc.). They also cause ulceration of the gastrointestinal tract, loss of

calcium from bones (causing oesteoporosis) and the retention of water and sodium in the body.

The non-steroidal anti-inflammatory drugs currently in use modify or diminish but do not arrest the inflammatory response. The main therapeutic actions of non-steroidal anti-inflammatory drugs in man include the lessening of fever (anti-pyresis), pain (analgesia) and inflammation.

Non-steroidal anti-inflammatory drugs such as salicylic acid, indomethacin and phenylbutazone share certain biochemical properties such as protein stabilization (Piliero and Colombo, 1967), uncoupling of oxidative phosphorylation (Whitehouse and Haslam, 1963; Lewis et at, 1972) and stabilization of lysosomal membranes (Mizushima et al, 1975; Lewis and Krygier, 1973). Some of these properties may well be involved in the anti-inflammatory action of non-steroidal anti-inflammatory drugs but the main mode of action of these anti-inflammatory drugs is the inhibition of prostaglandin biosynthesis.

A large number of non-steroidal anti-inflammatory drugs inhibit prostaglandin synthesis and there is a good correlation between their rank order of potencies in reducing oedema and prostaglandin concentrations in vivo (Higgs et al, 1976). The selective inhibition of cyclo-oxygenase by drugs such as aspirin and indomethacin prevents the synthesis of inflammatory mediators such as prostaglandin E_2 and prostacyclin (Ferreira and Vane, 1979).

A number of cyclo-oxygenase inhibitors have a differential

effect on leucocyte migration in vivo (Adams et al, 1977; Eskins et al, 1980a and b). Indomethacin, aspirin and flurbiprofen enhance the accumulaion of cells in inflammatory exudates at doses which significantly reduce prostaglandin production, but inhibit cell migration at higher doses (Eakins, 1980b). This observation can be explained if the inhibition of cycle-oxygenase diverts substrate towards the production of chemotactic lipoxygenase products, which then account for increased leucocyte migration. The subsequent inhibition of leucocyte migration at higher doses may be explained by non-specific inhibition of arachidonic acid peroxidation (Bragt and Bonta, 1980).

In vitro work has shown that non-steroidal anti-inflammatory drugs can inhibit the generation of superoxide anions from guinea-pig macrophages (Oyanagui, 1976b) and peritoneal exudate cells (Oyanagui, 1978). Studies by Simchowitz et al, (1979) have shown that superoxide anion generation in human neutrophils is inhibited by a number of non-steroidal anti-inflammatory drugs of concentrations comparable to those achieved therapeutically.

Non-steroidal anti-inflammatory drugs have also been shown to inhibit phosphodiesterase activity, thereby elevating the intracellular concentration of cyclic AMP (Weiss and Hait, 1977).

Non-steroidal anti-inflammatory drugs are not free of undesirable side-effects, and can produce tinnitus or deafness (Miller, 1978), gastric ulceration (Rainsford, 1977; Kivilaakso and Silen, 1979), decrease in renal function (Kimberly et al, 1978; Tan et al,

1979) and can also induce hypersensitivity reaction in a susceptible person (Szczeklik et al, 1977).

It is clear that the drugs which are currently used in the treatment of inflammation suffer from the disadvantage that their use may cause severe side-effects.

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

Therefore, efforts are continually being made to try to find a substance which has a potent anti-inflammatory activity but free from undesirable side-effects.

1.8 The Role of Polyamines in Inflammation

The polyamines putrescine, spermidine and spermine are simple aliphatic amines present in all living cells (Cohen, 1971). In general prokaryotes have a higher concentration of putrescine (1,4-diaminobutane) than spermidine and lack spermine. Eucaryotes usually have little putrescine and have spermine as well as spermidine. Polyamines are simple cationic compounds which are involved in a number of processes. Polyamine biosynthesis is intimately interrelated with the synthesis of nucleic acid and protein.

1.8.1 The Biosynthesis of Polyamines

The biosynthetic pathway for polyamines in eucaryotes is outlined in Figure 3. There are four enzymes involved in polyamine biosynthesis : two decarboxylases and two synthetases. Putrescine is formed following the decarboxylation of ornithine by ornithine decarboxylase (L-ornithine carboxy-lyase EC. 4.1.1.17). This is the rate limiting step since ornithine decarboxylase enzyme is present only in low concentration in most eucaryotic cells. Putrescine is incorporated directly into spermidine by the enzyme spermidine synthetase. The propylamine moiety is derived from methionine. The sequence involves the synthesis of S-adenosyl-methionine from methionine followed by decarboxylation of S-adenosylmethionine and transfer of the propylamine moiety to putrescine. Methionine is an essential amino acid in higher animals whereas it is synthesized from aspartic acid in plants and microbes.

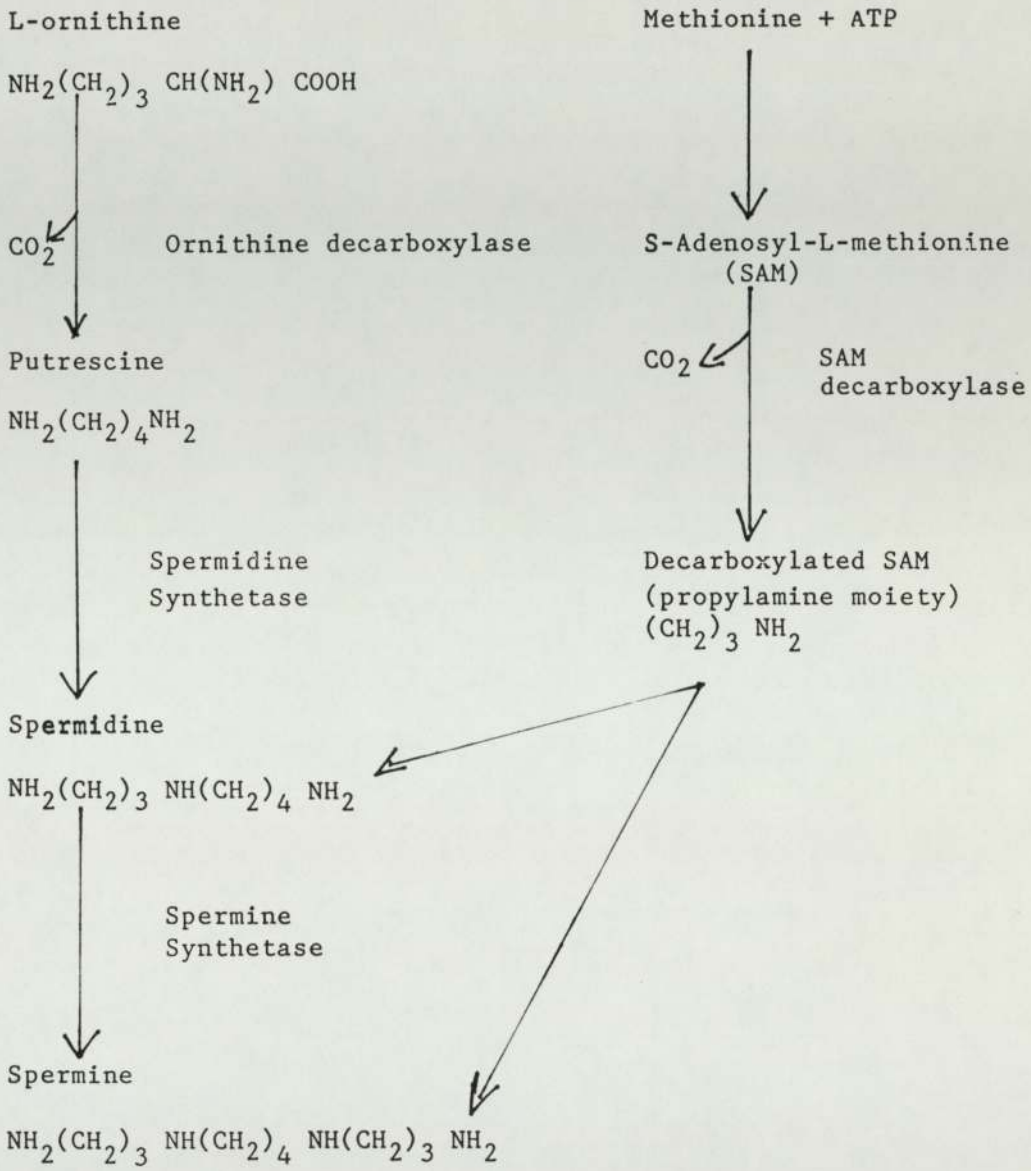

Figure 3. The Biosynthetic Pathway of Polyamines in Eucaryotes

S-adenosyl-L-methionine decarboxylase enzyme from higher organisms is stimulated by putrescine and similar diamines (Pegg and Williams-Ashman, 1968) whereas in lower organisms such as protozoa the enzyme is not influenced by diamines (Wickner et al, 1970). Spermine synthetase adds another propylamine moiety to spermidine to yield spermine.

In bacteria, putrescine may be formed either by ornithine decarboxylase or by the decarboxylation of arginine yielding agmatine (Morris and Fillingame, 1974; Morris and Pardee, 1966; Morris and Koffron 1969). Agmatine can then be converted to putrescine. Prokaryotes are in general incapable of spermine biosynthesis.

Cadaverine (1,5 diaminopentane) may be obtained by the decarboxylation of lysine present in E. Coli and in various Salmonella strains. Formation of cadaverine from lysine was also shown to occur in the kidneys of mice after the injection of an anabolic steroid (Henningsson, Persson and Rosengren, 1976; Persson, 1977).

Although the conversion of putrescine into spermidine and spermine is frequently thought to be irreversible, there is experimental evidence from the conversion in the rat of spermine to spermidine and of spermidine to putrescine. The conversion of spermidine to putrescine in rat liver is increased after partial hepatectomy or treatment with the hepatotoxins carbon tetrachloride and thioacetamide (Höltä, Sinervirta and Jänne, 1973).

1.8.2 Polyamines and Protein Synthesis

The relationship between polyamines and nucleic acids has been reviewed extensively (Cohen, 1971; Bachrach, 1972; Tabor and Tabor, 1976). These amines because of their polycationic nature, bind tightly to nucleic acids and have a variety of effects on nucleic acid biosynthesis and metabolism *in vitro*. Many studies have shown that polyamines (a) stimulate protein synthesis *in vitro* and *in vivo* (b) stimulate many of the intermediate steps in protein synthesis *in vitro* (c) bind tightly to substrates such as transfer ribonucleic acid (tRNA) and adenosine triphosphate (ATP) and (d) bind to ribosomes and stabilize ribosomal structure and function (Tabor and Tabir, 1972; Cohen, 1971; Bachrach, 1973; Stevens, 1970; Raina and Jänne, 1975). As Cohen (1971) has pointed out, the polyamines could play a role in almost every step from deoxyribonucleic acid to ribonucleic acid and protein.

1.8.3 Ornithine Decarboxylase

Ornithine decarboxylase has a molecular weight of 55000 daltons (Obenrader and Prouty, 1977). Mammalian ornithine decarboxylase requires pyridoxal phosphate as a co-factor and is strongly dependent on low molecular weight thiols for full catalytic activity (Ono et al, 1972).

Ornithine decarboxylase has a high turnover rate i.e. its rapid synthesis and degradation which allows rapid changes in activity (Snyder and Russell, 1970). It has the shortest half-life of any

known enzyme in eucaryotes. In regenerating liver, the half-life of ornithine decarboxylase is 10 minutes (Russell and Snyder, 1969).

Ornithine decarboxylase activity is normally very low in quiescent tissues but is dramatically higher in rapidly proliferating cells and in many other conditions, most of which involve increase in protein synthesis, for example, liver regeneration after hepatectomy (Russell and Snyder, 1968; Jänne and Raina, 1968; Fausto, 1969; Jänne and Hölttä, 1974), renal and cardiac hypertrophy (Brandt et al, 1972; Feldman and Russell, 1972), malignant growth (Russell and Snyder, 1968; Russell and Levy, 1971), administration of growth hormones (Russell, Snyder and Medina, 1970; Richman et al, 1973; Jänne and Raina, 1969), thioacetamide (Fausto, 1969) and a number of drugs (Russell, 1971). In almost all of these instances a peak of ornithine decarboxylase activity occurred 4 - 24 hours after the stimulus and lasted only a few hours. In some cases, a biphasic increase has been observed with peaks at 4 hours and 12 hours. The increase in enzyme activity appears to represent synthesis of new protein (Russell and Snyder, 1968). In most cases the rise in ornithine decarboxylase activity is followed by a rise in putrescine content.

The inhibition of ornithine decarboxylase by putrescine, spermidine, spermine and other related amines has been observed in a number of physiological systems as well as in a variety of cell cultures (Clark, 1974; Schrock et al, 1970; Jänne and Hölttä, 1974). This inhibition has been suggested to be due to the presence of a small protein called antizyme (Heller, Fong and Cannellakis, 1976). Ornithine decarboxylase antizyme has a molecular weight of 26000 daltons

and can be induced by exposing cells to polyamines. The antizyme reacts specifically with ornithine decarboxylase and inhibits its activity. It has recently been postulated that ornithine decarboxylase antizyme is normally attached to subcellular components (nucleus and ribosomes) and that polyamines at millimolar concentrations cause its release (Heller, Kyriakidis, Fong and Cannellakis, 1977). Polyamines may therefore regulate ornithine decarboxylase activity.

1.8.4 The Induction of Ornithine Decarboxylase by Cyclic AMP

The possibility that ornithine decarboxylase is controlled by a cyclic AMP dependent mechanism is supported by a number of observations. Many stimuli that can increase the intracellular level of cyclic AMP, for examples, hormones (Janne and Raina, 1969; Russell and Snyder, 1969, Russell et al, 1970), methyl xanthines derivatives (Byus and Russell, 1974) and dibutyryl cyclic AMP (Beck et al, 1972; Richman et al, 1973) increase ornithine dexarboxylase enzyme activity.

Figure 4 shows a model of major sequential biochemical events of a trophic response as proposed by Russell, Byus and Manen (1976). In response to trophic hormones, drugs administration, mitogens and various other growth stimuli, there is a series of biochemical events that result in transfer of stimuli from the cell membrane through cytoplasmic events to nuclear gene activation, ultimately resulting in the synthesis of new ribonucleic acid and protein species. Many of these stimulatory hormones have been shown to activate adenylate cyclase and increase the intracellular level of cyclic AMP (Perkins, 1973; Aurbach et at, 1975). The increase in intracellular level of

cyclic AMP leads to the activation of cyclic AMP-dependent protein kinase (Costa, Gerner and Russell, 1976; Byus and Russell, 1975; Keely et al, 1975). The cyclic AMP-deoendent protein kinase is then translocated to a nuclear site (Castagna et al, 1975; Costa, Kurosawa and Guidotti, 1976). The catalytic subunit of cyclic AMP-dependent protein kinase could then be responsible for phosphorylation of acidic nuclear proteins and/or histones which would result in the synthesis of new messenger ribonucleic acid for ornithine decarboxylase (Marks et al, 1973; Gurley et al, 1973; Karn et al, 1974; Gurley et al, 1974). The induction of ornithine decarboxylase would then lead to an increased rate of ribosomal ribonucleic acid synthesis and increased polyamine synthesis. Certain investigations have shown that a substantial concentration of spermidine is necessary in the nucleus in order for newly formed ribosomal ribonucleic acid to be transported to the cytoplasm. Otherwise, ribonucleic acid accumulates and inhibits further synthesis (Yu, Racevskis and Webb, 1972).

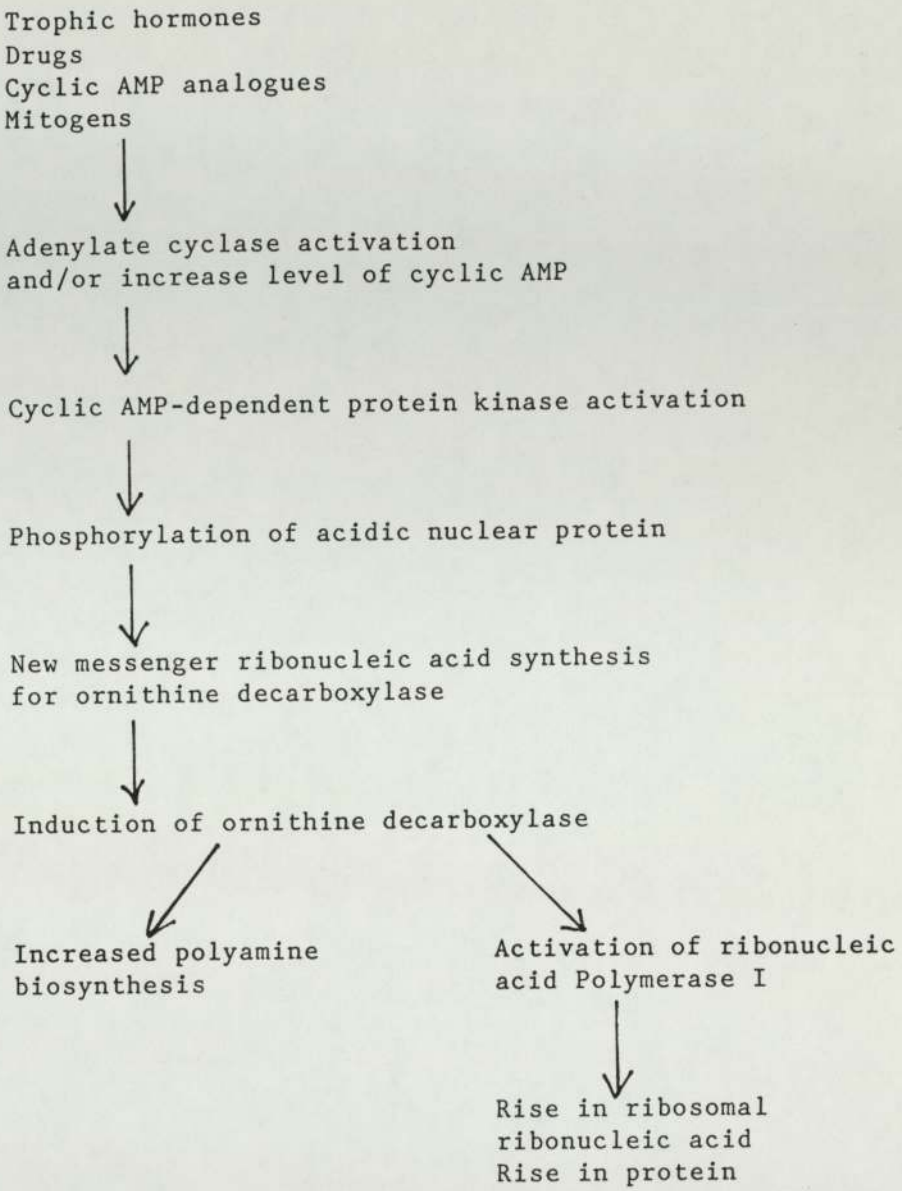

Figure 4. A Schematic Diagram of Major Sequential Steps in a Trophic Response

1.8.5 Polyamines and Inflammation

At the present time, the evidence for a direct involvement of polyamines in inflammation 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 (Flower and Blackwell, 1979; Tsurufuji, Sugio and Takamasa, 1979). Bartholeyns, Fozard and Prakash (1980) suggested that the diamine putrescine was necessary for the biosynthesis of these anti-inflammatory mediators. These workers found that dexamethasone induced rises in hepatic ornithine decarboxylase activity and putrescine concentration which was maximal at 4 hours. The concentrations of spermidine and spermine were unchanged. Using an irreversible inhibitor of ornithine decarboxylase (DL- α-difluoromethylornithine) the effects of dexamethasone could be inhibited, but the effects of indomethacin were unaltered.

The study was extended by Bartholeyns, Fozard and Prakash (1981) who found that pretreatment of rats 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-difluoromethylomithine in the carrageenan oedema but left unaltered the suppressive effects of actinomycin D. 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.

Liver disease has an ameliorating effect on inflammation (Hench, 1933) and it has been shown that there is a marked stimulation of ornithine decarboxylase activity in liver regeneration (Jänne and Raina, 1968). The administration of carbon tetrachloride (Hölttä, Sinervirta and Janne, 1973) and thioacetamide (Fausto, 1970) in rats causes an increase in polyamine biosynthesis. Thioacetamide has been found to inhibit the carrageenan paw oedema in rats (Bird, 1979) and carbon tetrachloride has also been found to inhibit inflammation (Mielens et al, 1969).

In both the human and rat, the levels of polyamines are elevated during pregnancy. In rat, the elevation of putrescine is greatest and spermine shows very little change (Andersson, Henningsson and Rosengren, 1978; Russell et al, 1978). Pregnancy is known to cause remissions in rheumatoid diseases (Fagerhol and Laurell, 1970).

An increase in ornithine decarboxylase activity and accumulation of polyamines have been demonstrated during the formation of granulation tissue induced experimentally by implanting viscose cellulose sponges subcutaneously in the rat (Raina, Jänne, Hannonen, Hölttä and Ahonen, 1973) and in healing wounds of rat skin (Mizutani, Inoue and Takeda, 1974).

The direct anti-inflammatory effect of the polyamines has been shown in animal models of inflammation (Bird and Lewis, 1979). Putrescine was found to be strongly anti-inflammatory. It was effective in the rat carrageenan oedema model as well as in the adjuvant arthritic rat at a dose of 50 µg/kg. Spermidine was also anti-inflammatory but at higher doses. Spermine and cadaverine were not anti-inflammatory at the doses used.

Polyester sponge exudate was also found to contain polyamines (Bird and Lewis, 1979). Bird et al, (1983) have shown that putrescine was ten times as potent as spermidine as an anti-inflammatory agent in carrageenan rat model of inflammation.

Polyamines have been shown to have an inhibitory effect on the reduction of cytochrome C by superoxide anion (Vanella et al, 1979). Vanella et al, (1980a) have also shown that spermine has a scavenging effect on superoxide anion.

Prevention of autohaemolysis of erythrocytes by polyamines indicates the protective action exerted by them against cellular oxidant injury (Vanella et at, 1980b). Khanna et al, (1982) have shown that putrescine, spermidine and spermine are able to significantly counter paraquat-induced augmentation of lipid peroxidation. Recently, Kafy and Lewis (1984) have shown that putrescine and spermidine protect lysosomes from the lytic action of superoxide radical.

1.9 Aims of Research

As early as 1956, anti-inflammatory activity was demonstrated in inflammatory exudates (Rindani, 1956). Anti-inflammatory activity has been reported in granuloma pouch exudates in intact and adrenelectomized rats (Di Pasquale et al, 1963), arthritic (Persellin, 1972), normal rat plasma (Lewis, Capstick and Best, 1976), human pregnancy serum (Hempel, Fernandez and Persellin, 1970), human peritoneal fluid (Billinghan, Robinson and Robson, 1969), extracts from rat livers damaged *in vivo* by dimethylnitrosamine (Pinals, 1973) and sponge-induced exudates in rats (Robinson and Robson, 1964).

The true nature of the anti-inflammatory factors is unknown. In sponge exudates, it has been suggested that they are proteins. There is also evidence that the liver and sponge exudates contain polyamines (Bird, 1979).

Cyclic AMP and putrescine have been implicated to be anti-inflammatory. The aims of the research were to test the anti-inflammatory activity of drugs that increase cyclic AMP and putrescine levels in the different animal models of inflammation, and to try to elucidate the mechanism of the anti-inflammatory action of putrescine.

2. MATERIALS AND METHODS

2.1 The Induction and Measurement of Carrageensn-induced Oedema

Carrageenan oedema was induced in 150-230 g male Wistar rats (Bantin and Kingsman, Hull) by the method of Winter, Risley and Nuss (1962). A 2% (w/v) solution of carrageenan (Type v lambda carrageenan, Sigma Chemical Company) was prepared in 0.9% (w/v) sterile saline. The carrageenan solution was prepared the day before use and left overnight in the refrigerator to get rid of the air bubbles. The oedema was induced by the sub-plantar injection of 0.05cm^3 of carrageenan solution into the left hind foot of the rats. The rats were anaesthetized during the injection of carrageenan into the footpads, by using a mixture of 3% halothane (Fluothane, I.C.I.) in oxygen (flow rate 3000 c.c. $minute^{-1}$) and nitrous oxide (flow rate 1500 c.c. $minute^{-1}$) produced by a Boyles veterinary anaesthetic trolley (British Oxygen Company, Limited).

The inflammatory response produced by the injection of carrageenan was estimated by measuring the increase in volume of the hind feet of the rats. The hind foot of each rat was immersed up to the hairline in a mercury bath which was linked to a blood pressure transducer and an oscillograph recorder. The recorder was calibrated before and checked during each experiment, using a glass plunger which had a mark on it corresponding to a volume of 2 cm^3. When the glass plunger was immersed in the mercury bath up to this mark, the recorder was adjusted to produce a recording of 2 cm. on the chart. The measurement of the hind foot of the rats were taken just prior to

the injection of carrageenan (time 0 hour) and at 3 hours and 5 hours after the injection of carrageenan. The rats were allowed access to food and water during the test.

To assess the anti-inflammatory activity of a drug on the carrageenan oedema, the increase in foot volume of the rats treated with a drug was compared with rats that were given only the vehicle in which the drug was dissolved. These rats served as the controls. The anti-inflammatory activity of a drug was expressed as the percentage suppression of the foot oedema compared with the controls. There were 6 rats per group in each carrageenan test.

The statistical difference between test groups was calculated by the use of Student's t-test. Results were considered significant if $P < 0.05$.

2.2 Drugs Used in the Carrageenan-Oedema Test

Two types of drugs that increased the level of cyclic AMP were tested for their anti-inflammatory activity in the carrageenan oedema test in the rats.

2.2.1 The Adenylate Cyclase Activators

2.2.1.1 Salbutamol

Salbutamol (salbutamol sulphate) at doses of 6 mg kg^{-1} and 10 mg kg^{-1} were given intraperitoneally in 1 cm^3 of 0.9% sterile

saline, 30 minutes before the injection of carrageenan. Salbutamol at a dose of 15 mg kg^{-1} was also given orally 45 minutes before given carrageenan. Control rats received 1 cm^3 of 0.9% sterile saline only.

2.2.1.2 Isoprenaline

Isoprenaline (1-) free base, (Sigma Chemical Company) was given intraperitoneally at doses of 4 mg kg^{-1} and 8 mg kg^{-1} in 0.5 cm^3 of 0.9% sterile saline, 30 minutes before the injection of carrageenan into the foot-pads of the rats. Control rats received 0.5 cm^3 of 0.9% saline only.

2.2.1.3 Phenobarbitone

Sodium phenobarbitone at a dose of 100 mg kg^{-1} was given by intraperitoneal injection 4 hours before the injection of carrageenan. Control rats received 1 cm^3 of water only.

2.2.2 Phosphodiesterase Inhibitor

0.5 cm^3 of 3-isobutyl-1-methyl xanthine (Sigma Chemical Company) was given intraperitoneally to the rats at a dose of 14 mg kg^{-1} in 0.9% saline/ethanol 5 : 1 (v/v) 2 hours before the injection of carrageenan. Control rats was given 0.5 cm^3 of 0.9% saline/ethanol 5 : 1 (v/v) only.

2.3 The Effect of Putrescine on Carrageenan-induced Oedema

Putrescine dihydrochloride (Sigma Chemical Company) was mixed with 2% carrageenan solution and 0.05 cm^3 of the mixture was injected into the left hind paw of each rat. Putrescine concentration was 85.5 µg. paw^{-1} (the concentration of putrescine refers to the free base). Control rats was injected with 0.05 cm^3 of 2% carrageenan only.

2.4 The Effect of 1,3 Diaminopropane on Carrageenan-induced Oedema

1.0 cm^3 of 1,3 diaminopropane (BDH) was given intraperitoneally at a dose of 15 mg kg^{-1}, 30 minutes before the injection of carrageenan.

In another experiment, 1.0 cm^3 of 1,3 diaminopropane was also given intraperitoneally at a dose of 5 mg kg^{-1}, 30 minutes before the injection of carrageenan and another dose at 1.5 hours after the carrageenan injection.

2.5 The Effect of 1,2 Diaminoethane on Carrageenan-induced Oedema

1.0 cm^3 of 1,2 diaminoethane (BDH) at a dose of 15 mg kg^{-1} was given intraperitoneally, 30 minutes before the injection of carrageenan.

2.6 The Effect of Putrescine on Serotonin-induced Oedema

Male Wistar strain rats in the range of 150-230 g were used for the experiment. Serotonin creatinine sulphate (Sigma Chemical Company) solution was prepared in sterile 0.9% saline at a concentration of 0.0092 %. The solution was prepared fresh on the day of experiment. 0.1 cm^3 of the solution was injected into the hind paw of each rat. This concentration gave good foot inflammation at 30 minutes and at 60 minutes after the serotonin injection. Foot measurements were taken before the injection of serotonin and at 1 hour after the serotonin injection, using exactly the same method as in the carrageenan oedema test. Putrescine dihydrochloride solution (prepared in 0.9 % saline) was given intraperitoneally to the rats at a concentration of 10 mg kg^{-1}, 30 minutes before the injection of serotonin into the paws of the rats (putrescine concentration refers to the free base). Control rats were given 0.9 % saline only.

2.7 The Effect of Propranolol on the Inhibition of Carrageenan Oedema by Isoprenaline or Salbutamol

1.0 cm^3 of propranolol (Sigma Chemical Company) at a concentration of 15 mg kg^{-1} was given concomitantly with either 1.0 cm^3 of isoprenaline (8 mg kg^{-1}) or 1.0 cm^3 of salbutamol (15 mg kg^{-1}) by intraperitoneal injection to the rats, 30 minutes before the injection of carrageenan. All the drugs were dissolved in sterile water. Control rats received 1.0 cm^3 of water only. 2 other group of rats were also used. One group was given 1.0 cm^3 of 15 mg. kg^{-1} propranolol only and the other group was given 1.0 cm^3 of isoprenaline

(8 mg kg^{-1}) or 1.0 cm^3 of salbutamol (15 mg kg^{-1}) only. All the drugs were given intraperitoneally 30 minutes fefore the injection of carrageenan.

2.8 The Effect of DL-α-difluoromethylornithine (DFMO) on the Anti-inflammatory Activity of Isoprenaline in the Carrageenan Oedema

2 doses of DL-α-difluoromethylornithine (DFMO) (a gift from Cetre de Reserche Mercel International, France) were used, 200 µg. kg^{-1} and 400 µg kg^{-1}. DFMO was dissolved in o.9 % sterile saline. 0.5 cm^3 of each dose of DFMO was given concomitantly with 0.5cm^3 of isoprenaline (8 mg kg^{-1}) by intraperitoneal injection, 30 minutes before the injection of carrageenan. One group of rats received 0.5 cm^3 of DFMO at the 2 doses only and another group of rats was given 0.5 cm^3 of isoprenaline (8 mg kg^{-1}) only. The control group was given 0.5 cm^3 of 0.9 % saline only. All the drugs were given intraperitoneally 30 minutes before the injection of carrageenan.

2.9 The Induction of Adjuvant Arthritis in the Rats

Adjuvant arthritis was induced in 150-230 g male Wistar rats using the method employed by Newbould (1963). The adjuvant consisted of human strains of heat-killed C, DT and PN tubercle bacilli (Ministry of Agriculture, Veterinary Laboratory, Surrey). 5 mg of the bacilli were placed in a roughened glass mortar (external diameter, 9 cm., Scientific Supplies) and 3 drops of liquid paraffin was added and the adjuvant ground to a paste over 30 seconds. More liquid

paraffin was added to a total of 5 cm^3 with continuous grinding over a further 60 seconds period. The rats were anaesthetized as previously described and 0.03 cm^3 of the adjuvant suspension was injected into the left hind paw of the rats. Foot volume measurements were made just prior to the adjuvant injection (day 0) to about day 25. The method of foot volume measurements was the same as in the carrageenan oedema test. There were 7 rats per group in each adjuvant arthritic test. The statistical difference between test groups were calculated by the use of Student's t-test. Results were considered significant if $P < 0.05$.

2.10 The Effect of Drugs that Increase the Intracellular Level of Cyclic AMP on the Development of Adjuvant Arthritis in the Rats

2.10.1 Theophylline

The doses used were 15 mg kg^{-1} and 30 mg kg^{-1}, given orally in 1.0 cm^3 volume daily. Control rats were given 1.0 cm^3 of water only daily.

2.10.2 Salbutamol

A dose of 15 mg kg^{-1} of salbutamol was given orally daily in 1.0 cm^3 volume daily. Control rats were given 1.0 cm^3 of water only.

2.11 The Preparation of Liver Samples for Polyamines Determinations from Adjuvant Arthritic Rats

4 of the adjuvant arthritic rats that were treated with theophylline and 4 rats from the control group, were killed on day 26 by cervical dislocation. The livers were quickly removed, weighed and homogenized in ice-cold 0.9 % saline (1 : 2 w/v) in a glass homogeniser. The homogenates were centrifuged at 2000 rpm for 15 minutes. The supernatants were taken out and recentrifuged at 100,000 g for 60 minutes. The final supernatants from each group of rats were pooled together and then freeze-dried. The freeze-dried samples were analysed for putrescine, spermine and spermidine contents.

2.12 The Preparation of Liver Samples for the Determination of Putrescine Levels from Rats Treated with Isoprenaline or Salbutamol

4 rats were dosed either with isoprenaline 8 mg kg^{-1} or salbutamol 15 mg kg^{-1} given intraperitoneally 3½ hours after been given the drugs, the rats were killed by cervical dislocation, the livers were removed and treated as in Section 2.11.

2.13 The Quantitative Estimation of Putrescine

The level of putrescine in tissue samples was determined using a modification of the method described by Seiler and Askar (1971).

The method involved the reaction of the diamine with 1-dimethylaminonaphthalene-5-sulphonylchloride (DANS-Cl), followed by 2 dimensional thin-layer chromatographic separation of the dansylated derivatives and fluorimetry of the DANS-putrescine.

0.1 g of freeze-dried liver sample was shaken with 2 cm^3 of 0.2 N perchloric acid for 15 minutes. The sample was then centrifuged for 10 minutes at 2000 r.p.m. and 1 cm^3 of the supernatant used for assay.

2.5 cm^3 of DANS-Cl solution (4mg cm^{-3} in acetone) was added to the sample and the reaction mixture was saturated with $Na_2 . CO_3 . 10 H_2O$. The mixture was then left in the dark at room temperature overnight for the dansylation reaction to complete. The next day, 10 mg. of D-proline dissolved in 0.2 cm^3 of water was added to the mixture, in order to react with the excess DANS-Cl. After 2 hours, the dansylated amine was 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 residue dissolved in 0.5 cm^3 of toluene/ethyl acetate (7 : 3).

20 µl of the sample was applied on 2 separate 20 x 20 cm. thin layer plates consisting of 250 µm layer of silica gel G (BDH). The 2 plates were duplicates. The plates were subjected to 2-dimensional thin layer chromatography. Putrescine standards were also applied to the plates.

The solvent system for the first dimension was trichloroethylene/methanol (19:1) and the solvent for the second dimension was chloroform-triethylamine (10:1) which was prepared about 1 hour before use.

After development, the thin layer plates were examined under an ultra violet lamp where the spots corresponding to putrescine were marked by comparison with the standards. The putrescine spots were scrapped 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, fluorescence was excited at a wavelength of 365 nm.

The amount of putrescine was determined from a previously prepared calibration curve obtained by using solutions containing known amount of putrescine and treating these in the same way as the samples (concentrations range from 0 to 100 mg. cm^{-3}).

2.14 The Estimation of Spermine and Spermidine

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

0.1 g of freeze-dried liver sample was shaken with 2 cm^3 of 0.2 N perchloric acid for 15 minutes. The sample was then centrifuged for 10 minutes at 2000 r.p.m. and 1.0 cm^3 of the supernatant used for assay.

0.4 cm^3 of DANS-Cl solution (30 mg. cm^{-3} in acetone) was added to each sample and the reaction mixture was saturated with $Na_2 CO_3 . 10 H_2O$. After completion of the dansylation reaction (by leaving overnight in the dark at room temperature), 0.1 cm^3 of proline solution (100 mg. cm^{-3}) was added to each sample to remove the excess DANS-Cl. After 30 minutes, the dansylated amines were extracted with 5.0 cm^3 of benzene.

20 µl of each sample was applied on to a thin-layer plate (20 cm x 20 cm) consisting of a 250 µm layer of silica gel G. The samples were applied 3 cm from the bottom of the plate and 2 cm apart. Spermine and spermidine standards were also applied.

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

After development, the thin layer plates were examined under an ultra violet lamp, where the spots corresponding to spermine and

spermidine were scraped out and the dansylated amines extracted from the carrier by shaking with 5.0 cm^3 of benxene/acetic acid (99 : 1).

After standing for 5 minutes, the fluorescent intensity of the extracts were measured at 500 nm, excitation wavelength 365 nm.

The amounts of spermidine and spermine were determined from previously prepared calibration curves, obtained by taking solutions containing known amounts of spermidine and spermine (concentrations range from 0 to 100 mg. cm^{-3}) and treating these in the same way as the samples.

2.15 The Determination of Plasma and Liver Cyclic AMP Levels in Rats Given Isoprenaline or Salbutamol

2.15.1 The Preparation of Rat Plasma

1.0 cm^3 of isoprenaline (8 mg. Kg^{-1}) or salbutamol (15mg. kg^{-1}) was given intraperitoneally to 4 rats. Blood was taken from the rats by cardiac puncture at 3½ hours and 5½ hours after being dosed with isoprenaline or salbutamol. The rats were anaesthetized during the cardiac puncture using a mixture of 3% halothane in oxygen and nitrous oxide produced by the Boyle's veterinary anaesthetic trolley.

2.0 cm^3 of blood was taken from each rat using a 5 cm^3 syringe (containing 0.05 cm^3 ethylene diamine tetraacetic acid (EDTA) 0.5 M ph 7.5). The blood from the 4 rats in a group was pooled together.

Blood from the control rats which was given 0.9% saline only was also taken.

The blood was centrifuged at 1000 g for 10 minutes at 4°C. The plasma was taken out and stored at -20°C until required. The cyclic AMP level in the plasma was assayed using a 'cyclic AMP assay kit' obtained from Radiochemical Centre, Amersham.

2.15.2 The Preparation of Liver Samples from Rats Treated with Isoprenaline or Salbutamol for Cyclic AMP Determination

The liver samples were prepared according to the method of Tovey et al, (1974). 2 rats were dosed either with isoprenaline (8 mg. kg^{-1}) or salbutamol (15 mg. kg^{-1}) given intraperitoneally. The rats were killed by cervical dislocation 3½ hours after been given the drugs. The livers were quickly removed, put in liquid nitrogen, weighed and homogenized in cold 0.1 M hydrochloric acid at a concentration of 0.1 g tissue per cm^3 of hydrochloric acid. The homogenate was heated at 100°C in a water bath for 2 minutes. After cooling, the suspension was centrifuged at 20000 g for 30 minutes at 4°C. The supernatant was taken out and freeze-dried. 0.1 g of freeze-dried liver sample was dissolved in 10.0 cm^3 of distilled water, adjusted to pH 7.5 with 1 M sodium hydroxide and diluted appropriately with assay buffer before assay. 50 µl of the liver sample was used for the cyclic AMP assay using the 'cyclic AMP assay kit'.

2.15.3 Summary of the Cyclic AMP Assay

The assay was based on the competition between unlabelled

cyclic AMP and a fixed quantity of the tritium labelled compound for binding to a protein which has a high specificity and affinity for cyclic AMP. The amount of labelled protein-cyclic AMP complex formed was inversely related to the amount of unlabelled cyclic AMP present in the assay sample. Measurement of the protein bound radioactivity enabled the amount of unlabelled cyclic AMP in the sample to be calculated.

Separation of the protein bound cyclic AMP from the unbound nucleotide was achieved by adsorption of the free nucleotide on to coated charcoal, followed by centrifugation. An aliquot of the supernatant was then removed for liquid scintillation counting. The concentration of unlabelled cyclic AMP in the sample was determined from a linear standard curve.

2.15.4 Reagents of the Cyclic AMP Assay

The kit contained the following reagents, all of which contained Tris/EDTA buffer and were in freeze-dried form.

1. Tris/EDTA buffer.
   This was dissolved in 25.0 cm^3 of distilled water to give an approximately 0.05 M solution at pH 7.5 containing 4 mM EDTA.

2. Binding protein, purified from bovine muscle.
   The binding protein was dissolved in 15.0 cm^3 of distilled water by gentle swirling.

3. (8 - ^{3}H) Adenosine 3',5' - cyclic phosphate, 180 pmoles containing approximately 5μCi (185kBq).
This was dissolved in 10.0 cm^3 of distilled water.

4. Adenosine 3',5' - cyclic phosphate standard, 160 pmoles.
The cyclic AMP standard was dissolved in 5.0 cm^3 of distilled water.

5. Charcoal adsorbent.
20.0 cm^3 of ice-cold distilled water was added to the charcoal adsorbent. The container was then put in an ice-bath and stirred continuously using a magnetic stirrer.

2.15.5 The Preparation of Cyclic AMP Standards

Serial dilutions of the standard cyclic AMP solution was prepared as follows :

4 small glass tubes (100 mm x 10 mm) were placed in a rack and 0.5 cm^3 of the buffer (reagent 1) was added to each tube. 0.5 cm^3 of cyclic AMP standard (reagent 4) was added to the first tube and mixed thoroughly. 0.5 cm^3 of this dilution was transferred to the next tube and mixed again. The procedure was repeated sucessively with each remaining tube. Together with the original solution, 5 levels of standard cyclic AMP solution were available. 50 μl from each solution will give 16, 8, 4, 2 and 1 pmol per assay tube.

2.15.6 The Assay Procedure

1. 14 assay tubes (100 mm x 10 mm) and additional tubes for unknowns, in duplicates, were placed in a rack which was kept

at 0°C in an ice/water bath. The unknowns referred to the plasma or liver samples of the rats. The tubes were labelled and arranged according to the protocol shown below.

The Cyclic AMP Assay Protocol

Tube No.	Reagent 1 Buffer	Standards	Unknowns	Reagent 3 (^{3}H) Cyclic AMP	Reagent 2 Binding Protein	
1, 2	150	-	-	50	100	Charcoal blank
3, 4	50	-	-	50	100	Zero dose
5, 6	-	50	-	50	100	1pmol standard
7, 8	-	50	-	50	100	2pmol standard
9, 10	-	50	-	50	100	4pmol standard
11, 12	-	50	-	50	100	8pmol standard
13, 14	-	50	-	50	100	16pmol standard
15 etc.			50	50	100	Unknowns

All volumes were in microlitres.

2. 150 µl of the buffer (reagent 1) was pipetted into the assay tubes 1, 2. These tubes were for the determination of the blank counts per minute for the assay.

3. 50 µl of the buffer (reagent 1) was pipetted into the assay tubes 3, 4 for determination of binding in the absence of unlabelled cyclic AMP

4. Starting with the lowest level of standard cyclic AMP, 50 µl of each dilution was put into each successive pair of assay

tubes (tubes 5 - 14).

5. 50 µl of each unknown or sample for the determination of cyclic AMP level, was added in duplicate, into the additional assay tubes as appropriate.

6. 50 µl of the labelled cyclic AMP (reagent 3), was added to every assay tubes.

7. 100 µl of the binding protein (reagent 2) was added to assay tubes 3 - 14 and to every assay tube containing an unknown.

8. All tubes were vortex mixed for about 5 seconds.

9. The ice bath containing the tubes was placed in a refrigerator, at 2 - 4^{o}C and left for two hours.

10. The ice bath was removed from the refrigerator and ice replenished, if necessary.

11. 100 µl of the charcoal suspension was added to all tubes that can be centrifuged in 1 batch. The assay solutions were centrifuged not less than 1 minute nor more than 6 minutes after the addition of charcoal to the last tube in a batch. The tubes were vortex mixed briefly and placed in a refrigerated centrifuge.

12. The tubes were centrifuged at 4°C at a speed of 8000 rpm for 5 minutes to sediment the charcoal.

13. After centrifuging each batch, a 100 µl sample was removed from each tube, taking care not to disturb the sediment and placed in scintillation vials which contained 10.0 cm^3 of toluene-triton X-100 scintillation cocktail. The radioactivity was determined by liquid scintillation counting in a Packard Tri-Carb liquid scintillation spectrometer for 5 minutes.

2.15.7 The Preparation of Toluene-triton x-100 Scintillation Cocktail

4.4 g of 2,5-diphenyloxazole (PPO) (Sigma Chemical Company) and 0.1 g of 1,4-bis (5-phenyl-2-oxazolyl)-benzene; 2,2'-p-Phenylene-bis (5-phenyloxazole) (POPOP) (Sigma Chemical Company) were dissolved in 1 litre of toluene (BDH). 500.0 cm^3 of triton X-100 (BDH) was then added and the solution was well mixed and stored in the dark. Before adding radioactive samples for counting, vials were prepared by adding 10.0 cm^3 aliquots of the scintillation cocktail and 1.0 cm^3 of distilled water to each vial and the vials were shaken to obtain a clear solution. All chemicals were of scintillation grade.

2.15.8 Calculations

1. To determine the blank counts per minute (c.p.m.) for the assay, the c.p.m. for tubes 1, 2 were averaged.

2. The c.p.m. for tubes 3 and 4 were averaged and the blank c.p.m. was subtracted from this. This result was the

c.p.m. bound in the absence of unlabelled cyclic AMP (Co).

3. The c.p.m. for each pair of duplicates in tubes 5 to 14 and the additional pairs of tubes which contain unknowns were averaged. The blank c.p.m. was subtracted from each result to give the c.p.m. bound in the presence of standard or unknown unlabelled cyclic AMP (C_x).

4. Co/C_x was calculated for each level of cyclic AMP and the unknowns.

5. A graph of Co/C_x was plotted against pmoles of standard cyclic AMP per tube on a linear graph paper. This gave the calibration or standard curve for cyclic AMP.

6. The reagents for cyclic AMP assay could be stored at $0^{o}C$ for 1 to 2 months without affecting the reagents. When the cyclic AMP assay kit was used on more than one occassion, the calibration curve was checked by redetermining Co and Co/C_x for 3 levels of standard cyclic AMP (2 pmoles, 4 pmoles and 8 pmoles).

7. From the Co/C_x value of the sample, the number of pmoles of cyclic AMP can be obtained from the calibration curve. The values of cyclic AMP obtained for plasma samples were converted to pmoles of cyclic AMP per cm^3 of plasma, and the values of cyclic AMP obtained for liver samples were converted to pmoles of cyclic AMP per mg. wet weight of liver.

2.16 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 Dessaules (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 15 lbs. sq. in^{-1}. The rats (male, Wistar strain, 250-300 g) were anaesthetized as previously described and the sterile pellets planted subcutaneously using a caponiser. There were 4 pellets per rat; one pellet in each axilla and groin. On the 7th day after implantation the animals were killed by a blow to the head and the pellets were 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. The anti-inflammatory effect of a drug was calculated as the reduction of the increase in weight of the cotton pellets in the treated group as compared with the untreated control group of rats.

2.17 The Effect of Putrescine on Cotton Wool Pellet Granuloma Test

Rats which had previously been implanted with four cotton wool pallets were given subcutaneous injections of 1.0 cm^3 of putrescine daily, for the duration of the experiment. 1 group of rats received putrescine at a dose of 50 $\mu g. kg^{-1}$ and another group of rats received putrescine at a dose of 5000 $\mu g. kg^{-1}$. Control rats were injected

with 1.0 cm^3 of 0.9% (w/v) saline alone daily. There were 6 rats per group.

2.18 The Effect of Putrescine on Superoxide Anions Generated From The Stimulation of Guinea-Pig Polymorphonuclear Leucocytes By N-formyl-methionyl-leucyl-phenylalanine (FMLP) in The Presence of Cytochalasin B

2.18.1 The Induction and Preparation of Guinea-pig Polymorphonuclear Leucocytes (PMNs)

The accumulation of polymorphonuclear leucocytes was induced in 2 (300 - 350 g) male guinea-pigs by the intraperitoneal injection of 0.1% (w/v) oyster glycogen (Sigma Chemical Company). The oyster glycogen solution was prepared in sterile distilled water. After 24 hours, the guinea-pigs were anaesthetixed by using a mixture of 3% halothane in oxygen and nitrous oxide and 50 cm^3 of sterile (filtered through a sterile 0.22 m membrane filter, Millipore Corporation), heparinized Hanks' Balanced Salt Solution (HBBS, 10 cm^{-3}) was injected into the peritoneal cavity of each of the guinea-pigs. The abdominal cavity was gently massaged for 5minutes to produce a homogenous cell suspension. The guinea-pigs were then killed by an overdose of halothane and the peritoneal cavities were opened by cutting along the ventral midline and the cell-rich fluid was collected with a plastic pasteur pipette and transferred to a plastic tube. Care was taken not to include any hairs in the sample by wiping away loose hairs with a damp tissue. About 15-20 cm^3 of fluid was obtained from each of the guinea-pig. The cell suspension was centrifuged in plastic centrifuge tubes (110 mm x 15 mm) at 200 g for 10 minutes at room temperature. The supernatants

were discarded and the cell pellets were resuspended in 0.85% ammonium chloride solution (w/v) to lyse any contaminating erythrocytes. After standing for 5 minutes at room temperature, the cells were washed 4 - 5 times with HBBS. Before the last centrifugation, the cell suspensions were pooled together into 1 tube and then centrifuged. The supernatant was discarded and 1 cm^3 of HBBS was then added to the resulting cell pellet and the cells were dispersed by means of a vorted mixer. Total and differential counts were performed on the cell suspension.

2.18.2 The Composition of Hanks' Balanced Salt Solution (HBBS)

HBBS has the following composition (mg. $litre^{-1}$) :

$CaCl.2H_2O$	185.5	Nacl	8000
KCl	400.0	$NaHCO_3$	350
KH_2PO_4	60.0	Na_2HPO_4	47.5
$MgSO_4.\ 7H_2O$	200.0	D-glucose	1000

2.18.3 Total and Differential Cell Count

For the differential cell count, a smear of the cell suspension was made on a glass slide and air-dried. The cells were fixed in methanol and stained with Wright's stain (BDH). The cells were examined under an oil immersion at a magnification fo x1000 and approximately 500 cells were counted in 4 or 5 random fields. The cells harvested 24 hours after the intraperitoneal injection of 0.1% glycogen were 80% PMNS and 20% mococytes.

For the total leucocyte count, the cell suspension was diluted 1 : 10 with a white cell diluting fluid and counted using an 'Improved Neubauer' counting chamber. The cell suspension was then diluted to give a concentration of 20×10^6 cells cm^{-3}. The cells were kept on ice until ready for use.

2.18.4 Stock Solution of FMLP

FMLP (Sigma Chemical Company) was dissolved in dimethylsulfoxide (DMSO) to give a stock solution of 10^{-3}M. Aliquots of the stock solution was stored at -20° until required. These stock solutions were then thawed and diluted in HBBS prior to each experiment.

2.18.5 Stock Solution of Cytochalasin B

Cytochalasin B (Sigma Chemical Company) was made up as a 1 mg. cm^{-3} stock solution in dimethylsulfoxide and aliquots stored at -20°C until used. Aliquots of the stock solution were diluted with HBBS to give a concentration of 0.5 mg. cm^{-3} prior to use.

2.18.6 Putrescine Concentrations Used in The Assay

Putrescine dihydrochloride concentrations were prepared in HBBS to give concentrations in the range of 10 mM to 100mM in a final assay volume of 1 cm^3, 0.25 cm^3 of the various putrescine concentrations was used in the assay of the superoxide anions.

2.18.7 The Effect of Putrescine on Superoxide Anions Production From Guinea-pig Polymorphonuclear Leucocytes

The Assay of the superoxide anions generated from the stimulation of guinea-pig PMN_s by FMLP in the presence of cytochalasin B, was essentially as described by Simchowitz et al, (1979). Superoxide anions generation was measured by the reduction of ferricytochrome C (horse heart, Type III, Sigma Chemical Company). All experiments were performed in duplicates in a final volume of 1 cm^3 in 110 mm x 15 mm plastic centrifuge tubes. 0.25 cm^3 of PMN_s suspension (5 x 10^6 cells cm^{-3}) were incubated with 20 µl of cytochalasin B (5 cm^{-3}) for 10 minutes at 37°C in a shaking water bath. Then serially added were : 0.25 cm^3 of HBBS (control) or putrescine solution, 0.25 cm^3 of ferricytochrome C solution (1 mg. cm^{-3}) and 0.25 cm^3 FMLP (0.25×10^{-4} M). The concentrations in brackets referred to the final concentrations in 1 cm^3 of solution. The tubes were further incubated at 37°C for 10 minutes. The blank tubes for the control and each of the putrescine concentration, which had all the reagents minus the cells, were also incubated. The tubes were then removed and put in an ice water bath. 10 µl of superoxide dismutase (20 cm^{-3}; from bovine blood, Sigma Chemical Company) was added to all the tubes to stop the reaction. When the tubes were cold, they were centrifuged at 200 g for 5 minutes at room temperature and the absorbance of the supernatant was read out at 550 nm in a spectrophotometer. The amount of superoxide anions produced was calculated from the absorbance values of the samples divided by the extinction coefficient for the change between ferricytochrome C and ferrocytochrome C at 550 nm. The effect of putrescine on superoxide anions was expressed as the percentage inhibition of superoxide anions generation in the presence of putrescine.

3. RESULTS

3.1 The Effect of Adenylate Cyclase Activators on the Carrageenan-induced Oedema

3.1.1 The Anti-inflammatory Effect of Salbutamol

The effects of salbutamol at doses of 10 mg. kg^{-1} and 12 mg. kg^{1-} given intraperitoneally, on the carrageenan-induced oedema in the rats, are shown in Figures 3.1 and Figures 3.2 respectively.

Salbutamol at the 2 doses used were strongly anti-inflammatory against the carrageenan oedema. Salbutamol at a dose of 10 mg. kg^{-1} produced a 52% suppression of the oedema compared to the control, at 3 hours after the injection of carrageenan. Salbutamol at a higher dose (12 mg. kg^{-1}) suppressed the oedema at 3 hours after the carrageenan injection, by 83% . Anti-inflammatory activity was still significant ($p < 0.005$) when measurement was made at 5 hours after the carrageenan injection, where the suppression of the oedema was about 50%.

The effect of salbutamol (15 mg. kg^{-1}) given orally is shown in Figure 3.3. The suppression of oedema at 3 hours and 5 hours after the injection of carrageenan were 83% and 51% respectively.

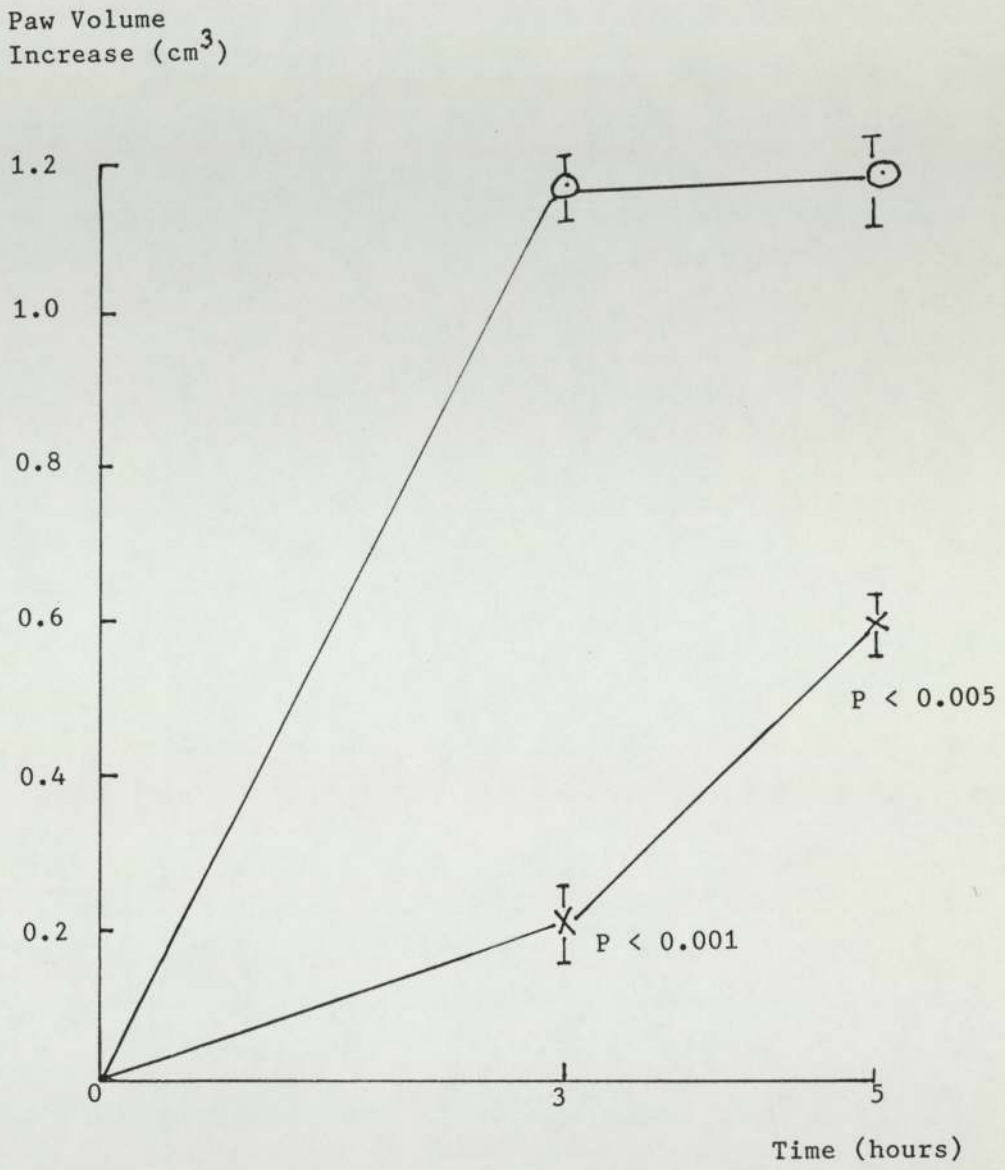

Figure 3.1 The effect of salbutamol (10 mg. kg^{-1}) on the carrageenan oedema x———x.

Salbutamol was given intraperitoneally 30 minutes before the injection of carrageenan into the hind paws of the rats.

Control group was given 0.9% saline only 0———0

Each point represents the mean of 6 animals ± SEM.

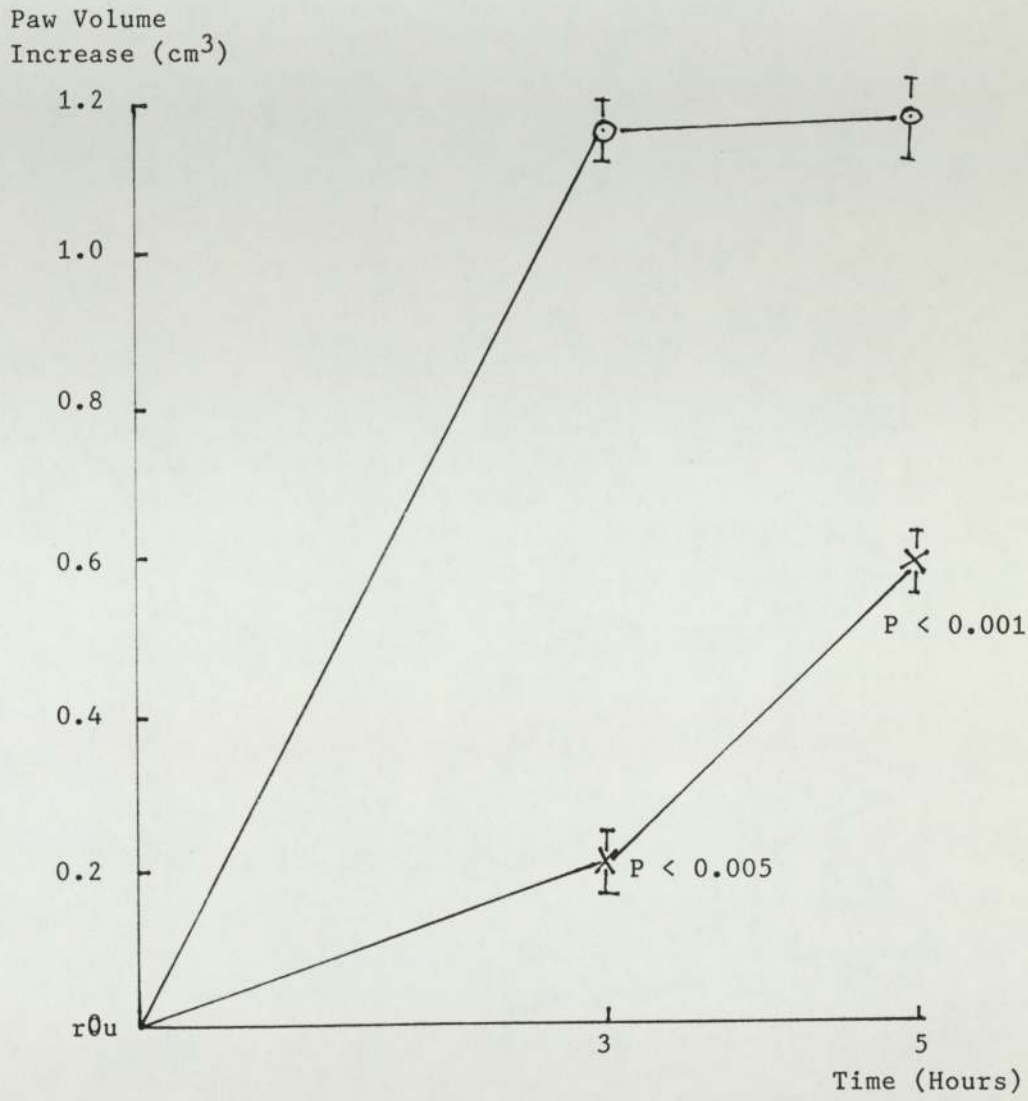

Figure 3.2 The effect of salbutamol (12 mg. kg^{-1}) on the carrageenan oedema x——x

Salbutamol was given intraperitoneally 30 minutes before the injection of carrageenan into the hind paws of the rats.

Control group was given 0.9% saline only 0——0

Each point represents the mean of 6 animals ± SEM.

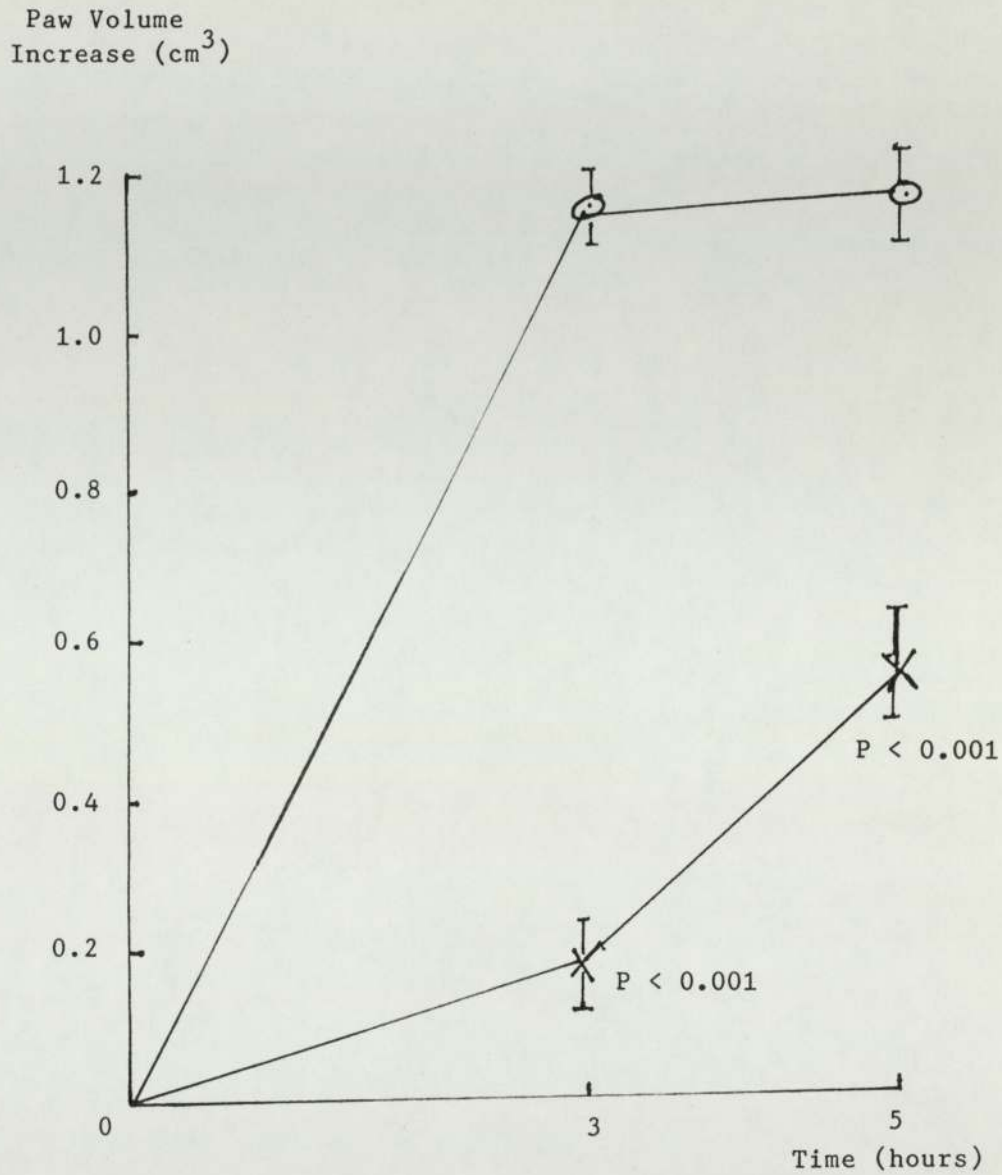

Figure 3.3 The effect of salbutamol (15 mg. kg^{-1}) orally x———x on the carrageenan oedema.

Salbutamol was given 45 minutes before the injection of carrageenan into the hind paws of the rats.

Control group was given 0.9% saline only o———o.

Each point represents the mean of 6 animals ± SEM.

3.1.2 The Anti-inflammatory Effect of Isoprenaline

Isoprenaline at the doses used (4 mg. kg^{-1} and 8 mg. kg^{-1}) strongly inhibited the carrageenan oedema (Figure 3.4). The suppression of the oedema at 3 hours after the injection of carrageenan, by isoprenaline 4 mg. kg^{-1} and 8 mg. kg^{-1} were 52% and 89% respectively. Isoprenaline at 8 mg. kg^{-1} almost abolished the oedema formation.

Isoprenaline did not suppress the carrageenan oedema when measurements were made at 5 hours after the carrageenan injection. However, when another dose of isoprenaline was given 3.5 hours after the first dose, the oedema formation at 5 hours was suppressed though not as much as that at 3 hours (Figure 3.4).

3.1.3 The Anti-inflammatory Effect of Sodium Phenobarbitone

Sodium phenobarbitone at a dose of 100 mg. kg^{-1} inhibited the carrageenan oedema at 3 hours and 5 hours after the injection of carrageenan by 59% and 27% respectively (Figure 3.5).

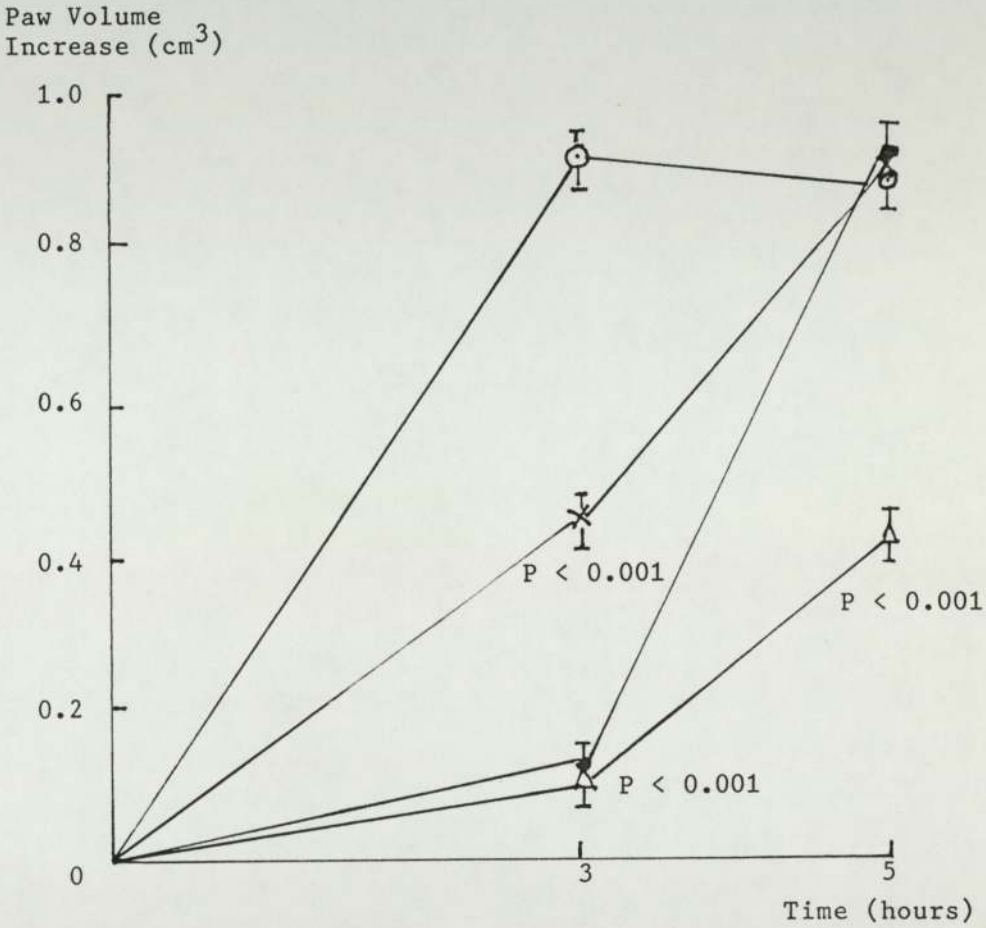

Figure 3.4 The effect of isoprenaline on the carrageenan oedema.

⊙——⊙ Control group (o.9% saline only)

x——x Isoprenaline 4 mg. kg^{-1} intraperitoneally.

●——● Isoprenaline 8 mg. kg^{-1} intraperitoneally.

Isoprenaline was given 30 minutes before the injection of carrageenan into the hind paws of the rats.

Δ——Δ Rats given another dose of 8 mg. kg^{-1} isoprenaline 3½ hours after the first dose.

Each point represents the mean of 6 animals ± SEM.

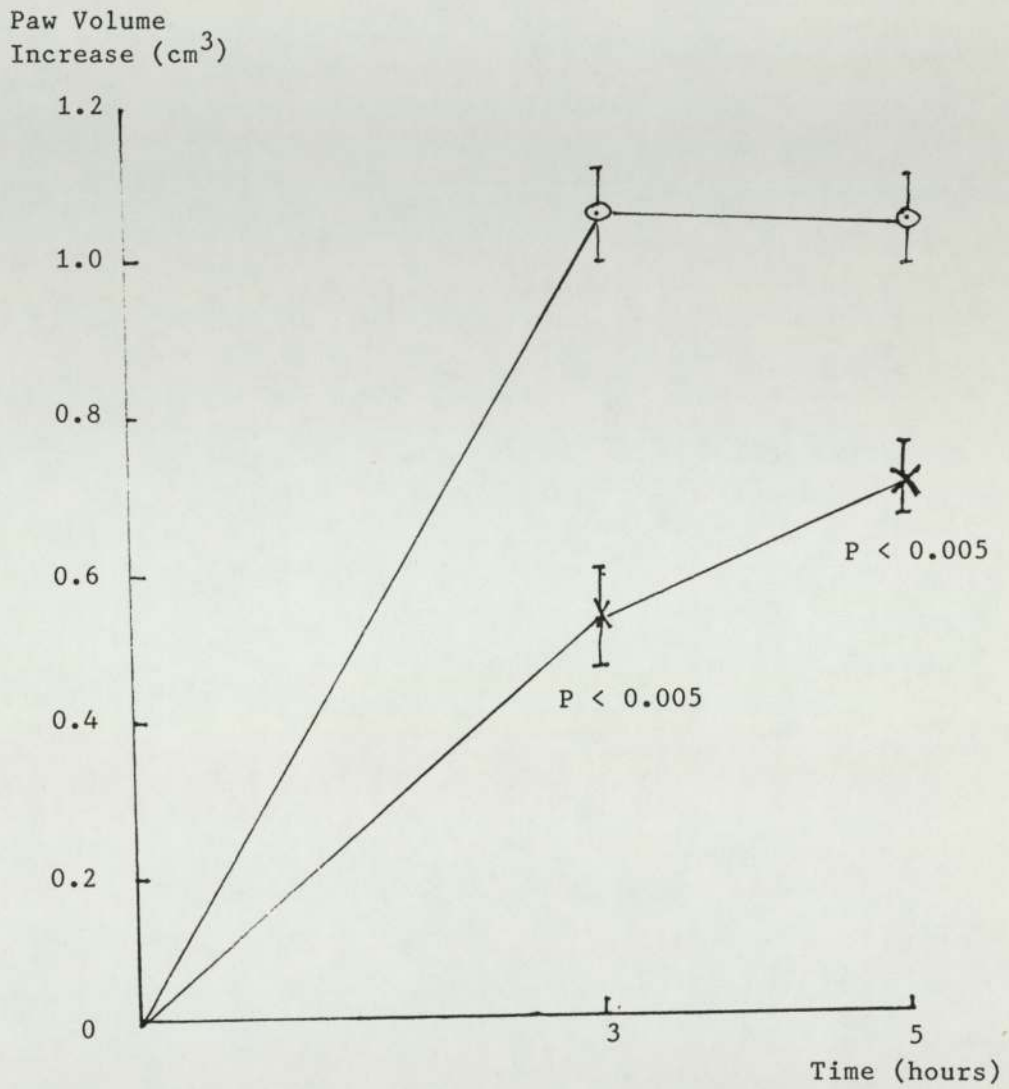

Figure 3.5 The effect of sodium phenobarbitone 100 mg. kg^{-1} on the carrageenan oedema ×——× Sodium phenobarbitone was given intraperitoneally 4 hours before the injection of carrageenan into the hind paws of the rats Control group was given 0.9% saline only ○——○ Each point represents the mean of 6 animals ± SEM.

3.2 The Effect of a phosphodiesterase Inhibitor on the Carrageenan Oedema

3-isobutyl-1-methyl xanthine when given intraperitoneally at a dose of 14 mg. kg^{-1} strongly inhibited the carrageenan oedema (Figure 3.6). The suppression of the carrageenan oedema at 3 hours and 5 hours when compared with the control group was 61% and 19% respectively. Rats that were given 0.9% saline : ethanol (5:1) and 0.9% saline only, showed no significant difference in oedema formation.

3.3 The Effect of Putrescine on the Carrageenan Oedema

The effect of putrescine when injected together with the carrageenan is shown in Figure 3.7. Putrescine at the concentration of 85.5 µg. paw^{-1} inhibited the oedema formed at 3 hours after the carrageenan injection by about 19% when compared with the control group. Even though the inhibition was small when compared with the other drugs used for examples, isoprenaline, it was significant ($P < 0.05$). Putrescine did not inhibit the oedema formed at 5 hours after the carrageenan injection.

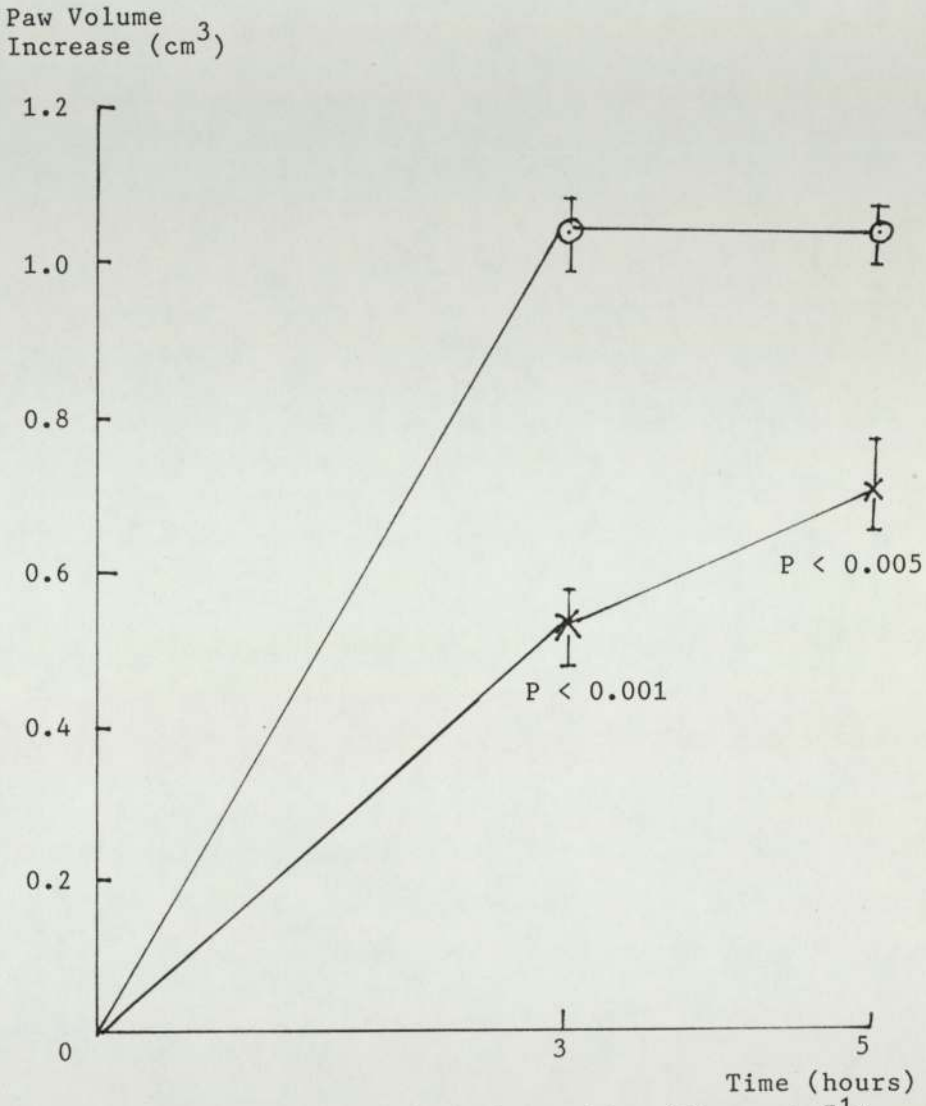

Figure 3.6 The effect of 3-isobutyl-1-methyl xanthine (14 mg. kg^{-1}) given intraperitoneally on the carrageenan oedema x——x. The drug was given 2 hours before the injection of carrageenan into the hind paws of the rats. Control group received 5:1 saline/ethanol 0——0 Each point represents the mean of 6 animals ± SEM.

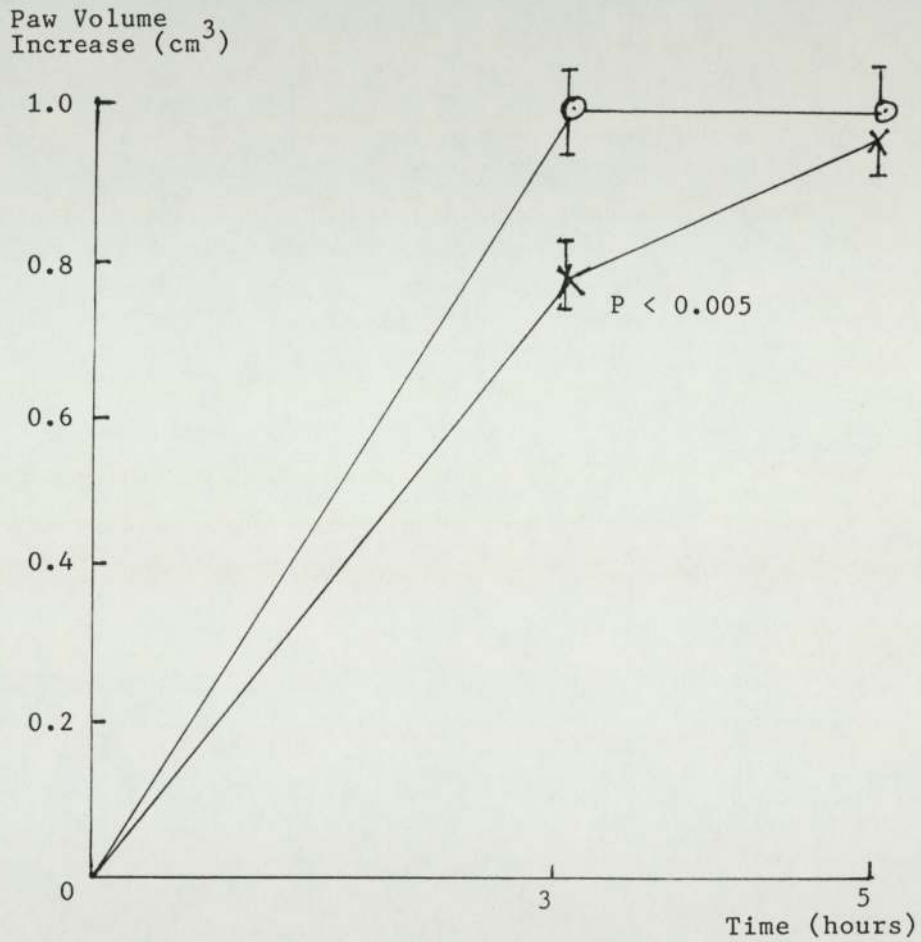

Figure 3.7 The increase in paw volume following the subplantar injection of 0.05 cm^3 of 2% carrageenan solution mixed with putrescine (85.5 µg. paw^{-1}) x——x compared to the controls (0.05 cm^3 carrageenan only) 0——0. Each point is the mean of 6 animals ± SEM

3.4 The Effect of 1,3 Diaminopropane on the Carrageenan Oedema

The results are shown in Figure 3.8 and Figure 3.9. 1,3-Diaminopropane at a dose of 15 mg. kg^{-1} significantly inhibited the oedema formation at 3 hours after the carrageenan injection by about 28%. Although there was still a slight inhibitory action at 5 hours, the result was not significant (Figure 3.8).

1,3 Diaminopropane at a dose of 5 mg. kg^{-1} given intraperitoneally 30 minutes before the injection of carrageenan and again at 1.5 hours after the carrageenan injection failed to inhibit the oedema at 3 hours but inhibited the oedema formed at 5 hours, by about 27% (Figure 3.9).

3.5 The Effect of 1,2 Diaminoethane on the Carrageenan Oedema

1,2 Diaminoethane at a concentration of 15 mg. kg^{-1} given intraperitoneally, did not inhibit the carrageenan oedema. The rats which received the drug produced the same amount of inflammation in the hind paws at 3 hours and 5 hours after the injection of carrageenan as the control group.

3.6 The Effect of Putrescine on the Serotonin-induced Oedema

The result is shown in Figure 3.10. Putrescine at a concentration of 10 mg. kg^{-1} given intraperitoneally to the rats 30 minutes before the serotonin injection significantly inhibited the oedema formed at 1 hour after the injection of serotonin, by about 18%.

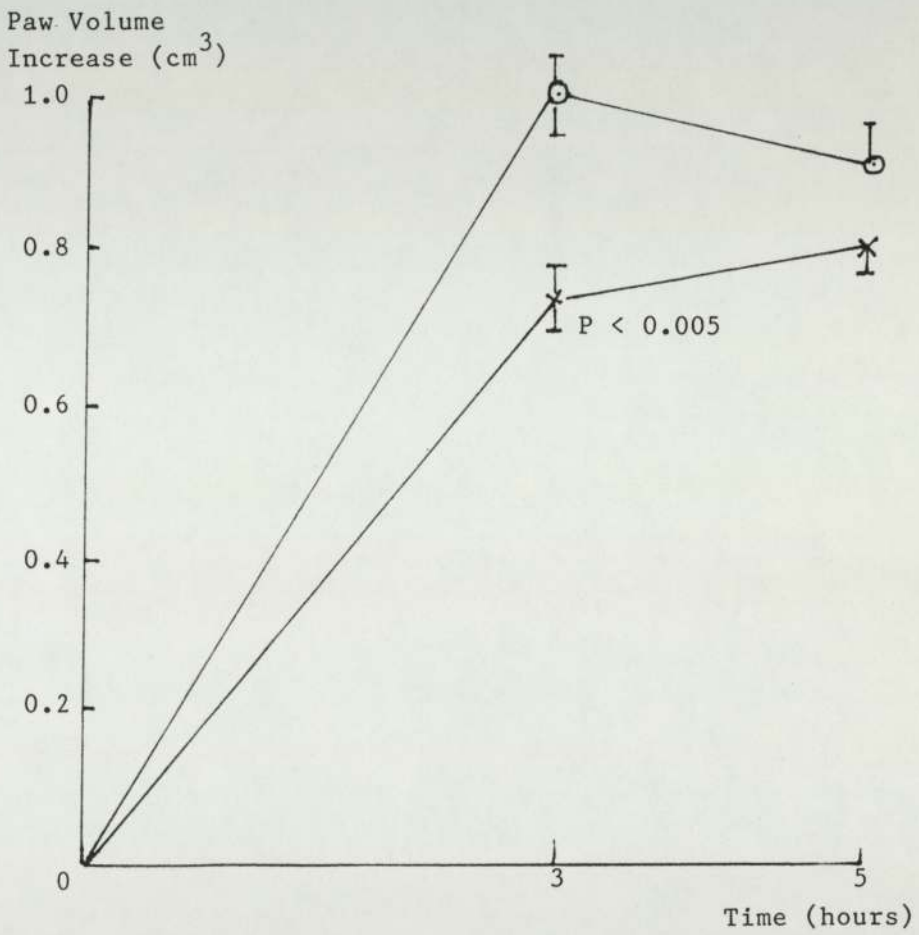

Figure 3.8 The effect of 1,3 diaminopropane at a dose of 15 mg. kg^{-1} on the carrageenan oedema x———x. 1,3 Diaminopropane was given intraperitoneally 30 minutes before the injection of carrageenan into the hind paws of the rats.
Control group received water only 0———0.
Each point represents the mean of 5 animals ± SEM.

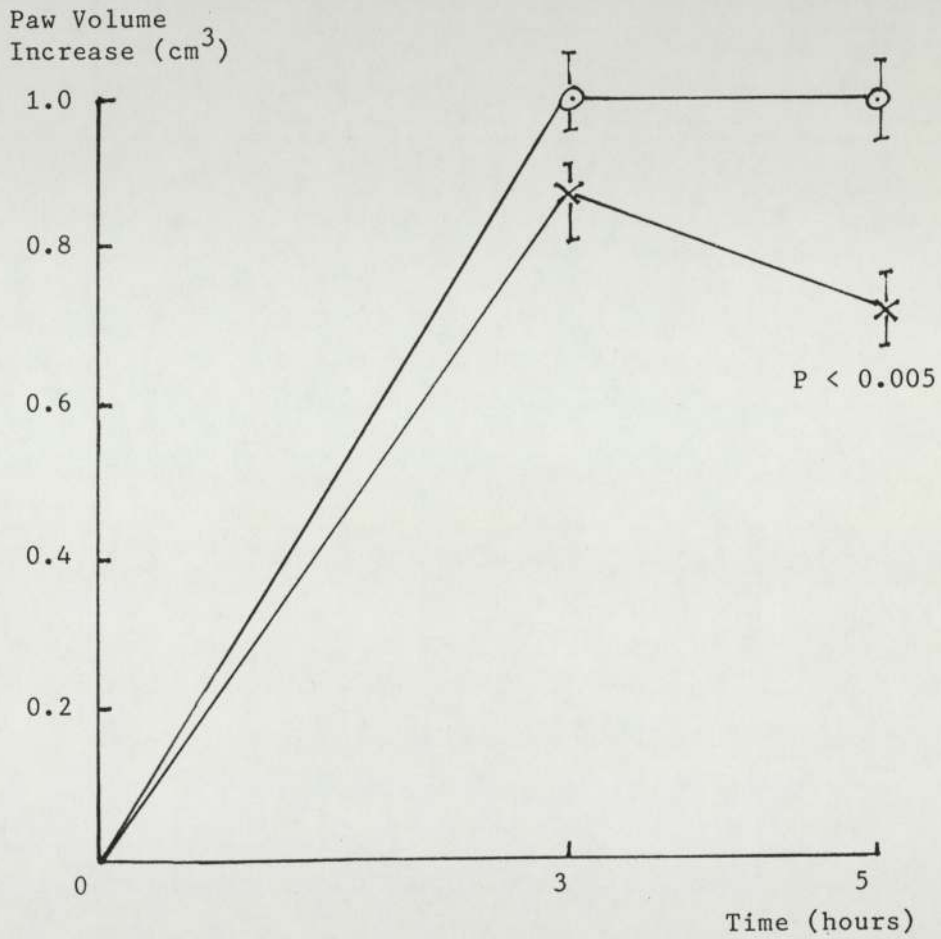

Figure 3.9 The effect of 1,3 diaminopropane at a dose of 5 mg. kg^{-1} on carrageenan the oedema x———x. 1,3 Diaminopropane was given intraperitoneally 30 minutes before the injection of carrageenan into the hind paws of the rats, and again at 1½ hours after the carrageenan injection. Control group was given water only 0———0. Each point represents the mean of 5 animals ± SEM.

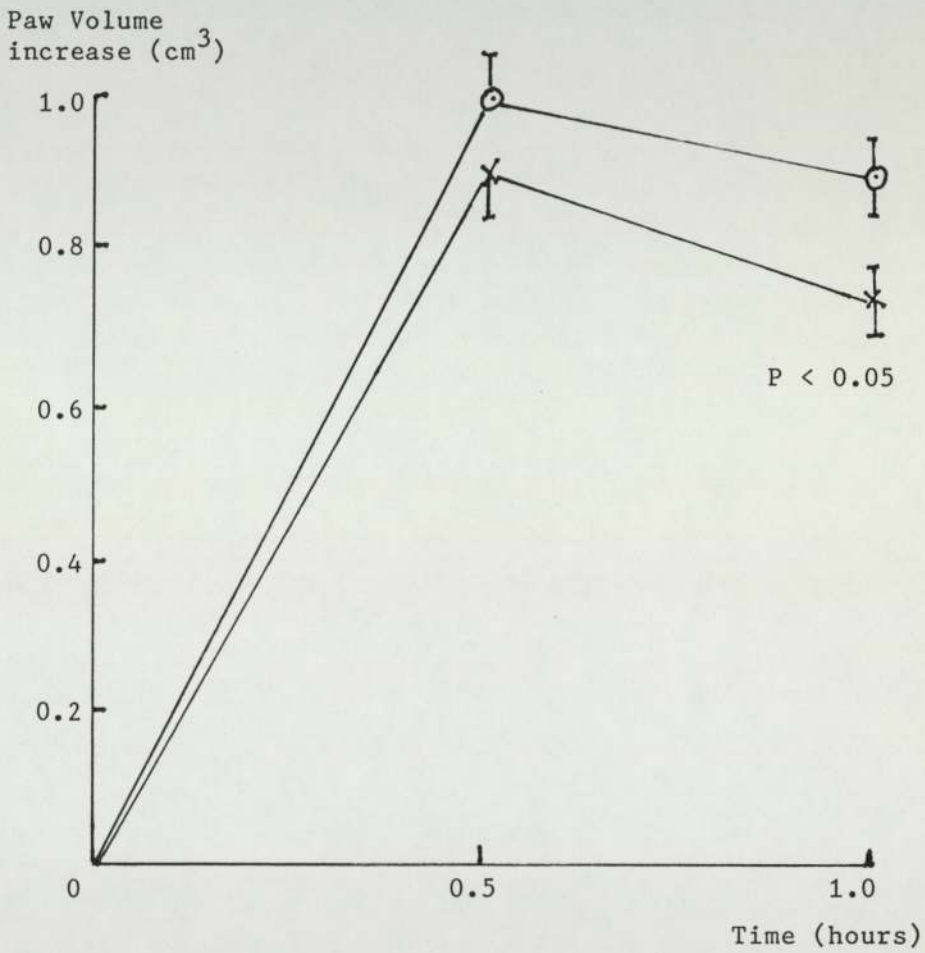

Figure 3.10 The effect of putrescine (10 mg. kg^{-1}) on the serotonin-induced oedema. Putrescine was given intraperitoneally 30 minutes before the injection of carrageenan into the hind paws of the rats. Control group was given 0.9% saline only 0———0. Each point represents the mean of 5 animals ± SEM.

3.7 The Effect of Propranolol on the Anti-inflammatory Activity of Isoprenaline or Salbutamol on the Carrageenan Oedema

The results are shown in Table 3.1 and Table 3.2. Propranolol (15 mg. kg^{-1}) completely abolished the anti-inflammatory effect of isoprenaline (8 mg. kg^{-1}) or salbutamol (15 mg. kg^{-1}) on the carrageenan oedema.

Table 3.1 The Inhibitory Effect of Propranolol on the Anti-inflammatory Activity of Isoprenaline on the Carrageenan Oedema

Treatment	Paw volume increase at: 3 hours after carrageenan injection (cm^3)	5 hours after carrageenan injection (cm^3)
Control	1.00 ± 0.04	0.98 ± 0.03
Isoprenaline (8 mg. kg^{-1})	0.10 ± 0.03	0.98 ± 0.04
Propranolol only (15 mg. kg^{-1})	0.98 ± 0.03	0.95 ± 0.05
Isoprenaline (8 mg. kg^{-1}) + Propranolol (15 mg. kg^{-1})	0.96 ± 0.04	0.95 ± 0.04

Each result represents the mean of 7 values ± SEM

Table 3.2 The Inhibitory Effect of Propranolol on the Anti-inflammatory Activity of Salbutamol on the Carrageenan Oedema

Treatment	Paw volume increase at: 3 hours after carrageenan injection (cm^3)	5 hours after carrageenan injection (cm^3)
Control	1.00 ± 0.04	0.98 ± 0.04
Salbutamol (15 mg. kg^{-1})	0.10 ± 0.03	0.60 ± 0.04
Propranolol (15 mg. kg^{-1})	0.98 ± 0.05	0.95 ± 0.04
Salbutamol (15 mg. kg^{-1}) + Propranolol (15 mg. kg^{-1})	0.98 ± 0.05	0.95 ± 0.05

Each result represents the mean of 7 values ± SEM.

3.8 The Effect of DL-α-difluoromethylornithine (DFMO) on the Anti-inflammatory Activity of Isoprenaline in the Carrageenan Oedema

The result is shown in Table 3.3

Table 3.3 The Effect of DFMO on the Anti-inflammatory Activity of Isoprenaline in the Carrageenan Oedema

Treatment	% suppression of oedema at 3 hours after carrageenan injection
Control	0.00
Isoprenaline (8 mg. kg^{-1})	92.00
DFMO only (200 mg. kg^{-1})	2.00
Isoprenaline (8 mg. kg^{-1}) + DFMO (200 mg. kg^{-1})	81.00
Control	0.00
Isoprenaline (8 mg. kg^{-1})	94.00
DFMO (400 mg. kg^{-1})	2.00
Isoprenaline (8 mg. kg^{-1}) + DFMO (400 mg. kg^{-1})	83.00

DFMO at doses of 200 mg. kg^{-1} and 400 mg. kg^{-1} prevented signifantly the isoprenaline inhibition of the carrageenan oedema by about 11% ($P < 0.005$). DFMO when given alone to the rats did not produce any significant effect on the carrageenan oedema when compared with the control.

3.9 The Anti-inflammatory Effect of Theophylline on Rat Adjuvant Arthritis

The results are shown in Figure 3.11 and Figure 3,12. Theophylline signigicantly ($P < 0.05$) inhibited the development of adjuvant arthritis on the injected paws of the rats during the acute phase (day 2) and when the systemic disease has begun (day 12 onwards). There was about 34% inhibition of foot swelling on day 2, 40% inhibition on day 12 and 37% inhibition of the foot swelling on day 19 (Figure 3.11).

Theophylline also significantly suppressed the development of inflammation on the uninjected foot of the rats on day 5 ($P < 0.05$), day 7 ($P < 0.05$), day 14 ($P < 0.05$) and day 26 ($P < 0.05$) (Figure 3.12).

The effect of theophylline 15 mg. kg^{-1} given orally and theophylline 30 mg. kg^{-1} orally, in the rat adjuvant arthritis was comparably the same.

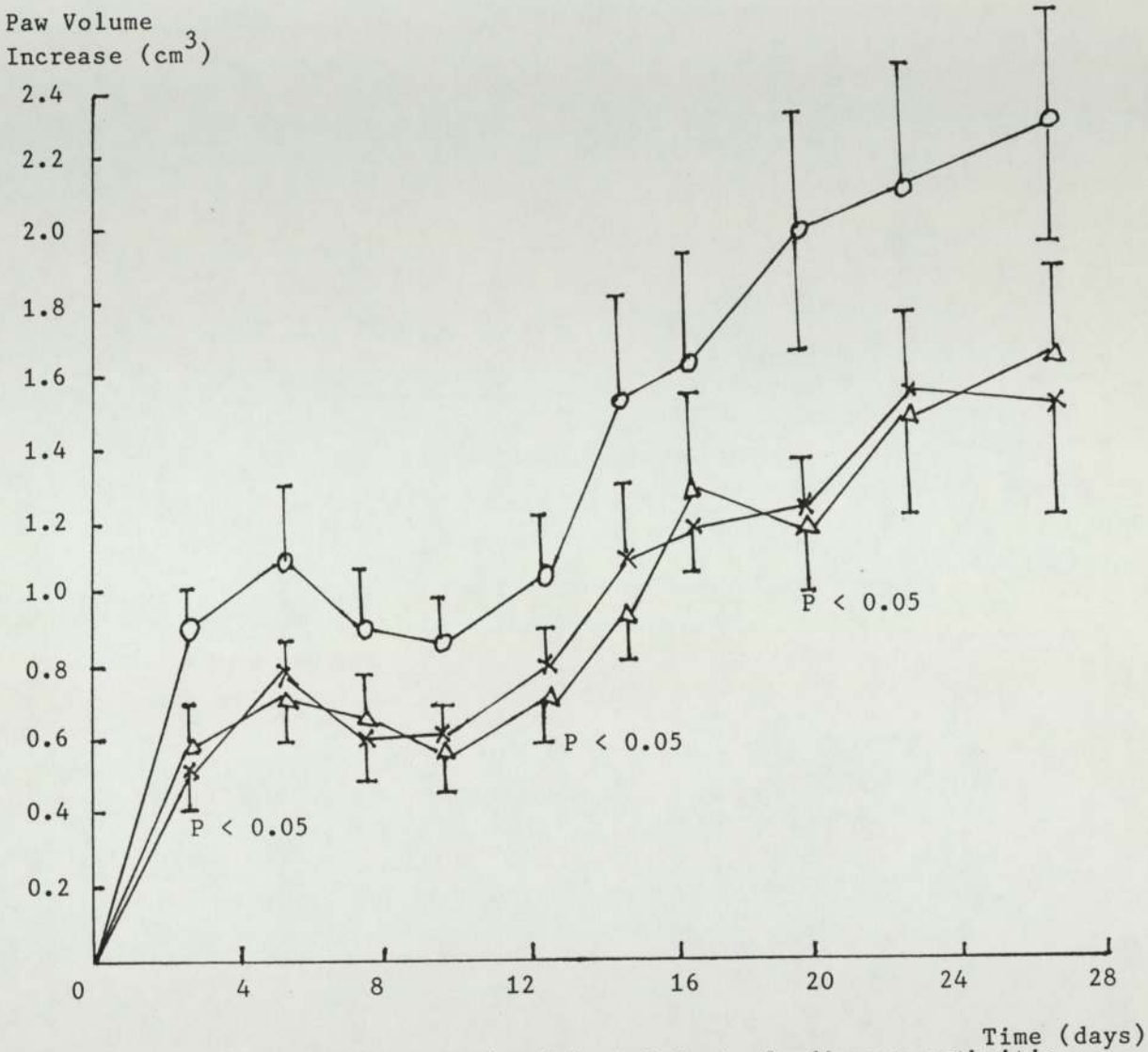

Figure 3.11 The effect on the injected foot of adjuvant arthritic rats given theophylline orally at doses of 15 mg. kg^{-1} Δ——Δ and 30 mg. kg^{-1} x——x compared to the controls O——O.
Each point represents the mean of 7 animals ± SEM.

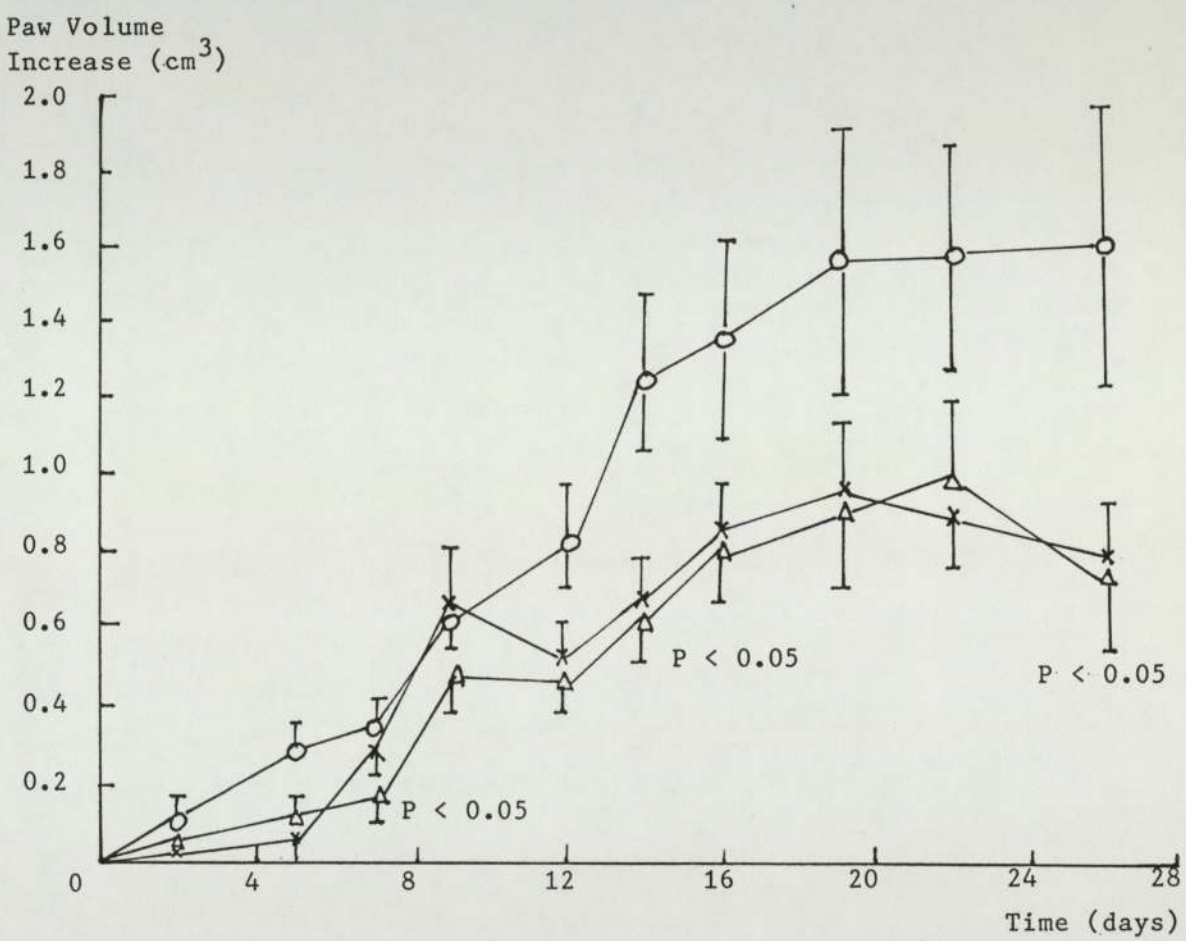

Figure 3.12 The effect on the non-injected foot of adjuvant arthritic rats given theophylline orally at doses of 15 mg. kg^{-1} Δ———Δ and 30 mg. kg^{-1} x———x compared to the controls 0———0.
Each point represents the mean of 7 animals ± SEM.

3.10 The Anti-inflammatory Effect of Salbutamol on Rat Adjuvant Arthritis

Salbutamol when given daily at a dose of 15 mg. kg^{-1} orally was able to suppress the development of adjuvant arthritis in the rats during the acute and chronic stage (Figure 3.13 and Figure 3.14).

During the acute stage, salbutamol significantly suppressed the inflammation on the injected paws on day 3 ($P < 0.005$), day 5 ($P < 0.005$) and day 7 ($P < 0.05$) (Figure 3.13). The percentage inhibition of inflammation on day 3, day 5 and day 7 were 33%. 42% and 36% respectively.

During the chronic stage, the development of the adjuvant arthritis was significantly suppressed when measured on day 17 ($P < 0.05$), day 20 ($P < 0.05$) and day 23 ($P < 0.05$). The percentage inhibition of inflammation on day 17, day 20 and day 23 were 18%, 25% and 26% respectively.

Salbutamol also significantly ($P < 0.05$) inhibited the development of inflammation on the uninjected paws of the rats on day 20 and day 23 (Figure 3,14).

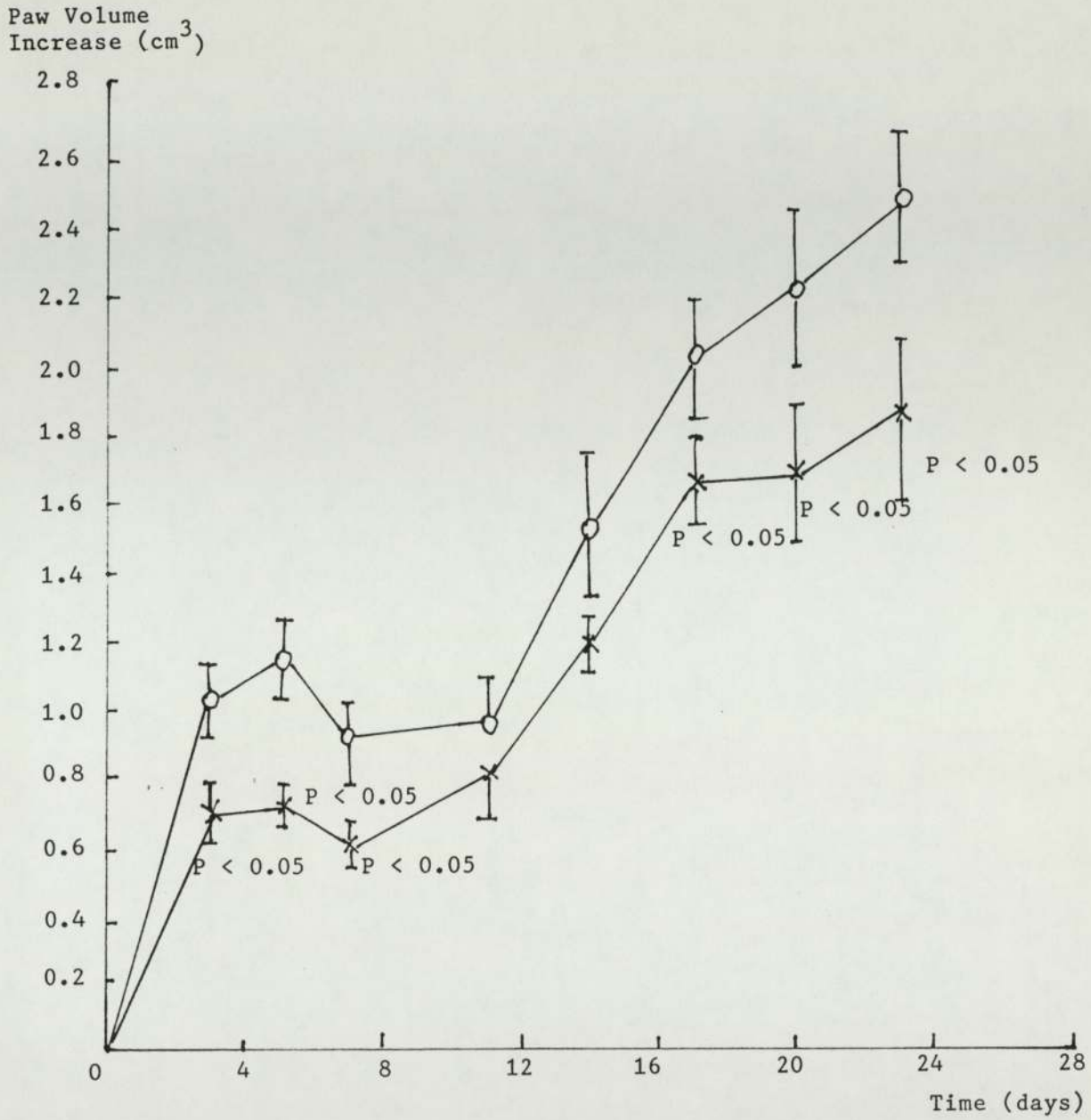

Figure 3.13 The effect on the injected foot of adjuvant arthritic rats given a daily oral dose of salbutamol 15 mg. kg^{-1} x———x compared to the controls 0———0.
Each point represents the mean of 7 animals ± SEM.

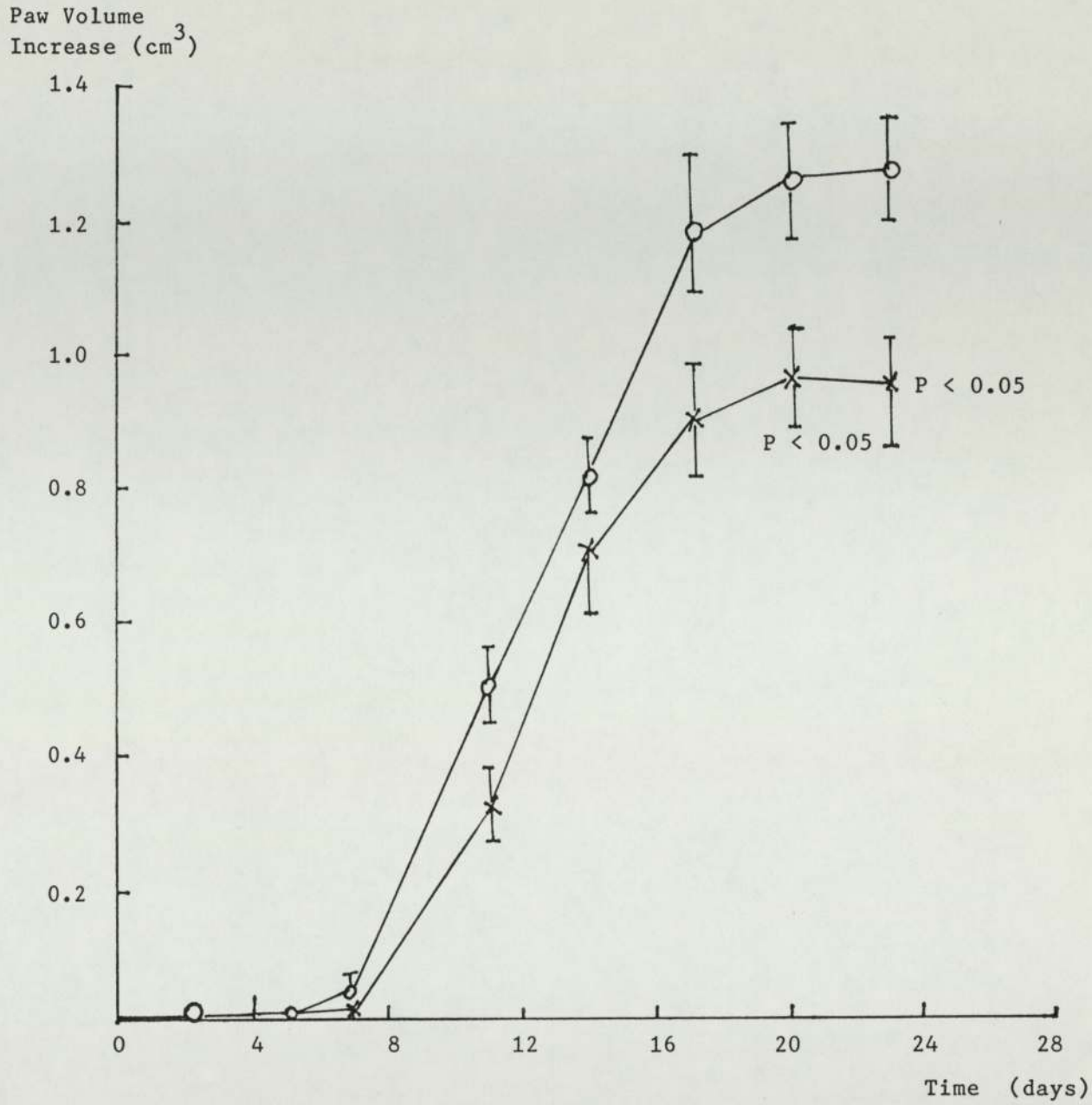

Figure 3.14 The effect on the non-injected foot of adjuvant arthritic rats given a daily dose of salbutamol 15 mg. kg^{-1} orally x———x compared to the controls o———o.

Each point represents the mean of 7 animals ± SEM.

3.11 Polyamine Levels in the Liver Extracts of Theophylline Treated Rats in Adjuvant Arthritis

The results are shown in Table 3.4. The liver putrescine levels in rats treared with theophylline were twice that of the controls. The amount of spermidine in the liver extracts of the theophylline treated rats were about 1½ times that of the control, but the amount of spermine was the same as the control.

Table 3.4 Polyamine Levels in the Liver Extracts of Theophylline Treated Rats in Adjuvant Arthritis, Killed on Day 26

Treatment	µg of putrescine per 100 mg. dry weight of liver	µg of spermidine per 100 mg. dry weight of liver	µg of spermine per 100 mg. dry weight of liver
Control	10.3 ± 0.55	2.5 ± 0.11	5.5 ± 0.12
Theophylline (15 mg kg^{-1})	20.1 ± 0.89	4.0 ± 0.13	5.5 ± 0.11
Theophylline (30 mg kg^{-1})	22.0 ± 0.47	4.0 ± 0.10	6.5 ± 0.13

Each result represents the mean of 5 determinations ± SEM.

3.12 The Plasma Cyclic AMP Levels of Rats Treated with Isoprenaline or Salbutamol

The result is shown in Table 3.5. There was an increase in the plasma cyclic AMP levels in the rats, 3.5 hours after been dosed with isoprenaline or salbutamol. The increase in the plasma cyclic AMP levels was about 3 times that of the control. At 5.5 hours after the administration of the drugs, the plasma cyclic AMP levels in rats treated with salbutamol were still high but the plasma cyclic AMP levels of rats treated with isoprenaline had returned to the control values.

Table 3.5 The Plasma Cyclic AMP Levels of Rats Treated With Isoprenaline or Salbutamol (pmoles cm^{-3})

Treatment	Time after dosing (hours)	
	3.5	5.5
Control	14.5 ± 1.0	12.1 ± 0.5
Isoprenaline (8mg. kg^{-1})	47.5 ± 0.3	14.0 ± 0.9
Salbutamol (15mg. kg^{-1})	39.3 ± 0.5	29.6 ± 0.8

Each result represents the mean of 5 determinations ± SEM.

3.13 The Liver Cyclic AMP Levels of Rats Treated with Isoprenaline or Salbutamol

The result is shown in Table 3.6. There was no difference in the liver cyclic AMP levels of rats determined at 3.5 hours after been given the drugs and the control rats.

Table 3.6 The Liver Cyclic AMP Levels in Rats 3.5 Hours After Been Given Isoprenaline or Salbutamol

Treatment	Liver cyclic AMP level (μg/mg. wet weight)
Control	0.74 ± 0.05
Isoprenaline (8 mg. kg^{-1})	0.63 ± 0.06
Salbutamol (15 mg. kg^{-1})	0.59 ± 0.06

Each result represents the mean of 5 determinations ± SEM.

3.14 The Liver Putrescine Levels of Rats Treated with Isoprenaline or Salbutamol

The result is shown in Table 3.7. There was an increase in the liver putrescine levels of rats 3.5 hours after been given isoprenaline or salbutamol.

Table 3.7 The Liver Putrescine Levels in Rats 3.5 Hours After Been Given Isoprenaline or Salbutamol

Treatment	Liver putrescine level (μg/mg. dry weight)
Control	3.9 ± 0.15
Isoprenaline (8 mg. kg^{-1})	14.9 ± 0.5
Salbutamol (15 mg. kg^{-1})	19.2 ± 0.7

Each result represents the mean of 5 determinations ± SEM.

3.15 The Effect of Putrescine on the Cotton Wool Pellet Granuloma Test

Daily subcutaneous injections of 1.0 cm^3 of putrescine at a dose of 50 g. kg^{-1} and 5000 g kg^{-1} had no effect on the formation of granulation tissue around cotton pellets as can be seen in Table 3.8

Table 3.8 The Effect of Putrescine on the Cotton Wool Pellet Granuloma Test

Treatment	Mean increase in pellet weight ± SEM (mg)
Control	10.19 ± 0.5
Putrescine (50 g. kg^{-1})	10.33 ± 0.6
Putrescine (5000 g. kg^{-1})	10.26 ± 0.4

Each result represents the mean of 4 determinations ± SEM.

3.16 The Effect of Putrescine on the Superoxide Anions Generated from the Stimulation of Guinea-pig Polymorphonuclear Leucocytes by FMLP in the Presence of Cytochalasin B

The result is shown in Table 3.9. Putrescine at concentrations of 10 mM to 40 mM showed very little scavenging action on the superoxide anions. At 100 mM, the percentage inhibition of superoxide anions was about 50%. The scavenging action of putrescine on the superoxide anions did not seem to be dose-dependent.

Table 3.9 The Effect of Putrescine on the Superoxide Anions Production from Guinea-pig Polymorphonuclear Leucocytes

Putrescine concentration (mM)	% inhibition of superoxide anions
10	12 ± 3.4
20	18 ± 3.6
40	22 ± 4.1
60	37 ± 7.6
80	44 ± 6.2
100	48 ± 6.9

Each value represents the mean of 4 determinations ± SEM.

4. DISCUSSION

It was found that the drugs that raised cyclic AMP and putrescine levels *in vivo* were anti-inflammatory against the carrageenan oedema rat model for acute inflammation. Isoprenaline and salbutamol which are in clinical use for the treatment of asthma were particularly effective. Salbutamol and theophylline were anti-inflammatory against adjuvant-induced arthritis in the rat which is a model for chronic inflammation. Bird et al (1983) showed that theophylline was anti-inflammatory against carrageenan-induced inflammation in the rat. All the drugs raise cyclic AMP either by blocking phosphodiesterase activity which breaks down cyclic AMP or by activating adenylate cyclase which synthesizes cyclic AMP. Since cyclic AMP stimulates ornithine decarboxylase activity, it follows that the increases in putrescine levels *in vivo* following administration of either isoprenaline or salbutamol were due to the stimulating action of these drugs on cyclic AMP levels. Therefore the anti-inflammatory action of the drugs was due to the *in vivo* production of cyclic AMP and putrescine, both endogenous substances with known anti-inflammatory activity. Recently, Haigh et al (personal communication) has shown that plasma cyclic AMP levels are depressed in patients with rheumatoid arthritis and osteoarthritis when compared with non-rheumatoid controls. The results showed that although isoprenaline was very effective in inhibiting the carrageenan oedema in the rats, it was very short acting. Isoprenaline failed to inhibit the oedema at 5 hours after the injection of carrageenan unless another dose was given about 3 hours after the first dose. This is not surprising since it is known that isoprenaline is rapidly broken down by enzyme catechol-O-methyltransferase. Isoprenaline inhibited the oedema in a dose dependent manner. The inhibition of

oedema at 3 hours after the carrageenan injection, by isoprenaline at a dose of 8 mg/kg was one and a half times of that produced by isoprenaline at a dose of 4mg/kg. Salbutamol was a long acting drug and inhibited the carrageenan oedema at both 3 and 5 hours after the carrageenan injection. Salbutamol was also effective in inhibiting the oedema when it was given orally indicating that it was well absorbed from the gastro-intestinal tract. Salbutamol had been shown to be anti-inflammatory when applied topically on croton-oil induced rat ear oedema (Seely and Glen, 1968).

An increase in the plasma level of cyclic AMP but not in the liver extracts was detected at 3½ hours after isoprenaline or salbutamol was given to the rats which corresponds to the anti-inflammatory action of the drugs in the carrageenan oedema. Marked increases in plasma cyclic AMP observed after β-adrenergic stimulation were not always associated with changes in their tissue contents in rats (Saito et al, 1976; Kunitada et al, 1978). Plasma cyclic AMP orginates from the cells of various tissues and plasma cyclic AMP concentrations are better index than the tissue concentrations for the response to β-adrenergic stimulation.

The inability of isoprenaline to inhibit the carrageenan oedema at 5 hours after the carrageenan injection coincided with the return of the rat plasma cyclic AMP level to normal at this time, whereas the plasma cyclic AMP concentration of salbutamol treated rats was still high at this time. Thus it seems that the anti-inflammatory action of isoprenaline and salbutamol is mediated through the increase

in cyclic AMP level.

The anti-inflammatory effect of isoprenaline and salbutamol was blocked by the use of the β-adrenergic antagonist, propranolol. This result further suggested that the anti-inflammatory effect of isoprenaline and salbutamol was due to the increase in cyclic AMP level.

The intracellular level of cyclic AMP can also be increased by inhibiting phosphodiesterase. Phosphodiesterase can be inhibited by the methyl xanthines (theophylline, aminophylline and 3-isobutyl-1-methyl xanthine). Theophylline (Bird, 1979) and aminophylline (Mckinney and Lish, 1964) had been shown to be anti-inflammatory in the carrageenan-induced oedema. The result showed that 3-isobutyl-1-methyl xanthine was also anti-inflammatory in the carrageenan oedema and on a weight to weight basis was the most effective in inhibiting the oedema among the methyl xanthines, which corresponds to it being the most effective in inhibiting phosphodiesterase.

Sodium phenobasbitone which increases cyclic AMP by activating adenylate cyclase directly was the least effective of the drugs used in inhibiting the carrageenan oedema. Thus the β-agonists seem to have greater anti-inflammatory activity in the carrageenan oedema.

On the chronic model of inflammation, salbutamol (15 mg/kg) and theophylline (30 mg/kg) both inhibited the development of adjuvant arthritis in rats. Other agents that can increase cyclic AMP level such as prostaglandin E, or a combination of prostaglandin E and

theophylline have been shown to potently suppress the development of adjuvant-induced arthritis in rats (Zurier and Quaglita, 1971; Parnham and Van Vliet, 1978). Seo and Saeki (1980) failed to inhibit adjuvant arthritis in rats using salbutamol alone at a dose of 3 mg/kg given subcutaneously daily but were able to inhibit the adjuvant arthritis using a combination of salbutamol and the phosphodiesterase inhibitor, aminophylline. They showed that significant increase in cyclic AMP content occured in the inflamed tissues in the adjuvant-injected paw, the spleen and the thymus.

Numerous studies suggest that β-adrenergic receptors are linked to adenylate cyclase and that the increase of intracellular cyclic AMP content is one of the early steps of the sequence generated by β-adrenergic stimulus (Robinson, Butcher and Sutherland, 1971).

β-adrenergic receptors are classified into two broad subclasses of β_1 and β_2 accordingly to pharmacological data obtained with intact tissues (Lands et al, 1967). Receptors found in the heart and adipose tissues are among β_1-type whereas those in the skeletal muscle, liver and trachea are among β_2-type receptors (Lands et al, 1967; Arnold et al, 1968).

Salbutamol is a specific β_2-adrenergic agonist and it increases the level of cyclic AMP through β_2-adrenergic agonist receptors (Brittain, Jack and Ritchie, 1970). Isoprenaline stimulates mostly β_1-adrenergic receptors.

Several properties of cyclic AMP are relevant to the anti-inflammatory action of this compound. In vitro studies have shown that the antigen-induced proliferation of lymphocytes (Bourne et al, 1974; Rocklin, 1976), migration inhibitory factor (MIF) secretion from lymphocytes (Rocklin, 1976), MIF activity on macrophages (Koopman et al, 1973), the release of histamine (Lichtenstein and Gillespie, 1973), lysosomal enzyme release (Zurier et al, 1973; Weissman, Zurier and Hoffstein, 1972) and superoxide anion production (Weissman, Zurier and Hoffstein, 1972; Tsan et al, 1976; Lehmeryer and Johnston, 1978) from leucocytes were all inhibited by an increase in cyclic AMP level. Such phenomena in the inflamed tissues as a whole would result in the inhibition of the inflammatory response. The inhibition of antigen-induced proliferation of lymphocytes by cyclic AMP may be important in preventing the development of delayed hypersensitivity reaction in adjuvant-induced arthritis.

In conclusion, cyclic AMP may play an important regulatory role in vivo in the inflammatory process.

Most drugs that increase cyclic AMP level also increase putrescine level through the induction of ornithine decarboxylase, the enzyme that synthesizes putrescine (Russell, 1980). Isoprenaline and salbutamol which increase cyclic AMP levels also increase putrescine levels. The rat liver putrescine level, when measured at 3½ hours after being dosed with isoprenaline or salbutamol, was more than twice the normal level. Catecholamines have been shown to cause the induction of ornithine decarboxylase in mouse parotid glands (Inoue et al, 1974). Even though the putrescine levels in livers of rats

treated with 3-isobutyl-1-methyl xanthine and sodium phenobarbitone were not determined, 3-isobutyl-1-methyl xanthine and sodium phenobarbitone have been demonstrated to induce ornithine decarboxylase and increase the putrescine level in rats within 4 hours after their administration (Russell, 1971; Levine, Leaming and Raskin, 1978).

Theophylline has also been shown to increase liver ornithine decarboxylase and putrescine levels (Byus and Russell, 1974; Beck, Bellantone and Cannellakis, 1972). Analysis of the livers of theophylline treated adjuvant arthritic rats showed that the putrescine level was twice that of the controls.

Putrescine and other polyamines have been found to be anti-inflammatory in animal models of inflammation (Bird et al, 1983; Oyanagui, 1948). In this investigation, the anti-inflammatory action of putrescine on carrageenan-induced oedema was confirmed. The sub-plantar injection of carrageenan mixed together with putrescine (85.5 μg/paw) was found to significantly decrease the carrageenan-induced oedema at 3 hours after the carrageenan injection. Putrescine when given to the rats has been shown to be almost completely metabolized within 4 hours of its administration (Janne, 1967). This rapid metabolism of putrescine might explain the failure of putrescine to inhibit the carrageenan oedema at 5 hours after the carrageenan injection. Due to its rapid metabolism, putrescine might not reach an effective concentration to exert an anti-inflammatory effect at this time Bird et al (1983) however found that putrescine was anti-inflammatory in the carrageenan model after 5 hours. Putrescine was also shown to inhibit serotonin-induced oedema,

suggesting that putrescine has some effect on the vasodilatation produced by serotonin. The length of the carbon chain separating the two amines may have some influence on the anti-inflammatory activity of polyamines because the putrescine analogue, diamino-propane which has 3 carbon chain separating the two amines was found to have some anti-inflammatory effect on the carrageenan oedema. Diaminoethane which has 2 carbon chain separating the two amines was not anti-inflammatory.

The anti-inflammatory activity of an agent that can increase the level of cyclic AMP can be due either to cyclic AMP itself or putrescine or both can be synergistic in their anti-inflammatory activity. In the investigation, the use of DL- α-difluoromethyl-ornithine, an inhibitor of ornithise decarboxylase, together with isoprenaline in the carrageenan oedema significantly blocked the anti-inflammatory action of isoprenaline by 11%. Therefore, it seems that some of the anti-inflammatory action of isoprenaline is due to putrescine.

In vitro studies have shown that putrescine stabilizes guinea-pig monocytes (Bird, 1979) and protects lysosomes from the lytic action of superoxide anions (Kafy and Lewis, 1984). The synthetic tripeptide F-formyl-methionyl-leucyl-phenylalanine (FMLP), which is a potent chemotactic factor for neutrophils (Schiffmann et al, 1975) has been shown to generate superoxide anions in the presence of cytochalasin B, in human neutrophils (Simchowitz et al, 1979). The result showed that FMLP in the presence of cytochalasin B also stimulated guinea-pig polymorphonuclear leucocytes to generate superoxide anion and putrescine had a scavenging effect on the superoxide anion. Other evidence in support of a scavenging action is that putrescine decreases superoxide

anion level in the xanthine oxidase catalysed conversion of xanthine to uric acid which is a reaction independent of neutrophils (Kafy and Lewis, 1984). Superoxide anion has also been shown to react directly with polyamines as demonstrated by electron spin resonance (Vanella et al, 1980a).

Although putrescine has a scavenging effect on superoxide, the concentrations of putrescine needed to obtain this effect were at non-phyciological concentrations. Similarly non-physiological levels of putrescine were required to stabilizeguinea-pig monocytes. Since extracellular concentrations of putrescine are very small (eg. 10 pmoles/ml in human plasma) and millimolar concentrations were needed to obtain scavenging effects on superoxide in the present work, it is unlikely that extracellular putrescine has a significant scavenging effect on superoxide. It is possible that putrescine may have some intracellular scavenging action since putrescine is normally an intracellular constituent and is present in cells at much higher concentrations (Tabor and Tabor, 1976).

Superoxide is produced by the membrane located nicotinamide adenine nucleotide oxidase system (Patriarca et al, 1971). Since the membrane is involved in forming phagosomes in phagocytosis, superoxide may accumulate in these vesicles. This is likely since the production of superoxide and other oxygen radicals is involved in the killing of micro-organisms ingested by phagocytes. The formation and accumulation of putrescine in these vesicles would effectively inhibit the action of superoxide. The removal of superoxide by putrescine inside these vesicles is important since during frustrated phagocytosis it is the expulsion of phagocytic contents that lead to tissue

damage in arthritis (Weissmann, Zurier and Hoffstein, 1972). The removal of superoxide in phagosomes will inhibit the build up of superoxide in the extracellular compartment due to stimulated phagocytic cells expelling less superoxide anion during phagocytosis. Rat polymorphonuclear leucocytes have the ability to convert ^{14}C ornithine to ^{14}C putrescine and therefore probably possess some ornithine decarboxylase activity (Haigh, Kafy and Lewis, 1984). Other cells, particularly macrophages have high levels of ornithine decarboxylase activity and these levels rise rapidly on stimulation of the cell.

Recent work by Kafy and Lewis (personal communication) showed that guinea-pig neutrophils convert putrescine rapidly to three product; pyrolline, γ-aminobutyric acid and a product which gave a strong Schiffs reaction. This compound is probably the unstable intermediate γ-aminobutyraldehyde. When putrescine was incubated separately with diamine oxidase or xanthine oxidase in the presence of xanthine, similar products were found. Aldehyde derivatives of oligoamines have powerful properties in inhibiting cell division. This maybe of interest as putrescine in vivo is anti-inflammatory against chronis inflammation which is associated with both macrophage and lymphocytic proliferation. Although putrescine is taken up by rat polymorphonuclear leucocytes, the more important source of intracellular amine is probably that produced in situ by ornithine decarboxylase and that the activity of this enzyme is controlled by cyclic AMP levels.

Current evidence suggests that superoxide anion may be involved in the pathogenesis of tissue injury in several rheumatic diseases.

Several agents known to activate polymorphs to generate superoxide anion appear relevant to the inflammatory process. These agents include the chemotactic factor C5a derived from activation of the complement system (Goldstein et al, 1975), aggregated lgG which reproduces several of the effects of immune complexes (Goldstein et al, 1975; Johnston and Lehmeryer, 1976) and phagocytosis of crystals such as sodium urate monohydrate or calcium pyrophosphate dihydrate (Simchowitz et al, 1979).

Superoxide dismutase which destroys superoxide, has been shown to have an ameliorating effect on several types of inflammation in vivo. These include such laboratory models as carrageenan-induced oedema (Oyanagui, 1976) and the reversed passive Arthus phenomenon (McCord and Fridovich, 1978). In early clinical trials, superoxide dismutase has been shown to exert beneficial roles in adult rheumatoid arthritis and osteoarthritis when injected parenterally (Menander-Huber and Juber, 1977). Therefore, removal of superoxide is anti-inflammatory.

Putrescine at concentrations of 50μg/kg and 5mg/kg did not inhibit granulation tissue formation in the cotton pellet in rats. Probably the doses were too small to show any effect. Putrescine at a concentration as high as 80 mg/kg was not found to be an irritant and inhibited carrageenan oedema in rats (Oyanagui, 1984).

The exact mechanism of the anti-inflammatory action of the polyamines is unknown. 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 the glucocorticoid, dexamethasone (Bartholeyns, Fozard and Prakash, 1981). This anti-inflammatory protein is suggested to inhibit the enzyme phospholipase A_2 which releases arachidonic acid, the precursor of prostaglandin biosynthesis, from membrane phospholipids (Flower and Blackwell, 1979).

Oyanagui (1984) confirmed that putrescine inhibits serotonin-induced oedema. It was found that the anti-inflammatory effect of dexamethasone on bradykinin-induced oedema was shown to be due to the synthesis of a new protein (Tsurufuji et al, 1980). Paw swelling induced by serotonin or bradykinin is principally dependent on an increase of vascular permeability caused by the opening of intercellular junctions of the vascular endothelium (Majno et al, 1969) and the prostaglandin is excluded (lack of inhibitory effect on the oedema by indomethacin). Oyanagui (1984) suggests that polyamines may act to reduce the vascular permeability through the induction of the synthesis of a certain protein in vivo which is given the name 'vascular permeability inhibiting protein' or 'vasoregulin'. Since polyamines inhibit the prostaglandin phase of carrageenan oedema, anti-inflammatory proteins may also affect prostaglandin generation or synthesis.

Another process by which the polyamines may exert their anti-inflammatory effect is through their interaction with calcium regulating processes.

Almost all secretory stystems studied has been shown to operate through a common calcium-dependent mechanism (Rubin, 1974; Douglas, 1974; Normann, 1976; Brownstein et al, 1980; Davis and Tai, 1980). Phagocytosis (Stossel, 1973) and lysosomal enzyme release (Goldstein, Hoffstein and Weissman, 1975) are stimulated by the presence of calcium in the medium. The histamine release from mast cells in anaphylactic reaction also depends on calcium-dependent process (Kazimierczak and Diarmant, 1978; Gompert, 1976) and the induction of superoxide anion production in polymorphonuclear leucocytes by the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP) has also been shown to depend on an influx of calcium ions into the leucocytes. In the presence of cytochalasin B, the chemotactic peptide induces a much larger influx of the calcium ions (Naccache et al, 1977) which causes an enhancement of superoxide anion production.

Polyamines have been shown to inhibit the calcium-dependent histamine secretion from sensitized mast cells (Theoharides, 1980). It has also been reported that mast cells from a mammary tumour which has a high concentration of polyamines fail to respond to compound 48/80 which is a classic histamine releasing agent of mast cells (Andersson et al, 1976).

Polyamines have also been shown to inhibit platelet aggregation in vitro (Rennert et al, 1976) and lymphocyte transformation (Allen et al, 1977; Byrd et al, 1977; Murray et al, 1977; Gaugas and Curzen, 1978). Lymphocyte activation is an indispensible part of cellular immunity ensuring that enough lymphocytes will be produced to combat any invading pathogen. Cytosolic calcium has been suspected to be involved in the antigenic stimulation and proliferation of lymphocytes

(Luckasen et al, 1974; Smith et al, 1975; Dianmantstein and Ulmer, 1975).

The polyamines may inhibit calcium-dependent processes through interference with calcium fluxes by either repelling calcium ions because both are organic cations, inhibiting protein phosphorylation or other enzyme mechanisms which affect calcium fluxes or by intercalating with membrane components thus altering membrane fluidity and hindering or procluding the critical association of calcium ions with appropriate elements of the membrane (Theoharides, 1980).

Another possible regulatory site for the polyamines could involve other steps such as the one recently shown in the mast cells where secretion was accompanied by calcium-dependent phosphorylation of specific proteins (Siegart et al, 1978; Theoharides et al, 1980) and polyamines have been reported to inhibit various protein kinases (Murray et al, 1976; Bachrach et al, 1978; Hochman et al, 1978).

In conclusion, cyclic AMP and putrescine are well established to be anti-inflammatory and drugs which can raise both cyclic AMP and putrescine levels may be useful in the treatment of inflammatory disease.

However, the exact mechanism by which putrescine exerts its anti-inflammatory effect is still unclear. Further work is needed to see whether putrescine has a direct inhibitory effect or an indirect effect via the synthesis of an anti-inflammatory protein, on the enzymes participating in prostaglandin synthesis.

Since polyamines may interfere with calcium regulating processes, future work is desirable to see if there is any direct involvement of polyamines with calcium fluxes in calcium-dependent processes in leucocytes and other inflammatory cells, for example, in lysosomal enzymes release and in superoxide anion production.

Prostaglandins have a dual role; they are pro-inflammatory (causing vasodilatation, oedema, intensifying the effect of other inflammatory mediators, etc) in the acute stage of inflammation but pharmacologic doses of prostaglandin E_1 and E_2 are anti-inflammatory during the chronic stage (Bonta and Parnham, 1978). Prostaglandin E has been shown to inhibit adjuvant arthritis in rats (Bonta, Parnham and Van Vliet, 1978), lymphocyte activation in vitro (Pelus and Strausser, 1977; Pastan et al, 1975), inhibit lymphokines release from activated lymphocytes (Stastny et al, 1973; Stastny et al, 1975) and inhibit growth of fibroblasts in vitro (Johnson and Pastan, 1971; Pastan et al, 1975). Therefore, prostaglandin E accumulating at the site of inflammation would exhibit a negative feedback mechanism.

The anti-inflammatory effect of prostaglandin E has been attributed to the increased level of cyclic AMP induced by prostaglandin E. Cyclic AMP has been shown to cause the induction of ornithine decarboxylase and the induction of ornithine decarboxylase by prostaglandin E has been reported in hamster kidney cells (Hogan et al, 1974), neuroblastoma cells (Bachrach, 1975), mouse mammary epithelium (Oka and Perry, 1976) and in rat ovary (Lampretcht et al, 1973). It is not known whether some of the anti-inflammatory effects of prostaglandin E

are due to an increase in polyamine level and an investigation into the relationship of the anti-inflammatory effect of prostaglandin to polyamine level would be of interest.

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