AN INVESTIGATION INTO POSSIBLE ANTI-INFLAMMATORY PLASMA PROTEINS IN ARTHRITIS.

A thesis submitted by

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ABSTRACT

1. It was confirmed that there are considerable overall changes in the levels of serum proteins in rats with adjuvant-induced arthritis. The concentrations of alpha-globulins and beta-globulins were found to increase whilst the concentrations of albumin decreased. These changes paralleled the severity of the inflammation. Cortisol administration reduced both the inflammation and appeared to normalise the levels of serum proteins.

2. Protease levels were elevated in the serum of adjuvant-induced arthritic rats and closely corresponded with increased lysosomal acid phosphatase levels.

3. The levels of the antiproteases, alpha-1-macroglobulin and alpha-2-acute phase globulin were increased during adjuvant-induced arthritis in the rat; but the trypsin inhibitory capacity (TIC) of the sera was decreased. Cortisol elevated the TIC levels of both normal and adjuvant arthritic rats and this may reflect an anti-inflammatory action of this drug.

4. A human fraction enriched with alpha-1-antitrypsin was found not to have anti-inflammatory activity in the model of carrageenan-induced oedema in the rat.

5. The transcortin levels were found to fall in both male and female rats with adjuvant-induced arthritis during the onset of chronic inflammation. The onset and severity of the disease was similar for both male and female rats despite the increased transcortin levels found in female rats.

6. It was confirmed that dimethylnitrosamine reduces the severity of adjuvant-induced arthritis and carrageenan-induced oedema in the rat. Saline extracts of livers from dimethylnitrosamine treated rats were found to have anti-inflammatory activity in carrageenan-induced oedema in the rat. TIC levels were found to be increased in both saline liver extracts from dimethylnitrosamine treated rats and the serum of dimethylnitrosamine treated rats.

7. A number of non-steroidal anti-inflammatory drugs were tested, at therapeutic concentrations, and found not to influence the stability of transcortin-bound 1,2-^{3}H-cortisol in human plasma. Therefore it appears that non-steroidal anti-inflammatory drugs do not act by causing the release of cortisol from its binding sites on transcortin.

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CONTENTS

1. INTRODUCTION.

1.1. The Aetiology of Rheumatoid Arthritis.

The cause of rheumatoid arthritis is unknown. Dressner (1955) has reviewed many of the early theories such as infection, metabolic disorders, endocrine malfunction, poor nutrition, allergy to certain foods, psychological disorders and damp or cold climates. He concluded that many of these theories had been largely disproved and were virtually untenable. At the present time it is widely believed that rheumatoid arthritis may well be initiated by a trigger, such as an infection or physical trauma, which may disappear leaving the disease in a self-perpetuating state (Hollander, McCarty, Astorga and Castro-Murillo, 1965; Zvaifler, 1965).

1.1.1. Bacterial Infections.

The search for infectious organisms in rheumatoid patients has continued for a number of years and is still in progress. Cecil, Nichols and Stainsby (1929) attracted attention to the possible involvement of micro-organisms by reporting that they had isolated streptococci from the blood of rheumatoid patients. However, their samples were probably contaminated since these findings were not confirmed (Dawson and Boots, 1933).

More recently claims have been made for the isolation of diptheroid type organisms from some rheumatoid samples (Duthie, Stewart, Stewart, Alexander and Dayhoff, 1967; Hill, McCormick, Greenbury, Morris and Kenningdale, 1967). Clasener and Biersteker (1969), however, demonstrated the possibility of tissue and fluid samples becoming contaminated by diptheroids, although it is probably

significant that they have been isolated more often from rheumatoid than non-rheumatoid patients (Bartholomew and Nelson, 1972). L-phase bacteria, which are organisms with little or no cell wall, have also been reported in rheumatoid tissue and fluids (Benedek, 1955). Roberts (1964) isolated the L-form of Bacillus lichenformis from fowls that had acute synovitis and Pease (1974) claimed finding the same organism in human rheumatoid samples.

Mycoplasmas, which are organisms only slightly larger than viruses, have been isolated from a number of animal species with synovitis (Sharp and Riggs, 1966). Pig mycoplasmas have also been used experimentally to induce arthritis in swine (Barden and Decker, 1971). There have been a number of attempts to isolate mycoplasmas from human rheumatoid material with little success (Person, Sharp and Rawls, 1973; Stewart, Duthie, Mackay, Marmion and Alexander, 1974). Only Williams (1972) and Jansson, Makisara, Vaninio K, Vaninio U, Snellman and Tuuri (1971) claim to have succeeded, but again there was the possibility of contamination of cultures

There has also been some interest shown in bedsonia organisms, since Schacter (1967) reported isolating them from rheumatoid patients.

Pearson and Wood (1959) induced arthritic lesions in some strains of rats by injection of mycobacterial adjuvant and Paronetto (1972) produced similar effects by injections of purified cell walls of Corynebacterium rubrum. These results therefore support the possibility of bacterial cell components causing an immune response, that might develop into rheumatoid disease in perhaps genetically sensitive individuals. However, until it can be shown that certain

bacterial organisms are consistently present at the onset of rheumatoid disease their possible role in the aetiology of the human disease will remain in doubt.

1.1.2. Viral Infections.

A viral infection could initiate rheumatoid disease by at least two mechanisms; either by altering the function of specific cells, such as synovial or plasma cells, or by producing an immune response against its antigenic components.

Castor (1971(a)) demonstrated the persistence of the altered metabolism of rheumatoid synovial cells in culture, including the increased rate of synthesis of hyaluronic acid. In some cell lines this type of persistent alteration in metabolism during tissue culture has been attributed to viruses (Hamerman, Todaro and Green, 1965; Ishimoto, Tenuri and Strominger, 1966).

Smith and Hamerman (1968) claimed that rheumatoid synovial cells in culture were resistant to infection by Newcastle virus disease and Grayzel and Beck (1970) claimed that they were resistant to Rubella virus. This resistance could indicate that the cells had been pre-infected by the same or similar virus. These results, however, were not confirmed in other laboratories since both viruses were shown to infect rheumatoid and non-rheumatoid synovial tissue cultures (Ford, 1972; Spruance, Smith, Krall and Ward, 1972). Clarris, Fraser and Rodda (1974) also found no significant difference between rheumatoid and non-rheumatoid cells to infection, but they discovered that the increased amounts of hyaluronic acid in rheumatoid cells interfered with the adsorption of viruses. However, Smith, Hamerman, Janis and Haberman (1974) claimed that the difference in results between the laboratories could be due to using different

techniques and/or different strains of viruses. They also reported transferring "viral resistance" from human rheumatoid synovial cells to rabbit synovial cells, which could indicate the transfer of a virus type particle.

Rubella virus has been shown to produce mild arthritis in humans (Weibel, Stokes, Buynak and Hilleman, 1969) and chronic synovitis in rabbits (Grayzel, Janis and Haberman, 1971). However, as yet no virus type particles have been isolated from rheumatoid samples (Smith and Hamerman, 1969; Wilkes, Simsarian, Hopps, Roth, Decker, Aptekar and Meyer, 1973). Shumacher (1975) however, has observed by electron-microscopy virus-like particles associated with the synovial vessel walls from rheumatoid patients, but further investigation is required.

1.2. The Pathogenesis of Rheumatoid Arthritis.

1.2.1. The Normal Joint.

Normal articular cartilage consists of collagen fibres that give the cartilage its strength and proteoglycans that give the cartilage its elasticity. The proteoglycans act as a "ground substance" or "cement" that helps to bind the collagen fibres and hold them in place. Collagen normally accounts for 50% of the dry weight of cartilage, although it is not uniformly distributed (Anderson, Ludoweig, Harper and Engelman, 1964). Proteoglycans account for 5-20% of the dry weight, but the actual amount varies roughly inversely with the local concentration of collagen (Muir, Bullough and Maroudas, 1970).

The structure of collagen has been reviewed by Kühn (1969). Its primary structure is quite unusual and consists of a polypeptide chain composed of approximately 33% glycine residues, 25% proline and

hydroxyproline residues and the remainder of other amino acids including hydroxylysine. There are both polar and apolar regions in the polypeptide, the polar regions have many repeating sequences of glycine-proline-hydroxyproline. Three polypeptide chains can form a tropocollagen molecule, the chains are held together by hydrogen bonds in the form of a triple helix. The tropocollagen molecules have a molecular weight of 300,000 daltons and are 2800 Å in length. In vivo these tropocollagen molecules associate linearly to form protofibrils which associate laterally forming collagen fibrils. There is a periodicity of 640 Å to 700 Å along the collagen fibrils due to a 25% displacement of the neighbouring protofibrils. Crosslinks are formed between the tropocollagen molecules producing an insoluble collagen fibril.

Proteoglycans are composed of a long protein core to which large chains of glycosaminoglycans are attached. The glycosaminoglycans have been reported to have molecular weights ranging from 2.3×10^5 daltons (Muir and Jacobs, 1967) to 3.2×10^6 daltons (Luscomb and Phelps 1967). In cartilage glycosaminoglycans are either chondroitin sulphate or keratin sulphate, although chondroitin sulphate has been found to be the major one (Tsiganos and Muir, 1967; Hoffman, Meyer, Mashburn and Bray, 1967). Chondroitin is composed of disaccharide repeating units of D-glucuronic acid and N-acetyl-D-galactosamine in an alpha (1-3) linkage, each disaccharide unit is attached to the next by alpha (1-4) linkages. Chondroitin sulphate has a sulphate ester group attached to either the 4 or 6 carbon position of N-acetyl-D-galactosamine. In keratin sulphate the D-glucuronic acid of chondroitin is replaced by D-glucose and N-acetyl-D-galactosamine by N-acetyl-D-glucosamine with a sulphate ester group attached to the carbon 6 position.

Many of the serine residues of the protein core have chondroitin sulphate chains attached (Muir, 1958). Keratin sulphate appears to be mainly attached to threonine residues (Tsiganos and Muir, 1967).

Joints are encapsulated by synovial tissue which is normally a thin and tough "membrane-like" material. King (1935) proposed that the cells lining the synovial membrane were responsible for synthesising and secreting synovial fluid, which is found in the joint space. However, normal synovial fluid is now considered as a dialysate of plasma to which hyaluronic acid and hyaluronate protein have been added (Sandson and Hamerman, 1962). In the normal joint the synovial membrane controls the influx of plasma proteins; the synthesis of hyaluronic acid and hyaluronate protein takes place in the synovial lining cells (Sandson and Hamerman, 1962). Barland, Novikoff and Hamerman (1962) demonstrated that human synovial tissue lining cells are of two types. Type A are phagocytic and type B are involved in the synthesis of hyaluronic acid and hyaluronate protein. Hyaluronic acid is similar in structure to chondroitin except that N-acetyl-D-galactosamine is replaced by N-acetyl-D-glucosamine. Hyaluronic acid is an important constituent of synovial fluid since it renders the fluid extremely viscous, making it an ideal lubricating agent for the relatively rough cartilage surfaces (Walker, Unsworth, Dowson, Sikorski and Wright, 1970).

1.2.2. The Rheumatoid Joint.

The affected joints of patients with rheumatoid arthritis are generally swollen and inflamed. This is mainly due to the proliferation of synovial tissue, the build up of oedema fluid and

the influx of inflammatory plasma cells. Proliferation of synovial tissue is a result of hyperplasia and hypertrophy of the synovial lining cells (Hamerman, Barland and Janis, 1969). This rheumatoid synovial tissue has increased metabolic activity and an increased rate of glycolysis, which leads to a build up of lactate and a fall in the pH of local tissues (Falchuck, Goetzl and Kulka, 1970). The synthesis of hyaluronic acid is also increased, but it is underpolymerised and has a lower molecular weight (Castor, Prince and Hazelton, 1966). This causes a fall in the viscosity of the synovial fluid and can lead to increased stress and friction on the cartilage in the joints (Walker, Unsworth, Dowson, Sikorski and Wright, 1970).

Synovial tissue can sometimes transform into a vascular granulation tissue, called pannus, this extends from the inflamed synovium between the joint space over the cartilage. Histologically it consists of proliferating fibroblasts, numerous small blood vessels, inflammatory plasma cells and the occasional collagen fibre (Hamerman, Barland and Janis, 1969). It has been suggested that the pannus is the cause of cartilage erosion in the rheumatoid joint (Parker and Keeper, 1935, Barland, Novikoff and Hamerman, 1964). However, it is worth noting that pathological changes in cartilage have also been found well away from the pannus (Hamerman, Janis and Smith, 1967).

There are several possible mechanisms whereby cartilage destruction can take place in the rheumatoid joint. Cartilage relies upon synovial fluid to supply the necessary nutrition so that it can maintain its state of metabolism. However, rheumatoid

synovial fluid is deficient in oxygen (Falchuk, Groetzl and Kulka, 1970), and glucose (Ropes and Bauer, 1953) and this may lead to changes in cartilage metabolism and turnover. It is also possible that pannus tissue overlapping onto cartilage competes for the available substrates thus causing cartilage destruction (Dettmer, 1971). The most likely cause of cartilage erosion, however, is its degradation by lysosomal enzymes, evidence for this has arisen from work by Fell and Mellanbye (1952). They discovered that an excess of vitamin A severely affected cartilage in chick embryos grown in tissue culture. Thomas, McClusky, Potter and Weissmann (1960) produced similar results in rabbits in vivo by administering them with large amounts of vitamin A. Dingle, Lucy and Fell (1961) confirmed the results of Fell and Mellanbye (1952) and further work indicated that the cartilage, after treatment with large quantities of vitamin A, contained a proteolytic enzyme (Lucy, Dingle and Fell, 1961). During the same era De Duvie, Pressman, Gianetto, Wattiaux and Applemans (1955) had discovered a new subcellular particle, that contained a series of hydrolytic enzymes. These particles were different from mitochondria and microsomes and were called lysosomes (De Duvie, Wattiaux and Wibo, 1962). It was finally proposed by Fell and Dingle (1963) that vitamin A caused an alteration of lysosomal membranes resulting in the release of lysosomal acid hydrolases, such as cathepsins, which could degrade and erode the cartilage matrix.

Hamerman, Janis and Smith (1967) were later able to demonstrate that the protein-polysaccharide portion of cartilage could be disrupted through cleavage of the polysaccharide chain by enzymes such as hyaluronidase and by proteolytic enzymes that attack the protein core.

Holt (1971) has suggested that primary lesions of cartilage take place in the "ground substance" with secondary involvement of collagen fibrils. Brandt and Muir (1971) have demonstrated that in vitro proteoglycans protect collagen from attack by collagenolytic enzymes. Evidence demonstrating that lysosomal enzymes are probably important mediators of both tissue destruction and inflammation was obtained by Weissmann, Spilberg and Krakauer (1969). They induced chronic inflammation and cartilage degradation by injecting lysates of purified lysosomes into the joints of experimental animals.

Cytochemical techniques have demonstrated that rheumatoid synovial lining cells and pannus have greatly increased numbers of lysosomes (Hamerman, Stephens and Barland, 1961). Other sources of lysosomes in the rheumatoid joint are polymorphonuclear leucocytes, macrophages and cartilage cells (Weissmann, 1966). However, polymorphonuclear leucocytes are probably a major source of lysosomes since it has been estimated that 10^9 leucocytes per day can infiltrate into the synovial fluid of a rheumatoid joint (Zvaifler, 1971). Barland, Novikoff and Hamerman (1964), using electron microscopy, discovered that rheumatoid synovial tissue had numerous altered lysosomes that contained "inclusions" or "granules". They suggested that these "inclusions" were partly phagocytosed material, probably particles from the erosion of cartilage matrix. Hollander, McCarty, Astorga and Castro-Murillo (1965) also found that polymorphonuclear leucocytes isolated from rheumatoid synovial fluid contained "inclusions" or "granules" within many of their lysosomes. They were able to demonstrate that in this case many of the phagocytosed particles were complexes of immunoglobulins.

Rheumatoid synovial fluid compared to normal synovial fluid has large quantities of lysosomal enzymes present (Luscombe, 1963). It has been shown that these enzymes are selectively released when polymorphonuclear leucocytes or macrophages take up or phagocytose particles such as immune complexes, micro-organisms or zymosan fragments (Weissmann, Zurier, Spieler and Goldstein, 1971; Wright and Malawista, 1973; Ringrose, Parr and McLaren, 1974).

Several mechanisms have been proposed to explain the selective release of lysosomal enzymes during phagocytosis. When particles are phagocytosed they are initially enclosed by a cellular membrane and can be identified as vacuoles, that are called phagosomes. These phagosomes merge with lysosomes to form secondary lysosomes in which the lysosomal enzymes normally digest the foreign particle (Cohn and Fedorka, 1969). It has been suggested that the selective release of lysosomal enzymes takes place if the lysosomes prematurely merge with phagosomes, while the phagosomal membranes are still incomplete and open at the cell surface. This mechanism has been described as "regurgitation during feeding" (Weissmann, Zurier and Hoffstein, 1972), electron microscope images consistent with such a mechanism have been produced by Zucker-Franklin and Hirsch (1964). Another proposed mechanism considers that during the process of digestion the membranes of the secondary lysosomes merge with the outer cell membranes again leading to the release of the lysosomal constituents. This has been referred to as "reversed endocytosis" or "frustrated phagocytosis" (Henson, 1971; Weissmann, Zurier and Hoffstein, 1972).

Although lysosomes contain a wide range of degradative enzymes, such as cathepsin D, it appeared at first that they all had

pH optima in the range pH 2.5 - 6.5 (Tappel, 1969). It seemed unlikely therefore that lysosomal enzymes would play a very active role when released into joint fluids that had pH values approaching neutrality. This problem was resolved, however, after recent discoveries of lysosomal enzymes that degrade tissue and have pH optima around neutrality. Evanson, Jeffrey and Krane (1968) described the release of collagenases in cultures of rheumatoid synovial tissue, capable of degrading undernatured collagen at neutral pH. Other studies have further characterised these collagenases from rheumatoid synovial tissue as two different enzymes that are distinct from collagenases of other sources (Harris, DiBona and Krane, 1969; Woolley, Glanville, Crossley and Evanson, 1972).

A variety of proteases capable of breaking down cartilage at neutral pH have been isolated from human polymorphonuclear leucocytes. They include a cartilage mucopolysaccharide degrading protease (Oronsky, Ignarro and Perper, 1973), a collagenase (Ohlsson, K and Ohlsson, I., 1974(a)), an elastase (Janoff, 1973; Ohlsson, K. and Ohlsson, I., 1974(b)) and chymotrypsin-like cationic proteins (Rindler-Ludwig and Braunsteiner, 1975). Lysosomes of human polymorphonuclear leucocytes have also been found to contain a cationic protein that disrupts most cells (Janoff and Zweifach, 1964), a protease which induces capillary permeability (Movat, Uriuhara, MacMorine and Burke, 1964) and a kinin generating enzyme (Movat, Steinburg, Habal and Ranadive, 1973).

Stimulated mouse macrophages have been shown to secrete collagenase (Werb and Gordon 1975(a)) and elastase (Werb and Gordon, 1975(b)), these are both active at neutral pH. Stimulated mouse

macrophages also contain a plasminogen activator (Unkeless, Gordon and Reich, 1974).

Other mediators of inflammation, beside lysosomal enzymes, have been found in rheumatoid synovial fluid. Kinins, which are small peptides, can produce vasodilation capillary permeability and pain (Armstrong, Jepson and Keele, 1957; Melmon, Webster, Goldfinger and Seegmiller, 1967). Kinins are generated from kininogen precursors, such as plasma alpha-2-globulin by kallikrein (a proteolytic lysosomal enzyme). However, no overall correlation has been found between the amount of kinin in synovial fluid and the clinical assessment of inflammation (Melmon, Webster, Goldfinger and Seegmiller, 1967).

Prostaglandins are other inflammatory mediators found in rheumatoid synovial fluid. They can produce vasodilation, oedema and pain, and may potentiate the effects of other mediators such as kinins (Ferreira and Vane, 1974; Robinson and Levine, 1974). Prostaglandin E_2 has been shown to be synthesised by rheumatoid synovial tissue in culture (Robinson, McGuire, and Levine, 1975). Both prostaglandin E_2 and prostaglandin E_1 are suspected of being involved in the processes of bone reabsorption (Robinson, McGuire and Levine, 1975).

1.2.3. The Role of Rheumatoid Factors.

Nichols and Stainsby (1931) discovered that certain strains of bacteria could be agglutinated by sera from patients with rheumatoid arthritis. Waaler (1940) later demonstrated that rheumatoid sera could also agglutinate sheep erythrocytes that had been previously sensitised with rabbit antisera. It is now known that the substances in rheumatoid sera responsible for the agglutination reactions are

immunoglobulins against native IgG. These are of the IgM, IgG or IgA type and are called rheumatoid factors. Both IgM and IgG rheumatoid factors have been shown to form soluble complexes with native IgG in sera and synovial fluid of rheumatoid patients (Kunkel, Muller-Eberhard, Fudenberg and Tomasi, 1961; Bland and Clark, 1963). The concentrations of these complexes are greater in the synovial fluids than the sera of rheumatoid patients and it has been found that the production of rheumatoid factors can take place in the rheumatoid synovial membrane of the joint (Smiley, Sacks and Ziff, 1968).

Since the discovery of rheumatoid factors there has been much discussion and conflicting evidence as to their role, if any, in the pathogenesis of rheumatoid disease. Christian (1961) considered that rheumatoid factors were probably byproducts of the disease as is the case for the Wassermann antibody found in syphilis. Some evidence has been found tending to support this view, for instance the transfusion of rheumatoid factor into healthy volunteers produced no ill effects (Harris and Vaughan, 1961); also rheumatoid factors have been found in the sera of some non-arthritic people (Wolfe 1968).

However, it has also been postulated that rheumatoid factors might play an important role in the pathogenesis of rheumatoid disease. Hollander, McCarty, Astorga and Castro-Murillo, (1965) proposed a cycle of events which they suggest is initiated by native IgG becoming denatured due to some unknown influence such as antigens or heat. Antibodies such as IgM would be formed against the denatured IgG leading to antigen-antibody complexes of IgG-IgM. These complexes would be phagocytosed by leucocytes causing a consequential loss of some lysosomal enzymes from the leucocytes. These lysosomal

enzymes would then give rise to inflammation and tissue destruction as well as denaturing more IgG and hence completing the cycle.

A similar hypothesis was proposed by Zvaifler (1965). He predicted that some unknown event initiates inflammation causing the disruption of lysosomes and leakage of lysosomal enzymes. These enzymes could alter leucocyte nucleoprotein and antibodies would be raised against the nucleoprotein. Antigen-antibody complexes would again be formed and in the presence of rheumatoid factor inclusion bodies could develop. These would be engulfed by leucocytes, leading to a further loss of lysosomal enzymes which would cause inflammation and tissue destruction and complete the cycle by altering more nuclear material.

There is a good deal of evidence to support the two latter theories especially with regard to the role of rheumatoid factors. Levels of complement in rheumatoid synovial fluid are depressed, although the levels are normal in rheumatoid sera (Hedburg, 1964). In synovial fluid the complement appears to be fixed by the IgG-rheumatoid factor complexes and there is a direct relationship between the amount of complexes and the depression of complement (Winchester, Agnello and Kunkel, 1970). The fixation of complement leads to the activation of the complement sequence and the release of a number of inflammatory mediators such as chemotactic substances that attract polymorphonuclear leucocytes (Ward and Zvaifler, 1971). Rawson, Abelson and Hollander (1965) discovered that leucocytes isolated from rheumatoid synovial fluid contained cytoplasmic inclusions of IgM rheumatoid factor complexed with IgG. It has also been demonstrated that polymorphonuclear leucocytes that are incubated with preformed complexes containing IgG and rheumatoid

factors will ingest the complexes and selectively release lysosomal enzymes (Astorga and Bollet, 1965; Weissmann, Zurier, Spieler and Goldstein, 1971). There is evidence that complexes containing lgG rheumatoid factor can be engulfed by synovial fluid leucocytes and synovial membrane macrophages whether or not they contain lgM rheumatoid factor. This could be the reason why rheumatoid patients with no serum lgM rheumatoid factor (seronegative) suffer from the disease, since they still have lgG rheumatoid factor in their serum (Mellbye and Natvig, 1971). Restifo, Lussier, Rawson, Rockey and Hollander (1965) produced inflammatory reactions in the previously unaffected knees of rheumatoid patients by injection of purified extracts of their own lgG. The leucocytes isolated from these joints had lgG and lgM complexes in their cytoplasm. Osteoarthritic patients were given the same treatment, but in this case no inflammation was produced neither was there any inflammation after the injection of lgG from healthy people into the unaffected knees of rheumatoid patients. These results tend to suggest that both rheumatoid factors and lgG from rheumatoid patients (probably denatured) are necessary for these inflammatory reactions. Rawson, Quismorio and Abelson (1969) were able to produce severe arthritis in rabbits by repeated injections of lgG fragments into the knee. This suggests that "rheumatoid factors" were raised against the initial injections, hence creating the right conditions for arthritis to develop upon subsequent ones. Further support for Zvaifler's hypothesis came from the discovery of antinuclear antibodies in the sera of rheumatoid patients (Barnett, Bienstock and Bloch, 1966) and the later finding of granulocyte specific antibodies in greatest concentration in rheumatoid synovial fluids (Elling, Graudal and Faber, 1968).

Extranuclear deposits of nucleoprotein have been identified in leucocytes taken from rheumatoid synovial fluids (Brandt, Cathcart and Cohen, 1968).

Pope, Teller and Mannik (1974) proposed that lgG complexes of intermediate size are formed as a result of the self-association of lgG rheumatoid factor molecules, that is each molecule acting as both an antigen and antibody and combining with other lgG molecules to form complexes. Natvig and Munthe (1975) have evidence that these lgG rheumatoid factor molecules may associate intracellularly at their site of synthesis in the plasma cells. They also suggest that the intracellular formation of these complexes may effect the control mechanisms for lgG synthesis by blocking any feedback mechanism. This would allow the lgG rheumatoid factor complexes to be continuously produced and they could then play a major part in the self-perpetuation of rheumatoid disease.

1.3. Animal Models for the Study of Rheumatoid Arthritis.

Animal models can be useful for studying the aetiology and pathogenesis of certain diseases, and for testing possible new drugs. Rheumatoid arthritis, however, appears to be a disease that occurs naturally only in humans. For this reason many attempts have been made to artificially induce rheumatoid disease in laboratory animals. Gardner (1960) reviewed the methods that had been tried and divided them into five main groups, infective, immunological, chemical, endocrinological and physical. He concluded, rather pessimistically, that not one form of experimental arthritis bore even a resemblance to human rheumatoid arthritis and suggested that further investigations should be directed on human tissues.

During the last decade the situation has changed. Micro-organisms, such as mycoplasmas, have been isolated from animals with joint disease (Sharp and Riggs, 1966). Infections of Erysipelothrix insidiosa have been shown to cause an erosive polyarthritis in swine and dogs. The diseased joints are histo-pathologically similar to those in human rheumatoid arthritis and high titres of rheumatoid factors are found (Sikes, Crimmins and Fletcher, 1969; Sikes, Causey, Jones, Fletcher and Hayes, 1971). Intravenous injections of Erysipelothrix insidiosa into rabbits produced a persistant polyarthritis again similar to the human disease. Rheumatoid factors were found in the serum and in leucocyte inclusions, there was also a reduction in the synovial fluid complement levels (Astorga, 1969). Synovitis has since been produced in rabbits by repeated intravenous injections of sterile cell-free extracts of Erysipelothrix insidiosa (White, Puls and Mirikitani, 1971).

Intraperitoneal injections of aqueous suspensions of Group A streptococcal cell wall fragments can cause peripheral arthritis in rats (Cromartie, Craddock and Schwab, 1968) and articular injections in rabbits lead to monoarticular arthritis (Schwab, Cromartie, Ohanian and Craddock, 1967).

Immunological methods for inducing arthritis in animals have been highly developed. Originally Stoerk, Bielinski and Budzilovich (1954) and later Pearson (1956) observed that intradermal injections of tissue homogenates in Freunds adjuvant (heat killed mycobacteria suspended in a water-in-oil emulsion) caused polyarthritis in the rat. Subsequently it was discovered that the presence of tissue was unnecessary and that a mixture of mycobacterial components in oil alone induced a reproduceable arthritis, especially if injected into

the foot pad (Pearson and Wood, 1959; Ward and Jones, 1962). Recently it has been shown that the severity of the arthritis depends upon the size of the mycobacterial components in the adjuvant (Liyanage, Currey and Vernon-Roberts, 1975). However, not all breeds of rat are equally susceptible; whereas the incidence of arthritis in Sprague-Dawley rats is almost 90%, Buffalo rats are resistant (Swingle, Jaques and Kvarn, 1969). The arthritis appears after a latent period of almost ten days from the injection of adjuvant. Articular lesions develop in the paws and tail, sometimes the spine may also be affected. The disease reaches a peak after about twenty-five days and may then regress in some animals, although in others it may become chronic (Pearson and Wood, 1959; Pearson and Wood, 1963). The synovial tissue of the affected joints proliferates and pannus tissue invades the joint space causing severe bone erosion. An accumulation of mononuclear cells in the synovium is also evident (Pearson and Wood, 1963; Pearson, 1963). Many of the early invading mononuclear cells appear to be macrophages derived from rapidly dividing bone-marrow cell precursors (Putnam, Lubraoff and Waksman, 1969). The histopathology of the diseased synovium viewed by light and electron microscopy has been described as indistinguishable from that in humans (Muirden and Peace, 1969). However, unlike the human disease there are few polymorphonuclear leucocytes present at the affected joints (Pearson, 1963) and there is no local synthesis of immunoglobulins by plasma cells (Jasin, Cooke, Hurd, Smiley and Ziff, 1973). There is much evidence that this disease is caused by a delayed hypersensitivity reaction to the disseminated antigenic constituents of the mycobacteria (Waksman, Pearson and Sharp, 1960).

This has been largely confirmed by the passive transfer of arthritis to healthy rats using lymphnode and spleen cells from arthritic rats (Pearson and Wood, 1964).

The active antigenic component of the mycobacterial adjuvant has been found to be due to a complex peptidoglycolipid called Wax D, that can be extracted from the bacterial cell wall (Wood, Pearson and Tanaka, 1969). Further work has shown that a certain peptidoglycan component of Wax D is probably responsible (Adam, Ciorbaru, Petit and Lederer, 1972). It is interesting that other organisms, such as Nocardia asteroids and Corynebacterium rubrum, have been found that can substitute for mycobacteria in the adjuvant (Flax and Waksman, 1963; Paronetto, 1970).

Dumonde and Glynn (1962) developed an excellent immunological model of a chronic antigen-induced arthritis in rabbits. The rabbits were initially immunised with fibrin in Freunds adjuvant, several weeks later fibrin was injected into the joints giving rise to chronic monoarticular arthritis. This type of arthritis has also been produced with soluble antigens such as ovalbumin (Consden, Doble, Glynn and Nind, 1971) and bovine serum albumin (Cooke and Jasin, 1972). The joint lesions closely resemble those of human rheumatoid arthritis with synovial hypertrophy and hyperplasia, pannus formation and erosion of articular cartilage and bone. There is also a considerable influx of polymorphonuclear leucocytes, lymphocytes and plasma cells into the affected joints (Dumonde and Glynn, 1962). A further resemblance to human rheumatoid arthritis is the prolonged local synthesis of immunoglobulins, which takes place in the inflamed synovium (Glynn, 1968; Glynn, 1969). Several mechanisms have been

proposed in attempts to explain the chronic nature of the disease. Dumonde and Glynn (1962) considered that the disease might be due to a delayed hypersensitivity reaction, since there is the need for the pre-immunisation of the rabbits. Phillips, Kaklamanis, and Glynn (1966) suggested that there might be an immune response against an autoantigen, such as a by-product of the inflammation. Another possibility is that the antigen persists in the joint for a considerable time, hence maintaining local antibody synthesis and inflammation (Glynn, 1968; Consden, Doble, Glynn and Nind, 1971). Recently it has been demonstrated that the antigen is selectively retained on the articular surfaces of the collagenous tissues of the joint (Jasin, Cooke, Hurd, Smiley and Ziff, 1973).

Winter, Risley and Nuss (1962) have used a preparation of carrageenan, which is a mucopolysaccharide derived from Irish sea moss, to produce oedema upon injection into the hind footpad of the rat. Carrageenan is not known to be antigenic and therefore the inflammatory response is entirely due to the local stimulus and is highly reproduceable (Gardner, 1960). The major use of this method appears to be for screening anti-inflammatory compounds, because the inflammation it induces responds well to therapeutic concentrations of widely used anti-inflammatory drugs; such as aspirin, phenylbutazone and hydrocortisone (Winter, Risley and Nuss, 1962).

In summary it would appear that although there is not yet an animal model of rheumatoid arthritis that is identical with the human disease, many aspects of the human disease can indeed be reproduced in animals. It is possible therefore that if the various animal models could be superimposed upon each other the product would be a disease profile almost identical to that of human rheumatoid arthritis.

1.4. Drugs Used in the Treatment of Rheumatoid Disease.

1.4.1. Steroidal Anti-Inflammatory Drugs.

Glucocorticoids can be very effective in reducing inflammation and tissue destruction in patients with rheumatoid arthritis, but because of possible dangerous side effects they are usually only prescribed for short intervals (Cope, 1972). Cortisol is the most active anti-inflammatory glucocorticoid that occurs naturally, although the synthetic glucocorticoids, prednisolone and dexamethasone have greater activity (Cope, 1972). The anti-inflammatory actions of glucocorticoids are polyvalent and do not yet appear to be fully understood.

Glucocorticoids have been found to inhibit capillary dilation, reduce the increased permeability of blood vessels and reduce the extravasation of fluid during inflammation (Ashton and Cook, 1952; Sutton, Feldmann and Maibach, 1971). These effects are often mediated by kinins (Kellermeyer and Graham, 1968) and it has been demonstrated that therapeutic levels of cortisol in plasma can prevent the formation of kinins, by both leucocytes and kallikrein in a cell-free system (Cline and Melmon, 1966).

Lewis and Piper (1975) have also recently reported that cortisol and prednisolone can inhibit prostaglandin-mediated vasodilation accompanying lipolysis in subcutaneous fat, by inhibition of prostaglandin release from the tissue.

Local administration of cortisone can depress the numbers of lymphocytes, macrophages and polymorphs that participate in inflammatory reactions (Rebuck and Mellinger, 1953; Allison, Smith and Wood, 1955). Makman, Dvorkin and White (1968) found that high concentrations of

of glucocorticoids at 10^{-3}M could suppress DNA, RNA and protein synthesis in rat and rabbit lymphoid cell cultures. Therapeutic concentrations of glucocorticoids have been shown to inhibit the antibody production of steroid sensitive animals such as rats and rabbits, however, there is no convincing evidence that this is the case for humans (Claman, 1972). Balow and Rosenthal (1973) were able to demonstrate that pharmacological concentrations of cortisol and dexamethasone significantly blocked the macrophage migration inhibitory factor activity of the guinea-pig. Weston, Claman and Krueger (1973) found that cell-mediated immunity reactions in the guinea-pig between antigen-sensitive lymphocytes and monocyte macrophages were inhibited, at therapeutic concentrations of cortisol, by preventing the non-immune macrophages from responding to macrophage aggregation factor.

There has been considerable interest generated in the possibility that glucocorticoids might inhibit the release of lysosomal enzymes by direct stabilization of lysosomal membranes. Fell and Dingle (1963) established that excess vitamin A could cause the release of hydrolytic enzymes from lysosomes. Weissmann and Thomas (1963) discovered that the release of hydrolases from rabbit liver lysosomes due to excess vitamin A could be retarded or antagonised by cortisol. Symons, Lewis and Ancill (1969) demonstrated that a wide range of glucocorticoids at concentrations 10^{-4}M could inhibit the release of lysosomal enzymes from isolated rabbit liver lysosomes during incubation at 37°. Ignarro (1972) reported that liver lysosomes could be stabilized <u>in vivo</u>, he found that lysosomes isolated from rats predosed with cortisol were more stable, during incubation in hypotonic solution, than lysosomes from untreated animals.

Ignarro (1971(a)) also found that rabbit polymorphonuclear leucocyte lysosomes were stabilized *in vitro* by cortisol at concentrations of 10^{-3}M, although rat polymorphonuclear lysosomes were not. Lewis and Day (1972) demonstrated that therapeutic concentrations of glucocorticoids (10^{-6}M) inhibited the release of lysosomal enzymes from rheumatoid synovial fluid cells and rheumatoid synovial membrane. They also reported that these cells and tissues concentrated the steroids; this may be the reason why high concentrations of glucocorticoids are required to stabilize isolated lysosomes *in vitro*. Persellin and Ku (1974) recently claimed that glucocorticoids did not stabilize lysosomes isolated from human polymorphonuclear leucocytes against lysis by heat or detergent, either *in vitro* or *in vivo*. Ignarro (1972), however, found that some experimental procedures for assessing lysosomal membrane stabilization, such as thermal lysis, gave conflicting results when compared to hypotonic lysis. This led to his suggestion that methods based on thermal lysis were probably invalid, but this requires further investigation. It is possible that the stabilization of lysosomal membranes is an artefact and this reflects the danger of equating *in vitro* results to the *in vivo* situation. This may explain why cortisone, which has little anti-inflammatory activity *in vivo*, stabilizers lysosomes *in vitro* (Symons, Lewis and Ancill, 1969; Ignarro (1971(a)).

Weissmann (1971) proposed that since excess vitamin A appears to induce lysosomal membranes to merge more easily, cortisol may have the opposite effect preventing the merger of lysosomes and hence the release of lysosomal enzymes. Wright and Malawista (1973) found that cortisol inhibited the normal mobilisation and release of granular enzymes from phagocytosing human polymorphonuclear leucocytes.

Perper and Oronsky (1974) demonstrated that lysosomal enzyme release from human leucocytes in contact with opsonised IgG attached to cartilage discs was suppressed by cortisol at 10^{-4}M. The release of lysosomal enzymes from cultured mouse peritoneal macrophages has also been found to be inhibited by therapeutic concentrations of prednisolone and dexamethasone, during zymosan induced phagocytosis (Ringrose, Parr and McClaren, 1975).

Cooper, DeChatelet and McCall (1972) reported that glucocorticoids inhibited oxygen uptake and affected the cellular metabolism of human polymorphonuclear leucocytes by inhibition of the hexose monophosphate shunt pathway. This may lead to a fall in the production of lysosomal enzymes and a decrease in the rate of their release, since both activities require cellular energy.

Castor (1971(b)) found that rheumatoid synovial tissue contains a peptide that can induce hypermetabolic activity in cultured synovial cells. Castor (1972) has shown that pharmacological concentrations of cortisol can inhibit this "connective tissue activation". Apparently cortisol does not inhibit the activating peptide directly, but probably acts at a later stage by affecting transcription or translation of messenger RNA.

1.4.2. Non-Steroidal Anti-Inflammatory Drugs.

Some of the common non-steroidal anti-inflammatory drugs used in the treatment of rheumatoid arthritis are aspirin, salicylic acid, indomethacin, phenylbutazone, oxyphenbutazone, ibuprofen, flufenamic acid, mefenamic acid, chloroquine, penicillamine and gold salts. There are numerous reports in the literature on possible biochemical mode of actions of these compounds.

Whitehouse and Haslem (1962) reported that some non-steroidal anti-inflammatory drugs could uncouple oxidative phosphorylation. However, 2,4-dinitrophenol is a more potent uncoupler of oxidative phosphorylation and this has no anti-inflammatory activity in the rat (Goldstein, DeMeo and Shemano, 1968). Non-steroidal anti-inflammatory drugs have been found to inhibit a number of enzyme systems such as dehydrogenases, aminotransferases, decarboxylases, aminoacyl-t RNA synthetases, proteases and ribonucleic acid polymerases; these functions have been reviewed by Smith and Dawkins (1971). These authors point out that the concentrations of drug required for enzyme inhibition are either well above the toxic dose or approach the toxic dose, which is also the case for the uncoupling effect of drugs on oxidative phosphorylation. Therefore it is highly unlikely that any of these mechanisms are important from an anti-inflammatory point of view.

Pielero and Colombo (1967) demonstrated that many non-steroidal anti-inflammatory drugs bound and partially inhibited the denaturation of plasma proteins by heat. They suggested that this might be a useful screening procedure to test new anti-inflammatory compounds. It has also been suggested that these compounds might compete with anti-inflammatory steroids, such as cortisol, for binding sites on plasma proteins, thus creating an increase in the "free" levels of cortisol (Brodie, 1965). However, Stenlake, Williams, Davison and Downie (1971) reported that there was no significant increase in the "free" levels of cortisol in plasma during incubation with non-steroidal anti-inflammatory drugs. MacArthur, Dawkins and Smith (1971), however, found that L-tryptophan was displaced from the binding sites of plasma proteins by non-steroidal drugs and they proposed that such drugs

might also displace certain peptides that could be anti-inflammatory in nature.

Therapeutic concentrations of non-steroidal anti-inflammatory drugs have been shown to inhibit the aggregation of human platelets (Stuart, 1970; Zucker and Peterson, 1970). Castor (1972) also found that some non-steroidal anti-inflammatory drugs at therapeutic concentrations were potent inhibitors of connective tissue activation during tissue culture. The active drugs did not appear to alter the activity of the activating peptide molecule, but interfered at a later stage.

Conflicting results have been obtained for the effects of non-steroidal anti-inflammatory drugs on the stability of lysosomal membranes. Brown and Schwartz (1969) reported increased labilization of rat liver lysosomes during incubation at 45° with some non-steroidal anti-inflammatory drugs in a cell-free system. Ignarro (1971(b)) found that the same drugs stabilized rat liver lysosomes against hypotonic solutions when incubated at 37°. Guinea-pig polymorphonuclear leucocyte lysosomes were also stabilized by some non-steroidal anti-inflammatory drugs against hypotonic lysis (Ignarro and Colombo, 1972), although rat and rabbit polymorphonuclear leucocyte lysosomes were labilized (Ignarro, 1971(a)). Lewis, Capstick and Ancill (1971) have shown that rat liver lysosomes are stabilised by phenylbutazone, oxyphenbutazone and azapropazone over a wide concentration range, but are labilized at high concentrations above 10^{-3}M. Pollock and Brown (1971) reported that liver lysosomes from rats predosed with phenylbutazone and indomethacin were more fragile than normal rat liver lysosomes when subjected to thermal labilization. Ignarro (1972) again demonstrated, however, that liver lysosomes from rats predosed

with some non-steroidal anti-inflammatory drugs and incubated in hypotonic solutions were more stable than normal rat liver lysosomes. Ignarro (1972) also suggested that the failure of non-steroidal drugs to inhibit lysosomes against thermal labilization probably indicates that it is not a valid method for assessing lysosomal membrane integrity. Unfortunately the mechanism of lysosome stabilization by anti-inflammatory drugs is unresolved and the anti-inflammatory affect this may have in vivo, if any, remains uncertain.

High levels of some non-steroidal anti-inflammatory drugs have been found to inhibit the release of lysosomal enzymes during phagocytosis of starch particles by rabbit polymorphonuclear leucocytes (Chang, 1972), and during phagocytosis of opsonised IgG on cartilage discs by human polymorphonuclear leucocytes (Perper and Oronsky, 1974). Gold thiomate appeared to inhibit neutral protease activity of human polymorphonuclear leucocyte lysosomes, but did not affect the release of lysosomal enzymes during phagocytosis (Perper and Oronsky, 1974). Phenylbutazone and flufenamic acid have also been found to inhibit the release of lysosomal enzymes from cultured mouse peritoneal-macrophages during phagocytosis of zymosan particles (Ringrose, Parr and McLaren, 1974).

Vane (1971) discovered that aspirin, salicylic acid and indomethacin could inhibit the synthesis of prostaglandin E_2 and prostaglandin $F_{2\,alpha}$ from arachidonic acid in cell-free homogenates of guinea-pig lung. Smith and Willis (1971) also found a similar inhibition of prostaglandin biosynthesis by human platelets during incubation with aspirin. Many of the non-steroidal anti-inflammatory drugs have now been demonstrated to inhibit prostaglandin synthesis, some in a wide variety of human and other animal tissues (Ferreira and Vane, 1974). The drugs are all active at low therapeutic

concentrations and their relative potency as inhibitors of prostaglandin synthetase largely correlates with their potency as inhibitors of carrageenan induced oedema (Paulus and Whitehouse, 1973). It is interesting that the administration of prostaglandins to humans can result in the production of erythema, pain and fever; since all these symptoms when they occur normally can be reduced by the administration of aspirin-like drugs (Ferreira and Vane, 1974).

1.5. Endogenous Anti-Inflammatory Proteins.

There are many reports in the literature on the existance of naturally occurring anti-inflammatory substances in inflammatory exudates of laboratory animals (Rindani, 1956) and in humans (Billingham, Robinson and Robson, 1969(a)). These substances have been found to inhibit granulation tissue deposition (Di Pasquale, Girerd, Beach and Steinetz, 1963), carrageenan-induced oedema (Billingham, Robinson and Robson, 1969(a); Billingham, Robinson and Robson 1969(b)) and delayed hypersensitivity reactions (Gaugas, Billingham and Rees, 1970).

Billingham, Gordon and Robinson (1971) demonstrated that rat anti-inflammatory exudates, from implanted sponges, contain an anti-inflammatory protein that like the majority of plasma proteins is synthesised in the liver. This protein was not found in the liver perfusates of injured rats until several days after sponge implantation and was not found in perfusates from normal animals. The synthesis of the protein can be blocked by actinomycin D, which inhibits the synthesis of mRNA. Billingham and Robinson (1972) have further purified the protein and have shown that it migrates electrophoretically between the alpha-glycoproteins and transferrin. It appears likely therefore that inflammation, due to either injury or disease, can

trigger a response in the liver causing the liver to synthesise anti-inflammatory proteins, which are then carried by the blood back to the inflammatory site.

The plasma protein patterns of rats with inflammation are altered, especially in chronic diseases such as adjuvant-induced arthritis (Lowe, 1964; Glenn, Bowman and Koslowske, 1968). Similar changes in plasma proteins have been reported in patients with rheumatoid arthritis, these changes include increases in the alpha-1-globulin and alpha-2-globulin fractions and decreases in albumin concentration (Ropes, Perlman, Kaufman and Bauer, 1954; Stidworthy, Payne, Shetlar, C. L. and Shetlar, M. R., 1957). The plasma proteins that increase in concentration during human rheumatoid arthritis and adjuvant arthritis in the rat have been termed acute phase reactants (APR); the levels of APR have been shown to correlate with the severity of the human disease (McConkey, Crockson, R. A. and Crockson, A. P., 1972). Other diverse inflammatory conditions such as surgical trauma (Neuhaus, Balegno and Chandler, 1961; Crockson, Payne, Ratcliffe and Soothill, 1966) and injection of turpentine into rats, can cause increases in APR (Maung, Baker and Murray; 1964). It is possible that some of these APR may be anti-inflammatory proteins. It is interesting that conditions as diverse as pregnancy and viral hepatitis, in which there are significant changes in the levels of certain plasma proteins, often result in the remission of rheumatoid arthritis (Hench, Kendall, Slocumb and Polley, 1949; Hench, 1933).

Pinals (1973) induced hepatic damage in rats with intra-peritoneal injections of dimethylnitrosamine. He found that rats injected with dimethylnitrosamine, four or five days after being given injections of mycobacterial adjuvant, developed a less severe form of arthritis. He also demonstrated that saline extracts of livers, taken

from rats injected two or three days previously with dimethylnitrosamine, could control adjuvant arthritis, whereas saline extracts from normal rat livers could not. The anti-inflammatory substance in the dimethylnitrosamine treated liver extracts was neither a corticosteroid nor dimethylnitrosamine, since it is rapidly metabolised, although it could be a peptide or protein.

Persellin (1972) reported the appearance of a protein in adjuvant arthritic rat plasma that migrated electrophoretically as an alpha-globulin and had an approximate molecule weight of 170,000 daltons. This protein was found to react with membranes of rat erythrocytes and rat liver granules and stabilize rat liver lysosomes in vitro against thermal lysis. Lowe and Turner (1973), however, found that plasma from normal rats could stabilize rabbit polymorphonuclear leucocyte lysosomes in vitro against lysis by Triton-X-100, and that during the development of adjuvant arthritis this ability decreased. Lowe and Turner (1973) have suggested that the fact that they used a different strain of rats to Persellin (1972) might explain the difference in their own and Persellin's results. It is also possible that the conflicting results are a consequence of using a different type of lysosomes and different methods of lysis.

Human sera has also been shown to inhibit the lysis of rabbit polymorphonuclear leucocytes by Triton-X-100; this ability increases during pregnancy and is not due to increased corticosteroid levels (Hempel, Fernandez and Persellin, 1970). Lewis, Capstick and Cosh, (1975) demonstrated that rat liver lysosomes were stabilized in vitro by human rheumatoid synovial fluid. This stabilizing activity was destroyed by pre-incubating the synovial fluid with trypsin and has been shown to be probably due to a high molecular weight plasma protein

that migrates electrophoretically with the alpha/beta globulins (Lewis, 1976). Lewis, Capstick and Best (1976) have isolated a high molecular weight protein fraction from the sera of normal Wistar rats that has anti-inflammatory activity in both carrageenan and arthritic rat models. However, further work is needed to identify the protein(s) responsible and discover their mode of action.

Ford-Hutchinson, Insley, Elliott, Sturgess and Smith (1973) discovered that normal human plasma contains a small molecular weight substance (below 1000 daltons) that has anti-inflammatory properties against carrageenan-induced oedema; it is resistant to acid and proteolytic digestion and can be extracted by solvents. Further work by Badcock, Ford-Hutchinson, Smith and Walker (1975) has shown that the anti-inflammatory substance has a molecular weight of below 500 daltons and that it can inhibit the release of chemotactic and anaphylactic factors from rat and guinea-pig serum.

1.5.1. Antiproteases.

Other potentially anti-inflammatory substances in plasma are antiproteases. These are proteins that bind and inhibit proteolytic enzymes (proteases), some of which are directly involved in inflammation and tissue destruction. The major antiprotease present in human plasma is alpha-1-antitrypsin; its concentration is in the region of 2g/L and it can be responsible for up to 90% of the trypsin inhibitory capacity of plasma (Ganrot, P.O., 1972).

Alpha-1-antitrypsin was first isolated by Schultz, Göllner, Heide, Schönenberger and Schwick (1955) who used organic solvents and low pH precipitation techniques. Schultz, Heide and Haupt (1962) later discovered its capacity for inhibiting trypsin and gave it the name

alpha-1-antitrypsin. However, the original methods used for its isolation led to a considerable loss in activity. Therefore more gentle isolation procedures such as polyacrylamide gel electrophoresis, ion-exchange chromatography and affinity chromatography have recently been adopted (Crawford, 1973; Musiani and Tomasi, 1976). Alpha-1-antitrypsin has been characterised as an alpha-1-glycoprotein composed of a single polypeptide chain that contains 12% carbohydrate and has a molecular weight in the region of 50,000 daltons (Crawford, 1973; Musiani and Tomasi, 1976). There are a number of genetic variants called Pitypes, these have been designated according to their mobility in electrophoresis, the most common phenotype is Pi MM although so far a total of twenty three Pi alleles have been identified (Fagerhol and Laurell, 1967).

As well as trypsin, alpha-1-antitrypsin has been found to inhibit a number of other serine proteases these include chymotrypsin, plasmin, thrombin (Rimon, Shamash and Shapiro, 1966), kallikrein (Haberman, 1968), elastase (Kaplan, Kuhn and Pierce, 1973), granulocytal elastase (Ohlsson, 1971) and granulocytal collagenase (Harris, DiBona and Krane, 1969). Alpha-1-antitrypsin appears to bind all proteases in a 1:1 molar ratio (Ohlsson, 1975) and although the protease-antiprotease complexes are tightly bound the type of inhibition is probably competitive (Cohen, 1975). Cohen (1973) proposed that alpha-1-antitrypsin has two overlapping active sites; one active site contains an arginyl residue that binds serine proteases that cleave peptides at arginyl residues, and the other site contains an aromatic amino acid or leucyl residue that binds serine proteases that cleave peptides at either of these types of residues.

The other major antiprotease found in human plasma is alpha-2-macroglobulin; its concentration can vary from 2-4g/L and it is responsible for nearly 10% of the trypsin inhibitory capacity of plasma (Ganrot, P. O. and Schersten, 1967). Alpha-2-macroglobulin was first isolated as a macromolecular alpha-2-glycoprotein by Brown, Baker, Peterkofsky and Kauffman (1954), they used ultracentrifugation and ammonium sulphate precipitation methods to extract it from Cohn Fraction III-O of human plasma. Purification techniques used in other laboratories have included "Rivanol" precipitation combined with DEAE-cellulose chromatography (Steinbuch, Quentin and Pejaudier, 1964). More recently gel filtration of serum in 6% agarose (Bio-Gel A-5m) columns, after the initial removal of low density lipoproteins and IgM from the serum by precipitation, have been adopted (Frenoy, Razafimahaleo and Bourrillon, 1972). The molecular weight of alpha-2-macroglobulin has been estimated in the range of 725,000-820,000 daltons (Schönenberger, Schmidtberger and Schultz, 1958; Roberts, Riesen and Hall, 1974). The quaternary structure is unknown, although electron-microscope studies present a picture of two beans facing each other (Bloth, Chesebro and Svehag, 1968). Limited thiol reduction leads to the formation of subunits of 196,000 daltons (Frenoy, Razafimahaleo and Bourrillon, 1972). Jones, Creeth and Kekwick (1972) suggest an eight chain subunit structure with dimers of these chains forming the 196,000 dalton subunits, four of these subunits may form an alpha-2-macroglobulin molecule.

Alpha-2-macroglobulin has a broad spectrum of activity and can inhibit proteases from all four groups of endopeptidases. These include trypsin, chymotrypsin, plasmin, thrombin, kallikrein, granulocytal elastase, granulocytal collagenase, cathepsin B_1, cathepsin D, thermolysin and chlostridiopeptidase A (Barrett and Starkey, 1974).

There is conflicting evidence with respect to the number of protease that are bound by each molecule of alpha-2-macroglobulin. Some researchers have reported finding two binding sites per molecule and others one (Barrett and Starkey, 1973; Ohlsson, 1975). Barrett and Starkey (1973) have proposed a model for the interaction of alpha-2-macroglobulin with proteases. They suggest that the protease attacks a peptide bond in the alpha-2-macroglobulin molecule resulting in a conformational change that traps the protease. The binding appears to be irreversible, although it has been reported, that during prolonged incubation, elastase can digest the alpha-2-macroglobulin from within and escape (Baumstark, 1970).

Woolley, Roberts and Evanson (1976) discovered another potentially important antiprotease in human plasma, which migrates electrophoretically as a beta-1-globulin and specifically inhibits neutral collagenases. This antiprotease has a molecular weight of approximately 40,000 daltons and has been called beta-1-anticollagenase. It is probably important since it can inhibit collagenases that are normally only inhibited by alpha-2-macroglobulin, while its lower molecular weight will enable it to reach inflammatory sites much more easily than alpha-2-macroglobulin.

There are other antiprotease in human plasma, such as inter-alpha-trypsin and alpha-1-antichymotrypsin; but these are quantitatively less important than alpha-1-antitrypsin and alpha-2-macroglobulin and do not as yet appear to have any unique functions. Inter-alpha-trypsin has been characterised by Heide, Heimburger and Haupt (1965) and has been found to inhibit trypsin and chymotrypsin. Alpha-1-antichymotrypsin can inhibit chymotrypsin, but not trypsin (Heimburger and Haupt, 1965).

Plasma antiproteases have been found in every animal species studied (Nakamura, Ogata and Suzuno, 1972). The plasma antiproteases

of the rat are similar to those found in humans. The major plasma antiprotease in the rat is again alpha-1-antitrypsin, its biochemical characteristics and properties are similar to human alpha-1-antitrypsin, although it is immunologically different (Huttunen and Korhonen, 1973; Blackwood, Moret, Keller, Fierer and Mandl, 1974). Rat alpha-1-antitrypsin can bind and inhibit both trypsin and chymotrypsin (Huttunen and Korhonen, 1973), and it is possible that it can inhibit a similar range of serine proteases to human alpha-1-antitrypsin. In normal rat plasma there is little or no alpha-2-macroglobulin present, but its role appears to be taken over by alpha-1-macroglobulin (Ganrot, K. 1973). In rats with inflammation, however, a high molecular weight alpha-2-acute phase globulin also appears in large concentrations and this is immunologically similar to human alpha-2-macroglobulin (Heim, 1968). Both alpha-1-macroglobulin and alpha-2-acute phase globulin have been found to bind and inhibit trypsin and chymotrypsin in a similar manner to human alpha-2-macroglobulin (Ganrot, K., 1973; Huttunen and Korhonen, 1973). A third antiprotease is present in normal rat plasma. This has an intermediate molecular weight between alpha-1-antitrypsin and alpha-1-macroglobulin, but its trypsin inhibitory capacity in plasma is less than that of alpha-1-antitrypsin and alpha-1-macroglobulin (Huttunen and Korhonen, 1973).

The importance of antiproteases in combating inflammatory diseases has been well established, for instance genetic deficiencies of alpha-1-antitrypsin are associated with pulmonary emphysema (Eriksson, 1965) and juvenile liver cirrhosis (Sharp, Bridges, Krivit and Freier, 1969). Cox and Huber, 1976, have reported that genetic deficiencies of alpha-1-antitrypsin can also contribute to the development of rheumatoid arthritis and tissue destruction.

The levels of alpha-1-antitrypsin, like other acute phase proteins, have been found to rise during acute inflammation (Werner and Odenthal, 1967). Increased levels of alpha-1-antitrypsin in plasma and synovial fluid are also found in patients with rheumatoid disease (Swedlund, Hunder and Gleich, 1974; Brackertz, Hagmann and Kueppers, 1975). Lewis and Capstick (1976) have reported that the concentration of alpha-1-antitrypsin in synovial fluid varies inversely with the protease activity. Therefore even though the increased levels of alpha-1-antitrypsin are often unable to stop the progress of the disease they may reduce its severity. Plasma alpha-1-antitrypsin and alpha-2-macroglobulin levels have also been shown to rise rather dramatically during pregnancy (Fagerhol and Laurell, 1970; Schumacher and Schlumberger, 1963) and alpha-1-antitrypsin increases during hepatitis (Kindmark and Laurell, 1972); both these conditions can lead to remissions in rheumatic disease.

Ohlsson (1975) used ^{125}I labelled elastase to demonstrate the removal of protease-antiprotease complexes from the human circulation. The alpha-2-macroglobulin-protease complexes were rapidly removed with a half life of twelve minutes and broken down by the kuppfer cells. Elimination of the alpha-1-antitrypsin-protease complexes was less rapid; approximately 50% were removed within one hour. At least some of the labelled protease appeared to be transferred from alpha-1-antitrypsin to alpha-2-macroglobulin, whereupon it was rapidly removed again by the kuppfer cells. These results suggest that alpha-2-macroglobulin may have an important function in the removal of proteases from the circulation.

1.5.2. Transcortin.

In normal human plasma 75% cortisol is bound by a specific alpha-1-glycoprotein, that is referred to either as transcortin or corticosteroid binding globulin; 15% of the remaining cortisol is bound to albumin and 8% is unbound or free (Rosenthal, Slaunwhite and Sandberg, 1969). Transcortin has a high affinity for cortisol with an association constant of $10^{7}M^{-1}$ - $10^{8}M^{-1}$, there is one binding site per molecule. The binding of cortisol by albumin is fairly weak, association constant $10^{3}M^{-1}$ - $10^{4}M^{-1}$, and non-specific with a large number of binding sites per molecule (Westphal, 1970).

Transcortin has been purified from human plasma in several laboratories and its molecular weight is reported as low as 51,700 daltons and as high as 58,500 daltons (Seal and Doe, 1962; Muldoon and Westphal, 1967; Rosenthal, Slaunwhite and Sandberg, 1969). The concentration of transcortin in normal human plasma is in the region of 0.7 μM (36 μg/ml) and is higher than the cortisol concentration (Rosenthal, Slaunwhite and Sandberg, 1969). Transcortin has a half life of four or five days and there is little or no daily variation in plasma levels (Sandberg, Woodruff, Rosenthal, Nienhouse and Slaunwhite, 1964). In humans three or four fold increases in the levels of transcortin take place during oestragen administration and two or three fold increases take place during the latter stages of pregnancy (Sandberg, Slaunwhite and Carter, 1969).

Specific corticosteroid binding globulins have been found in the plasma of every vertibrate animal species studied (Seal and Doe, 1963). Transcortin from rat plasma has been purified and characterised by Rosner and Hochberg, (1972). Its association constant for cortisol is reported as $3 \times 10^{6}M^{-1}$ and one binding site per molecule has been

established, the concentration of transcortin in pooled male rat plasma was approximately 100 µg/ml (Rosner and Hochberg, 1972). Gala and Westphal (1965) demonstrated that the transcortin concentration in female rats was twice that of male rats, although the transcortin levels can be increased 50-70% by oestragen administration. The transcortin levels in female rats are unaffected by oestragen administration or during pregnancy, but can be depressed by testosterone.

Rosenthal, Slaunwhite and Sandberg (1969) have shown that the increased transcortin levels of humans during pregnancy and oestrogen administration lead to two or three fold increases in the total cortisol levels and also in the unbound cortisol levels. However, the percentages of bound and unbound cortisol remain the same. The increase in total cortisol concentrations takes place because transcortin-bound cortisol is not metabolised by the liver, nor can it act as a feed back mechanism on the pituitary to inhibit adrenocorticotrophin release (Sandberg and Slaunwhite, 1971). Any increase in total cortisol levels during pregnancy or oestrogen administration, does not result in the effects of hyperadrenocorticism such as Cushings syndrome, because the bound cortisol appears to be inactive. Milder affects such as impaired glucose metabolism or suppression of the pituitary can sometimes occur, however, due to the increased levels of unbound cortisol (Sandberg and Slaunwhite, 1971).

Transcortin-bound cortisol has been found to be inactive in other situations. Slaunwhite, Lockie Back and Sandberg (1962) discovered that mice failed to respond to a dose of transcortin-bound cortisol in a standard glycogen maintainence assay, but responded to an equivalent dose of unbound cortisol. Blecher (1966) measured the inhibition by gluco-corticords of ^{14}C-glucose metabolism to carbon dioxide and showed that

transcortin could block the effects of 5×10^{7}M cortisol on isolated rat adipose tissue. Doe, Lohrenz and Seal (1965) were able to find several families with genetic deficiencies of transcortin, these all had low plasma cortisol levels but did not show any evidence of glucocorticoid insufficiency.

However, transcortin does appear to have some functions. Keller, Richardson and Yates (1969) suggested that the protein binding of cortisol may alter some of its biological functions. They found that cortisol administration to male rats induced alanine transaminase in the liver and pancreas, but in female rats and male rats treated with oestragens alanine transaminase was induced only in the liver. Dexamethasone which is a synthetic glucocorticord not bound by transcortin, induced alanine transaminase in the liver and pancreas of all the animals. Since female rats and male rats treated with oestragen have higher levels of transcortin, Keller and his associates proposed that the differences in transcortin levels were probably responsible for the selective action of cortisol on female rats and male rats treated with oestragens.

Recently it has been discovered that the binding association constants of some glucocorticoid receptors are similar to those of transcortin (Baxter and Tomkins, 1971). Therefore it is possible that transcortin-bound cortisol could be selectively transferred to high affinity receptors in certain target tissues. Reif-Lehrer and Chader (1969) have demonstrated that chick embryo retina in tissue culture could take up transcortin-bound cortisol and leave free transcortin behind in the medium.

In conclusion, total cortisol levels are influenced by transcortin levels. A large increase in the transcortin concentration will only result in a relatively small two or three fold increase in the free

cortisol concentration, although this may have an anti-inflammatory effect. Any increase in the transcortin concentration will result in an increase in transcortin-bound cortisol, however, and this may result in a greater uptake of cortisol by some specific tissues.

1.6. Aims of Research.

It is not normally possible to study the complex biochemical changes that take place in humans during the onset of rheumatoid arthritis, simply because the disease is difficult to diagnose in its early stages. It is also difficult to study the natural development of rheumatoid disease in humans without the added complications of the effects of drugs, since it is unethical to stop drug treatment for more than short periods of time. Therefore it was proposed to study some of the biochemical changes that take place in the blood of rats during the onset and development of adjuvant-induced arthritis.

It is known that during inflammatory states in humans and in rats there are increases in the levels of some blood proteins, called acute phase proteins, and decreases in the levels of others (Crockson, Payne, Ratcliff and Soothill, 1966; Glenn, Bowman and Koslowske, 1968). It was proposed to study the changes in serum protein patterns of the adjuvant arthritic rat and to investigate the levels of inflammatory serum proteins, such as neutral proteases, and possible anti-inflammatory proteins, such as antiproteases. It was also planned to study the effects of cortisol administration on the serum protein patterns and on the levels of antiproteases during adjuvant-induced arthritis. Total cortisol levels are controlled by transcortin levels and since cortisol is a naturally occurring and potent anti-inflammatory substance it was also proposed to investigate the levels of transcortin in adjuvant arthritic rat serum.

It has been reported that certain compounds such as dimethylnitrosamine that cause liver damage can induce the synthesis of anti-inflammatory substances in the liver (Pinals, 1973). Experiments were designed to confirm these findings and to study the serum and liver of rats treated with dimethylnitrosamine, to try and establish if the anti-inflammatory activity can be correlated with increased antiprotease levels.

It was also proposed to separate from plasma a fraction enriched with alpha-1-antitrypsin in order to try to demonstrate whether or not this antiprotease can directly inhibit inflammation in the rat.

Finally, it was planned to investigate the suggestion that non-steroidal, anti-inflammatory drugs may act by promoting the release of cortisol from its binding sites on albumin and transcortin in human plasma (Brodie, 1965).

2. MATERIALS AND METHODS.

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

Adjuvant arthritis was induced in both male and female 150-200g Wistar rats (Bantin and Kingman, Hull). The procedure followed was similar to that adopted by Newbold (1963). The adjuvant consisted of human strains of heat-killed C, DT and N tubercle bacilli (Ministry of Agriculture Veterinary Laboratories, Weybridge, Surrey). The bacilli were finely ground using a porcelain mortar and pestle and carefully suspended in liquid paraffin to a concentration of 5 mg/ml. A mixture of 3% halothane in oxygen (flow rate 300 cc/min.) and nitrous oxide (flow rate 1,500 cc/min.) produced by a Boyles apparatus (British Oxygen Company Limited) was used to anaethetise the rats. The left hind foot-pad of each rat was then carefully injected with 0.03 ml of the adjuvant. Control animals were injected with sterile 0.9% saline.

Carrageenan oedema was induced in 150-200 g male Wistar rats by the method of Winter, Risley and Nuss (1962). A 2% (w/v) solution of the calcium salt of carrageenan (Sigma Chemical Company Limited, U.S.A.) was prepared in sterile 0.9% saline. The rats were anaethetised, as previously described, and 0.05 ml of the carrageenan solution was injected into the left hind foot-pad. Control animals were injected with sterile 0.9% saline.

The inflammatory response to either the adjuvant or carrageenan was estimated by measuring the increase in size of the hind feet of the rats. The size of the hind feet was estimated either by measuring their volume or joint diameter. The volume was measured by immersing the hind feet separately, up to the hair line, in a mercury bath, linked to a

pressure transducer and a Devices recorder. The joint diameter of the feet was measured using a micrometer screw-gauge. The measurements of the hind feet of the rats were taken just prior to the injection of adjuvant or carrageenan and again at various intervals during the experiments.

2.2. The Administration of Substances to Rats with Adjuvant-Induced Arthritis.

2.2.1. Cortisol-Acetate.

Normal and adjuvant arthritic rats were given daily subcutaneous injections of 0.5 ml cortisol-acetate suspension (Upjohn Limited). (5 mg/Kg bodyweight) in 0.9% saline, from the day prior to the injection of adjuvant until the end of the experiment. Normal and adjuvant arthritic control rats were given daily injections of 0.5 ml 0.9% saline alone.

2.2.2. Dimethylnitrosamine.

Adjuvant arthritic rats, that had been injected with adjuvant five days earlier, were given intra-peritoneal injections of 0.5 ml dimethylnitrosamine. (Aldrich Chemical Company, Middlesex). (25 mg/Kg body weight) in 0.9% saline. Control adjuvant arthritic rats were injected with 0.9% saline alone.

2.3. The Administration of Substances to Rats with Carrageenan-Induced Oedema.

2.3.1. Dimethylnitrosamine.

Three days prior to injection of carrageenan rats were given intra-peritoneal injections of 0.5 ml dimethylnitrosamine (25 mg/Kg

body weight) in 0.9% saline. Control rats were injected with 0.5 ml 0.9% saline alone.

2.3.2. Liver Extracts from Dimethylnitrosamine-Treated Rats and Normal Rats.

Liver extracts were prepared from livers of rats that had been given intra-peritoneal injections of 0.5 ml dimethylnitrosamine (25 mg/Kg body weight) in 0.9% saline three days earlier and from the livers of normal rats. 20g liver from each source were separately homogenised in 40 ml ice-cold 0.9% saline for 30 sec. using an electrically driven Potter homogeniser. The cell debris was removed by centrifugation at 100,000g in an M.S.E. Superspeed 50 centrifuge. The clear saline extracts were decanted and part of each was dialysed overnight against 0.9% saline at 4°. The remaining part of each extract was kept overnight at 4°.

Rats were given intra-peritoneal injections of one of the following: 1.5 ml of saline liver extract from rats pre-treated with dimethylnitrosamine, 1.5 ml of dialysed saline liver extract from rats pre-treated with dimethylnitrosamine, 1.5 ml of saline liver extract from normal rats and 1.5 ml of dialysed saline liver extract from normal rats. Control rats were given injections of 1.5 ml of 0.9% saline alone. After treatment with the above extracts the rats were immediately injected with carrageenan in the left hind foot-pad.

2.3.3. A Protein Fraction Containing Human Alpha-1-Antitrypsin.

A partially purified fraction of human alpha-1-antitrypsin (see later text for method of preparation) was mixed with carrageenan solution to give final concentrations of 60 mg protein/ml and 30 mg protein/ml. 0.05 ml of these carrageenan solutions, containing the protein fraction,

were immediately injected into the left hind foot-pad of rats. Control rats were given either injections of carrageenan solution that contained no protein fraction or injections of protein fraction in 0.9% saline alone (60 mg protein/ml).

2.4. Preparation and Storage of Plasma and Serum.

Blood was withdrawn from Wistar rats and New Zealand White rabbits (Hylyne Rabbits Limited, Northwich) by the technique of cardiac puncture. This method allows the withdrawal of relatively large volumes with minimum stress to the animal and avoids the possibility of dilution by extravascular fluids. During the procedure the animals were anaethetised, using a Boyles apparatus, as previously described.

When plasma was required the blood was drawn into heparinised syringes, for serum the blood was allowed to clot. The plasma or serum was separated from the whole blood by centrifugation at 2,000g for 20 min. on an M.S.E. bench centrifuge.

Fresh human plasma was obtained from volunteers who visited the National Blood Transfusion Centre, Edgbaston, Birmingham. This plasma contained a small amount of citrate buffer, pH 7.4.

All plasma and serum was stored in sealed containers at -20^{o} until required for use.

2.5. Polyacrylamide Gel Electrophoresis of Rat Serum Proteins.

The polyacrylamide gels were made by a similar method to that developed by Davies (1964). A small pore 7.5% (w/v) gel was prepared from two solutions. The first consisted of 4.2 M acrylamide, 0.052 M NN^{1}-methylenebisacrylamide and 0.46 mM potassium ferricyanide. The second solution consisted of 0.3 M tris (hydroxymethyl) aminomethane

(Tris), titrated to pH 8.5 with hydrochloric acid, plus 0.032 M NNN^{1}N^1-tetramethylethylenediamine. Two volumes of the first solution were mixed with one volume of the second and one volume of distilled water. Four volumes of a gelation initiator, 6.1 mM ammonium persulphate, were then added.

A large pore gel was prepared from two solutions. The first consisted of 1.4 M acrylamide and 0.16 M NN^1-methylenebisacrylamide. The second solution consisted of 0.47 M Tris buffered at pH 8.5 with phosphoric acid. Two volumes of the first solution were mixed with one volume of the second and four volumes of distilled water. One volume of gelation initiator, 17.5 mM ammonium persulphate, was then added.

The small and the large pore gel solutions were de-aerated in vacuo to remove excess oxygen that inhibits gelation. The small pore gel was then pipetted into cylindrical glass tubes 70 mm x 6.5 mm diameter, sealed at the lower ends with rubber caps, until the tubes were two-thirds full. The large pore gel solution was carefully layered on top to a depth of approximately 1 cm. Water was layered on top of the large pore gel to exclude oxygen and the gels allowed to set.

1 µl Samples of rat serum were accurately pipetted onto small circular discs of filter paper 6 mm in diameter. After spotting with bromophenol blue the discs were placed on top of the large pore gel and covered with a few grains of washed sand to keep them in place. The rubber caps were removed from these tubes and the tubes placed in a Shandon disc electrophoresis apparatus. The electrode buffer, pH 8.5, consisted of 50 mM Tris and 0.38 M glycine. The electrophoresis was performed at a current of 4 m. amp per tube and the serum proteins allowed to migrate towards the anode, until the bromophenol blue marker reached the end of the tube. The gels were then removed and stained with a solution of 1% (w/v) amido-black in 7% (v/v) acetic acid for

approximately 2 h. The amido-black stain is specific for proteins and fixes them in the gel. The background stain of the amido-black in the gels was removed electrophoretically, again using the Shandon disc electrophoresis apparatus. 7% (v/v) Acetic acid was used as electrode buffer and a voltage of 150 volts applied. Upon completion of destaining the gels were stored in 7% (v/v) acetic acid at room temperature. The gels were finally scanned for stained protein using a microdensitometer (Joyce Loebl Chromoscan 200) and photographed.

2.6. Assay Methods for Serum Proteins.

2.6.1. Acid Phosphatase Activity.

Acid phosphatase activity was determined by the method of Symons, Lewis and Ancill (1969). 0.1 ml Serum was incubated with 0.5 ml of 15 mM p-nitrophenylphosphate and 0,5 ml of 90 mM sodium citrate buffer, pH 4.8, for 30 min. in a shaking water bath at 37°. The reaction was stopped by the addition of 5 ml of 0.1 M sodium hydroxide solution. The p-nitrophenol released was determined by measuring the absorbance of the solution at 410 n.m. against a blank in a Pye Unicam S.P.500 spectrophotometer. The blank was prepared by addition of the serum to the incubation medium after the addition of sodium hydroxide. A standard curve of the absorbance of p-nitrophenol at 410 nm was also prepared.

2.6.2. Protease Activity.

Protease activity was determined by a method similar to that described by Rinderknecht, Geokas, Silverman, Lillard and Haverback (1968). 0.5 ml Serum and 2 ml of 0.1 M Tris-HCl buffer pH 7.4 were incubated with 20 mg of azure blue impregnated hide powder (Calbiochem, U.S.A.) for 18 h at 37° in a shaking water bath. The solutions were then filtered to remove the remaining hide powder and the blue dye

released was read at 595 n.m. on a Pye Unicam S.P. 500 spectrophotometer against a blank. For the blank the serum was incubated separately and added to the hide powder and buffer suspension immediately prior to filtration. A standard curve of the absorbance at 595 n.m. of completely dissolved azure blue impregnated hide powder was also prepared.

2.6.3. Trypsin Inhibitory Capacity (TIC).

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

10 µl serum were added to 2 ml trypsin solution (20 µg/ml in 0.1 M Tris-HCl buffer pH 8.2 and 0.02M calcium chloride). 10 µl of a 40 mg/ml bovine serum albumin solution (Fraction V) (Sigma Chemical Company, U.S.A.) were added to the trypsin solution as a control. The solutions were then allowed to stand at room temperature for 10-15 min. to activate the trypsin inhibitory proteins. 0.5 ml of the above solution was added to 2.5 ml BAPNA substrate (0.45 mg/ml in 0.1 M Tris-HCl buffer pH 8.2 and 0.02 M calcium chloride) and the mixture incubated for 10 min. in a shaking water bath at 37°. The reaction was stopped by the addition of 0.5 ml of 30% (v/v) acetic acid. The samples were read against a blank at 400 n.m. on a Pye Unicam S.P. 500 spectrophotometer. The blanks were prepared by adding the serum-trypsin solution to the BAPNA substrate after the addition of the 30% (v/v) acetic acid. A standard curve of the absorbance at 400 n.m. of p-nitroaniline was also prepared.

2.6.4. Trypsin-Protein Esterase (TPE) Activity.

A method to determine the TPE activity of serum was developed by Ganrot, P. O. (1966), it enables the concentration of certain large molecular weight protease inhibitors to be assessed. These can bind and inhibit trypsin against large molecular weight substrates, such as proteins, but cannot inhibit trypsin against small molecular weight substrates such as BAPNA. The activity of the bound trypsin is unaffected by soybean trypsin inhibitor.

25 µl Serum were added to 0.5 ml trypsin solution (100 µg trypsin/ml of 0.1 M Tris-HCl buffer, pH 8.2,and 0.02 M calcium chloride), 0.5 ml Tris-HCl buffer without trypsin were added to the blank. After 5-10 sec. 1.5 ml BAPNA solution containing soybean trypsin inhibitor (Koch-Light Laboratories, Bucks.) (0.9 mg BAPNA and 33.4 µg soybean trypsin inhibitor per ml 0.1 M Tris-HCl buffer pH 8.2 and 0.02 M calcium chloride) were also added. The mixture was incubated for 10 min. at 37° in a shaking water bath. The reaction was stopped by the addition of 0.5 ml of 30% (v/v) acetic acid. The samples were read against the blank at 400 n.m. in a Pye Unicam S.P. 500 Spectrophotometer.

2.6.5. Estimation of Protein Concentration.

Protein concentrations were estimated by the biuret procedure of Gornall, Bardawill and David (1949). The biuret reagent consisted of a solution of 6 mM copper sulphate, 2.1 mM sodium potassium tartrate, 0.8 M sodium hydroxide and 6 mM potassium iodide. 5 ml Biuret reagent were added to 0.1 ml serum, 0.1 ml standard protein solution (80 mg protein/ml) (Sigma Chemical Company Limited, U.S.A.) and 0.1 ml distilled water to act as blank. After standing for 30 min. at room temperature the absorbance of the solutions were read against the blank at 540 n.m. on a Pye Unicam S.P. 500 spectrophotometer and compared with the standard protein solution.

2.6.6. Transcortin Levels.

Rat serum transcortin levels were estimated by a method described by Milgrom and Baulieu (1969). 0.5 ml Serum were incubated with 2.3 µg, specific activity 1.9 µCi, 1,2-^{3}H-cortisol (Radiochemical Centre, Amersham, Wilts.) for 15 min. at 37°. The 1,2-^{3}H-cortisol was present in a large excess and therefore displaced any endogenous steroid from the transcortin binding sites. The plasma was then allowed to stand for 2 h in a cold room at 4°, all further procedures were carried out at this temperature. 2 ml Ice-cold dextran-coated charcoal suspension (0.25 g charcoal and 0.025 g dextran in 100 ml Tris-HCl buffer, pH 7.4) were added and the mixture shaken vigorously at constant speed. At various intervals during a period of 2 h., 0.3 ml samples of the suspension were removed and centrifuged for 2 min. in a small Eppendorf bench centrifuge. 0.2 ml Charcoal-free supernatant were added to 5 ml scintillation fluor and the radioactivity counted in a liquid scintillation counter (counting efficiency 40%). The scintillation fluor was based on that used by Bray (1960) and consisted of 100 g naphthalene, 4 g 2,5 diphenyloxazole (PPO), 200 mg [1,4-di(2-(5-phenyloxazolyl)) benzene] (POPOP) and 100 ml absolute methanol made up to 1L with dioxan.

The charcoal free supernatant contained only transcortin-bound 1,2-^{3}H-cortisol, since any "free" or albumin-bound cortisol is rapidly absorbed by the dextran-coated charcoal (Milgrom and Baulieu, 1969).

2.7. An Estimation of the Stability of Transcortin-Bound 1,2-^{3}H-Cortisol to Non-Steroidal Anti-Inflammatory Drugs.

1 ml Samples of human plasma were incubated with 1,2-^{3}H-cortisol (1.25 µCi, 8.2 ng) and the appropriate drug at therapeutic concentrations for 30 min. at 37°. The samples containing drugs were incubated in

duplicate, control samples containing no drugs were incubated in triplicate. Blank samples which contained drugs and heat inactivated transcortin (obtained by heating the plasma for 1 h at 60°) were also incubated in triplicate. All samples were then allowed to stand for 2 h in a cold room at 4°, all further procedures were carried out at this temperature. 3 ml Ice-cold charcoal suspension (0.25 g charcoal plus 0.025 g dextran in 100 ml of 0.01 M Tris-HCl buffer, pH7.4) were added to each sample. The samples were shaken vigorously at a constant speed and 0.3 ml aliquots removed at various intervals for a period of 120 min. These were immediately centrifuged in a small Eppendorf bench centrifuge and 0.2 ml charcoal free supernatant were added to 5 ml of modified Bray's scintillation fluor (see method 2.6.6.), the radioactivity of the sample was measured in a Beckman Scintillation counter (Counting efficiency 40%).

The charcoal free supernatant contained only transcortin-bound 1,2-^{3}H-cortisol since any "free" or albumin-bound cortisol is rapidly absorbed by the dextran-coated charcoal (Milgrom and Baulieu, 1969).

The following drugs were assayed in the above system: aspirin and sodium salicylate (B.D.H. Limited), phenylbutazone and oxyphenbutazone (Geigy Pharmaceuticals Limited), indomethacin and D-penicillamine (Merck, Sharp and Dohme Limited), ibuprofen (The Boots Company Limited) dapsone (I.C.I. Limited) and prednisolone (Sigma Chemical Company Limited, U.S.A.).

2.8. The Preparation of Plasma Protein Fractions.

2.8.1. Human Alpha-1-Antitrypsin.

A fraction containing human alpha-1-antitrypsin was prepared using disulphide exchange chromatography, the method has been described by Laurell, Pierce, Persson and Thulin (1975).

Human plasma was diluted with two volumes 0.03 M 2-mercapto-ethanol to reduce plasma protein disulphides. An initial protein fraction containing alpha-1-antitrypsin was prepared by ammonium sulphate precipitation, between 50-72% saturation at room temperature and pH 6. The precipitate was collected by centrifugation at 20,000 g for 30 min. in a high speed M.S.E. 18 centrifuge. It was dissolved in distilled water and dialysed against distilled water for 48 h at 4^{o}, followed by dialysis against 0.1 M Tris-HCl buffer pH 8.1 containing 5 mM ethylene-diaminetetraacetic acid (EDTA) for 12 h at 4^{o}.

The next stage involved disulphide exchange column chromatography. The column (30 cm x 3 cm diameter) was packed with approximately 60 ml of activated thiol-sepharose 4B. The activated thiol-sepharose 4B was obtained as a glutathione 2-pyridyl disulphide derivative of sepharose 4B (Pharmacia Fine Chemicals, Sweden). This was taken up in distilled water and the 2-pyridylmercaptan cleared by eluting with 200 ml of 0.02 M 2-mercaptoethanol. The column was washed with 300 ml 0.1 M Tris-HCl buffer pH 8.1 containing 5mM EDTA. 75 ml Tris-HCl buffer containing 2.5 mM 5,5^{1}-dithiobis-(2-nitrobenzoate) (Nb S_2) were passed through the column to form Nbs derivatives on the terminal cysteine of the thiol-sepharose 4B. The initial dark yellow band of Nbs was eluted and the Nbs_2-Tris-HCl buffer recirculated through the column for a minimum of 1 h. The column was then washed with 300 ml of Tris-HCl buffer alone.

200-400 mg of the protein fraction were applied to the column and recirculated through it over night at room temperature with a flow rate of approximately 1.5 ml/min. The column was then washed with 300 ml Tris-HCl, buffer pH 8.1, containing 0.5 M sodium chloride, 3 mM sodium azide and 10 mM EDTA; before finally washing with 150 ml Tris-HCl buffer alone.

Part of the protein-bound fraction was displaced from the column by 10 ml Tris-HCl buffer pH 8.1 containing 2.5 mM Nbs and 1.26 mM Nbs_2 (10 mg Nbs_2 in 10 ml Tris-HCl buffer reduced by 2 mg dithiothreitol). The remainder of the protein bound to the column was released with 25 ml 0.02M 2-mercaptoethanol in Tris-HCl buffer. The eluted fractions were collected in 5 ml aliquots using an LKB 7000 Ultrorac fraction collector.

The protein fractions released initially from the column contained a high percentage of TIC activity and were pooled. The free Nbs_2 and Nbs in the solution were removed by dialysis and absorbed by charcoal in the dialysate (0.5 mg/ml). The protein-bound Nbs was released by reduction with dithiothreitol. The protein fraction was finally dialysed for 48 h against distilled water at 4^{o} and then freezedried.

2.8.2. Rat Alpha-1-Antitrypsin.

An attempt was made to prepare an alpha-1-antitrypsin fraction from pooled rat serum using the previously described method of disulphide exchange chromatography. However, this method was unsuccessful for rat serum, therefore an alternative ion-exchange chromatography procedure reported by Bellet and De Bornier (1975) was adopted.

Pooled rat serum was precipitated by ammonium sulphate in the range 50-72% of saturation at pH 6 and room temperature. The precipitate was collected by centrifugation for 30 min. at 20,000 g using a high speed M.S.E. 18 centrifuge and dissolved in distilled water. This fraction was dialysed initially against distilled water for 48 h at 4^{o} and finally against Tris-HCl buffer pH 8.5 (0.1 M Cl^{-} + 0.08 M sodium

chloride) for 24 h at 4°.

300 mg of the dialysed protein fraction (10 ml) were applied to a Pharmacia column type K26/40 containing a diethylaminoethyl derivative of Sephadex (DEAE Sephadex) A-50-120 (Pharmacia Fine Chemicals, Sweden), equilibriated in Tris-HCl buffer pH 8.5. The elution was performed at room temperature with a buffer of constant pH but increasing ionic strength, using a flow rate of approximately 30 ml per h. An ionic strength gradient was prepared from three cells, cells I and II each contained 120 ml Tris-HCl buffer pH 8.5 (0.1 M Cl^- + 0.08 M sodium chloride) and cell III 120 ml Tris-HCl buffer pH 8.5 (0.1 M Cl^- + 0.15 M sodium chloride). The protein concentration of the eluate was monitered by its absorption at 260 n.m. on an LKB 8300 UVICORD II. The eluate was collected in 5 ml fractions in an LKB 7,000 Ultrorac/fraction collector. The fractions with the highest TIC activity over a narrow range were pooled. The pooled fractions were rechromatographed and again the fractions with the highest TIC activity were pooled. The pooled fraction was dialysed against distilled water for 24 h at 4° and freeze-dried.

2.8.3. Rat Alpha-1-Macroglobulin.

Low density lipoproteins and very low density lipoproteins were precipitated from normal rat serum by a method described by Burstein and Scholnick (1973). 0.5 ml 10% (w/v) Dextran sulphate and 5 ml 1M manganese chloride were mixed with 100 ml rat serum. The lipoprotein precipitate was removed by centrifugation at 6,000 g for 10 min. in an M.S.E. bench centrifuge. The high density lipoproteins were removed from the rat serum supernatant by a further method of Burstein and Scholnick (1973). The lipoproteins were precipitated by the addition of 6 ml 10% (w/v) dextran sulphate and 15 ml 1 M manganese chloride. After standing

for 2 h the precipitate was removed by centrifugation at 20,000 g for 30 min. in a high speed M.S.E. 18 centrifuge.

10 ml of the serum supernatant were then applied to a Pharmacia column (SR 25/100) filled with Bio-Gel A-0.5, 100-200 mesh (Bio-Rad., U.S.A.) in 50 mM sodium barbitone-sodium acetate buffer, pH 8.6. The flow rate was approximately 2 ml/min. The column eluate was monitored for protein by an LKB 8300 UVICORD II and collected in 5 ml samples by an LKB 7000 Ultrorac fraction collector. The fractions collected corresponding to the first protein peak gave a positive TPE assay test and were pooled. These pooled fractions were concentrated by dialysis against aquacide (flake polyethylene glycol) (Calbiochem, U.S.A.) and rechromatographed in an identical column filled with Bio-Gel A-1.5 m, 100-200 mesh (Bio-Rad. U.S.A.), in 50 mM sodium barbitone-sodium acetate buffer, pH 8.6. The eluate fractions with high TPE activity were again pooled and dialysed for 24 h at 4° against distilled water. Alpha-1-macroglobulin was found to be unstable if stored frozen in solution and during freeze-drying. It was therefore concentrated by aquacide and used immediately.

2.8.4. Rat Alpha-2-Acute Phase Globulin.

Alpha-2-Acute phase globulin was induced in the serum of rats by the method of Ganrot, K. (1973). Male Wistar rats were given intra-muscular injections of 1 ml oil of turpentine B.P. (Evans Medical Limited, Liverpool) two days prior to removal of blood.

The alpha-2-acute phase globulin was partially purified by eluting 20 ml of the above serum through a Pharmacia column (K 50/100) packed with Sephadex G-200 (Pharmacia, Sweden), using 0.05 M sodium phosphate buffer as the elutant. The flow rate was very slow approximately 5 ml/h

of normal rat serum. Equal volumes of normal rat sera and rabbit antisera were mixed and allowed to stand overnight at 4°. The antigen-antibody precipitates that had formed were then removed by centrifugation for 20 min. at 900 g on an M.S.E. bench centrifuge.

and the column was run in a cold room at 4° to reduce any deterioration of the protein. The column eluate was collected in 5 ml fractions and these were tested against antibodies to human alpha-2-macroglobulin (Dako-immunoglobulins, Denmark) by immunodiffusion (see methods 2.10.). The fractions which assayed positively contained alpha-2-acute phase globulin, since it is immunologically similar to human alpha-2-macroglobulin, and these fractions were pooled. The pooled fractions were dialysed for 48 h at 4° against distilled water and then freeze-dried.

2.9. The Induction and Partial Purification of Rabbit Antibodies to Rat Serum Proteins.

An attempt was made to raise rabbit antisera against the rat alpha-1-antitrypsin, alpha-1-macroglobulin and alpha-2-acute phase globulin fractions previously described. Approximately 10 mg of each rat protein fraction were dissolved in 1.5 ml sterile saline (0.9%). 1.5 ml Adjuvant (0.5 mg finely ground heat-killed tubercle bacilli per ml of liquid paraffin) were added to the protein solution. A stable water in oil emulsion was formed by repeatedly drawing the mixture through a narrow gauge needle with a syringe. 0.5 ml Emulsion were injected intramuscularly into each rabbit at four different sites over the back. The treatment was repeated after two weeks and again after four weeks in order to booster the circulating antibody levels. The rabbit sera were tested for antibodies against the rat serum proteins by immunoelectrophoresis (see methods 2.10.).

Since the rat alpha-2-acute phase globulin fraction was only partially purified the rabbit antiserum raised against it contained anti-bodies against a number of normal rat serum proteins. These anti-bodies were removed by precipitating them out of solution by the addition of normal rat serum.

The immunoglobulin fraction of the rabbit serum was partially purified by ion-exchange chromatography on DEAE-cellulose (Sigma Chemical Company, U.S.A.). A chromatography column (30 cm x 3 cm diameter) was packed with 15 g DEAE cellulose taken up and equilibriated at pH 7.5 with 0.01 M potassium phosphate buffer, pH 7.5. 10 ml Serum, that had been dialysed overnight at 4° against 0.01 M potassium phosphate buffer, pH 7.5, were applied to the column and eluted with the same buffer. The column eluate was monitered for protein using an LKB UVICORD II and collected in 5 ml fractions by an LKB Ultrorac fraction collector. The fractions of the first protein peak contained mainly immunoglobulins and these were pooled, and dialysed against aquacide to reduce the volume to about 3 ml. The fraction was then dialysed for 24 h against a solution of 0.1 M sodium chloride and 15 mM sodium azide (to inhibit bacterial growth) and stored at 4° until required for use.

The column was restored by eluting through it 200 ml of a solution containing 1.5 M sodium chloride and 0.3 M potassium phosphate buffer, pH 7.0, to release the remaining bound protein. The column was then re-equilibriated with 0.01 M potassium phosphate buffer, pH 7.5.

2.10. Immunodiffusion and Immunoelectrophoresis of Plasma Protein Fractions.

1% (w/v) Agarose gel was prepared by heating agarose (Sigma Chemical Company, U.S.A.) in 6 mM sodium barbitone - sodium acetate buffer pH 8.6 with constant stirring until dissolved. The agarose solution was then allowed to cool to 58° and poured onto a Shandon immunoelectrophoresis tray fitted with microscope slides. The excess agarose was removed before it had time to set with a Shandon perspex scraper. After setting a layer of agarose gel approximately 1 mm thick covered the slides.

For immunodiffusion studies a slot 30 mm X 1 mm was cut out of the agarose gel down the centre of a slide. Holes 1 mm diameter and 5 mm apart were cut along both sides of the slot at a distance of approximately 3 mm from its edge. 20 µl Rabbit antiserum were placed in the slot and 1 µl samples of each of the protein fractions to be assayed were placed in each hole. The antiserum and protein fractions were allowed to diffuse through the agarose gel for 12 h. The protein fractions that contained antigen specific for the antiserum formed small arcs of precipitated antigen-antibody complexes between the holes and the slot. The agarose gels were then immersed in 0.9% saline for 12 h to remove any non-precipitated protein. The arcs were stained by immersing the gels in a solution of 1% amido-black in 7% (v/v) acetic acid for 1 h. The background stain in the agarose gel was removed by washing the gel in 7% (v/v) acetic acid until the small arcs of stained protein complexes were clearly visible.

For immunoelectrophoretic studies 1% (w/v) agarose gel was prepared on a Shandon immunoelectrophoresis slide tray, as described above. A slot was again cut in the agarose gel down the centre of a slide. Two holes were cut opposite each other either side of slot 3 mm from its edge and about one third of the distance from one end. 1 µl Serum protein fraction (approximately 1 µg protein) was applied to each hole. The slide tray was then placed in a Shandon electrophoresis tank which contained 6 mM sodium barbitone-sodium acetate electrode buffer, pH 8.6. A voltage of 100 volts was applied across the agarose gel by a Shandon power pack so that the protein fraction migrated parallel with the edge of the slot towards the anode. The migration of the protein was visualised by a drop of bromophenol blue, the electrophoresis was stopped when the bromophenol blue reached the end of the slot. The slot was then filled with rabbit antiserum, which was allowed to diffuse into the agarose gel for 12 h. Any antigen-antibody precipitin arcs that had

formed in the agarose gel were visualised by staining with amido-black, as described above.

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

2.11. The Estimation of the Levels of Rat Alpha-1-Macroglobulin and Rat Alpha-2-Acute Phase Globulin by Electro-Immuno Assay.

The method of "rocket electrophoresis" developed by Laurell (1966) was used. 1% (w/v) Agarose gel was prepared by heating agarose, with constant stirring until dissolved, in 0.07 M barbital buffer, pH 8.6, containing 2 mM calcium lactate. The agarose solution was allowed to cool to 40° and the requisite amount of antiserum added. The antiserum concentration chosen was the minimum one that had previously been found to form a distinct precipitin line with the antigen. The antisera to rat alpha-1-macroglobulin and rat alpha-2-acute phase globulin prepared as previously described were mixed with agarose in ratio 1:40. 15 ml Agarose containing the antiserum were then carefully poured into a mould consisting of two identical rectangular glass plates 20 cm x 10 cm held approximately 1 mm apart by a 1 cm wide strip of polythene around three of the four edges. The mould was clamped and held upright until the agarose had set. The clamps were then removed and one glass plate slid away to reveal a slab of gel of uniform thickness 18 cm x 8 cm x 1 mm. Holes 4 mm in diameter were cut in the gel along the longest edge 2 cm from the edge with the centres of the holes a minimum of 8 mm apart. The gel was then placed in a Shandon electrophoresis tank with the line of holes perpendicular to the electric field and furthest from the anode. 8 µl Each protein solution to be assayed were accurately

pipetted into separate holes. For this procedure rat serum was diluted by 1:15 to assay alpha-1-macroglobulin and alpha-2-acute phase globulin. Dilutions were made with electrode buffer which consisted of 0.07 M barbital buffer pH 8.6. A voltage of 70-80 volts was then applied across the gel by a Shandon power pack. The electrophoresis was allowed to proceed for 18 h by which time the height of the "rockets" remained constant. The power pack was switched off and the height of the "rockets" was measured from the centre of the holes to the tips of the peaks. The height of the peaks were proportional to the concentration of the antigen that precipitated with the antisera. Standard antigen solutions were assayed during each electrophoretic run so that the results for different sera on different runs could be directly compared.

3. RESULTS.

3.1. The Purification of Plasma Protein Fractions and Production of Antisera.

3.1.1. Alpha-1-Macroglobulin.

The elution profile of normal rat serum, after the removal of alpha and beta-lipoproteins, following gel chromatography on Bio-Gel A-0.5 m is shown in Fig. 1. The fractions indicated, that had high TPE activity, were pooled and rechromatographed on Bio-Gel A-1.5 m (Fig. 2). The fractions with high TPE activity were again pooled and these were used to raise antisera in rabbits. Assay of the rabbit antisera by immunoelectrophoresis gave one strong precipitin line against whole rat serum (Fig. 3).

The specificity of the rabbit antisera for alpha-1-macroglobulin was investigated on fractions of whole rat serum, produced by gel filtration on Bio-Gel A-1.5 m (Fig. 4). Fractions were assayed by "rocket electrophoresis" using the above rabbit antisera preparation (Fig. 5). The "rocket" electrophoresis gave a peak of activity that directly corresponded with the TPE activity of the fraction (Fig. 4), the rabbit antisera was therefore considered to be specific for alpha-1-macroglobulin.

3.1.2. Alpha-2-Acute Phase Globulin.

The fractions produced by gel filtration of injured rat serum through Sephadex G-200, that gave precipitin lines when tested by immunodiffusion with rabbit antisera against human alpha-2-macroglobulin, were pooled. The protein from these pooled fractions, that was used to raise antisera in rabbits, produced rabbit immunoglobulins against a number of normal rat serum proteins. After removal of these immunoglobulins by precipitation with normal rat serum, immunoelectrophoresis

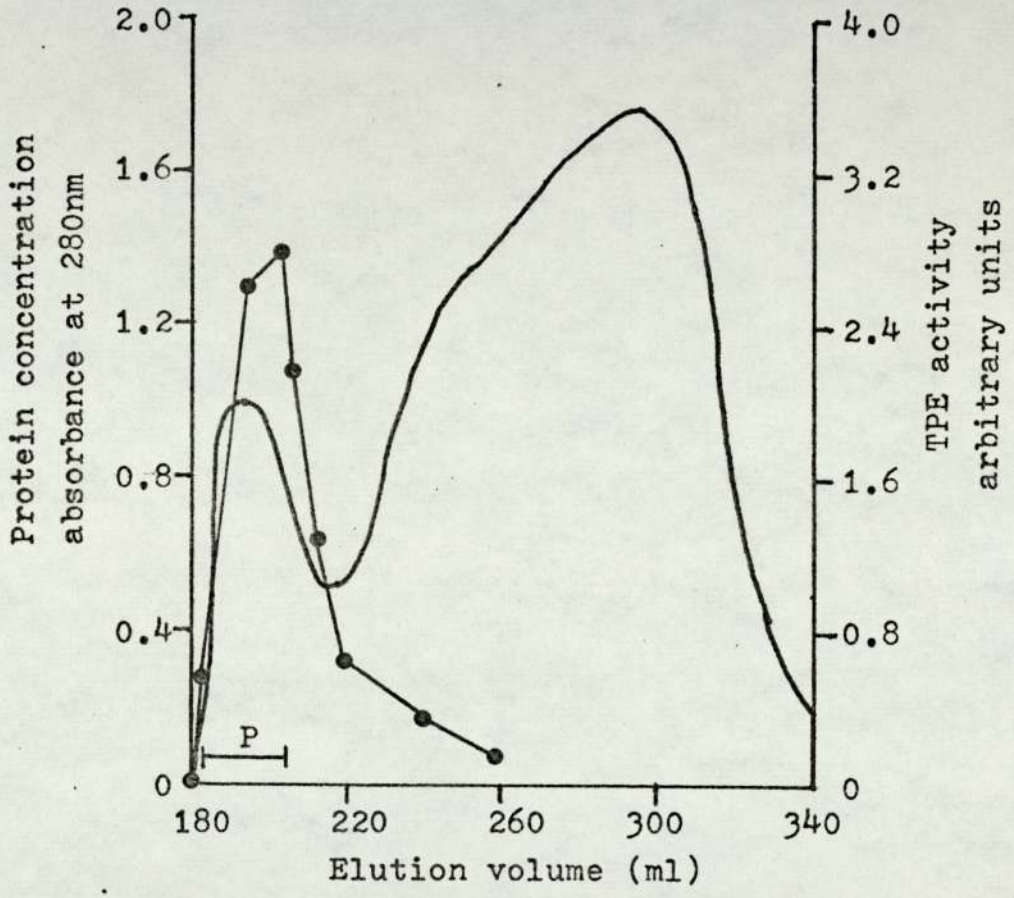


Fig.1. The protein elution profile of rat serum through a Bio-Gel A-0.5m column in 50mM sodium barbitone - sodium acetate buffer, pH 8.6, after initial removal of alpha and beta-lipoproteins. The absorbance at 280nm ——— and the trypsin-protein esterase activity ●———● of the eluted protein were measured. Fractions pooled are indicated by P.

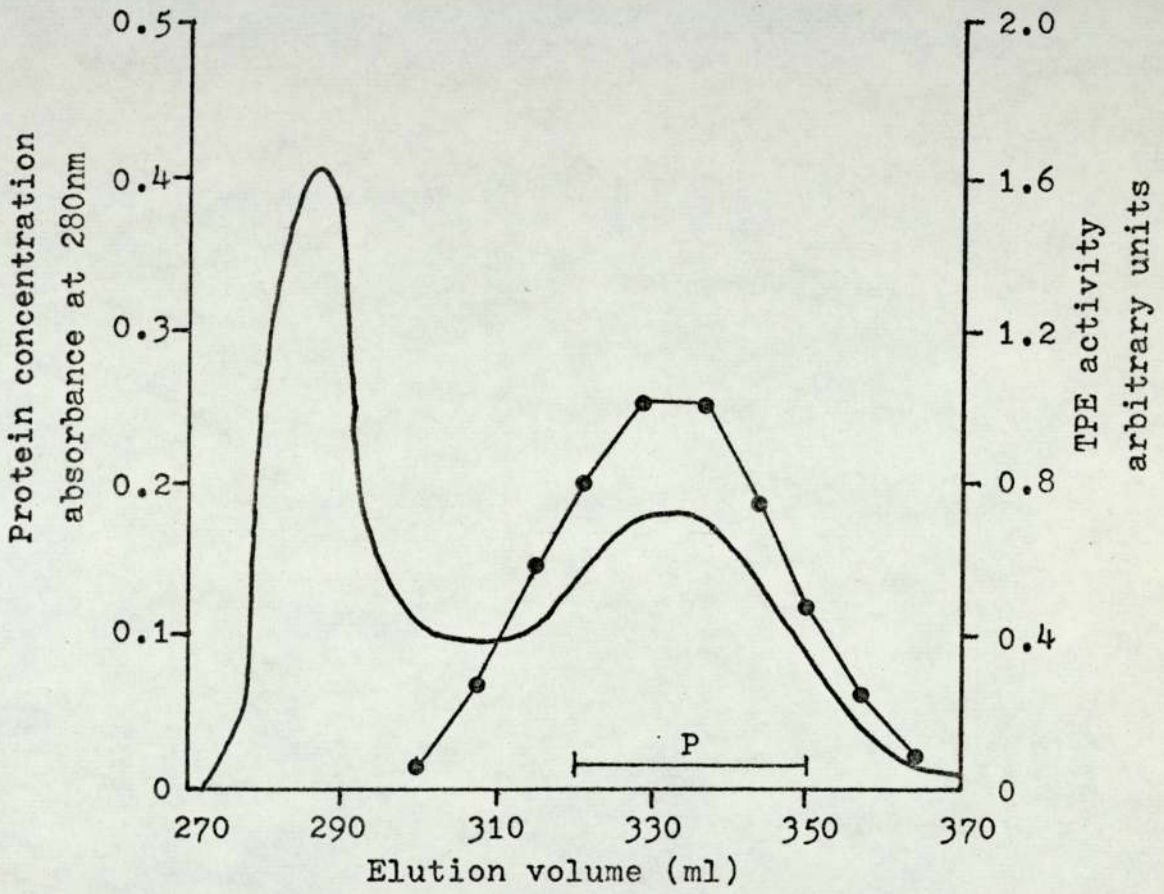


Fig.2. The elution profile of the previously pooled protein fractions (indicated in Fig.1.) through a Bio-Gel A-1.5m column in 50mM sodium barbitone - sodium acetate buffer, pH 8.6. The absorbance at 280nm ——— and the trypsin-protein esterase activity ●———● of the eluted protein were measured. Fractions pooled are indicated by P.

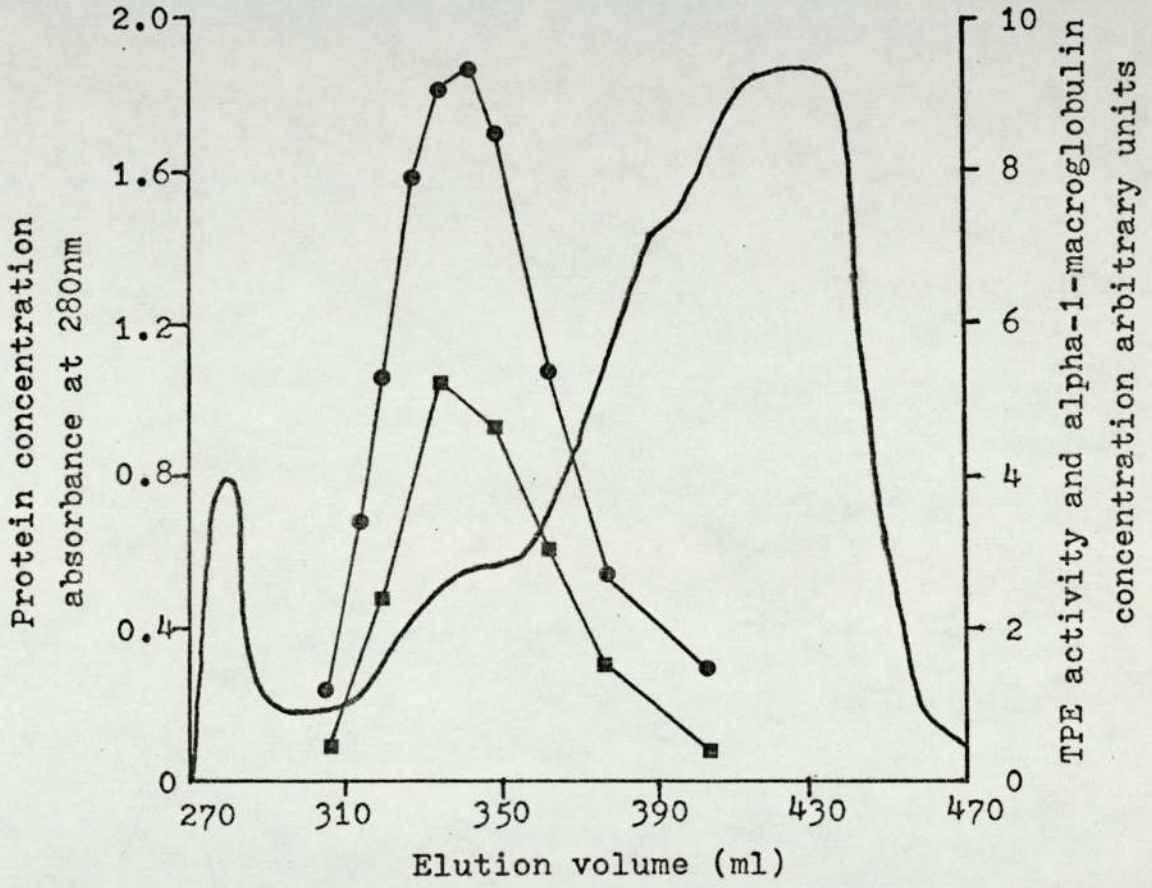
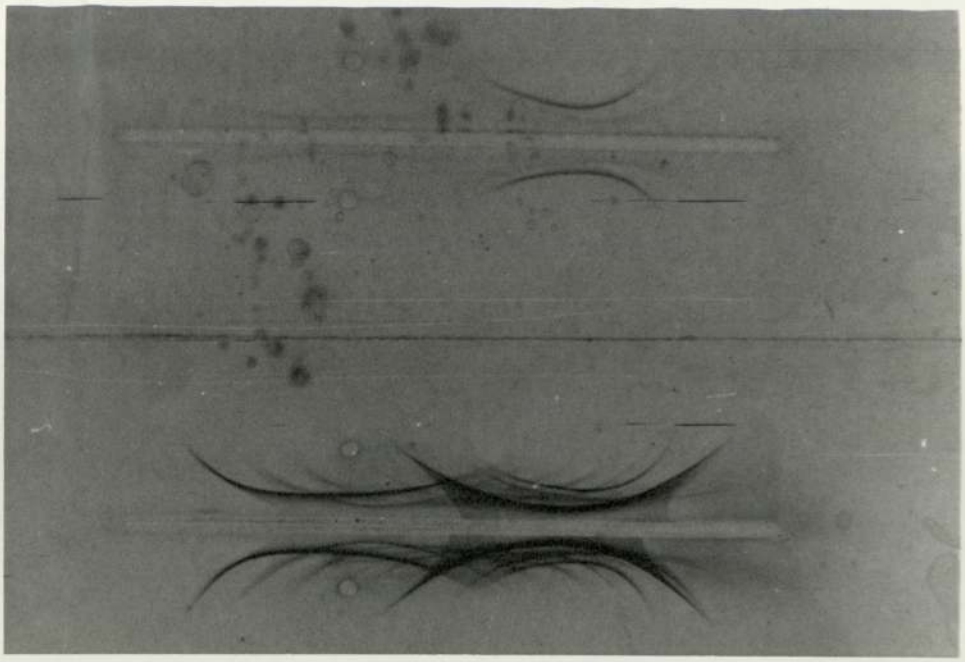


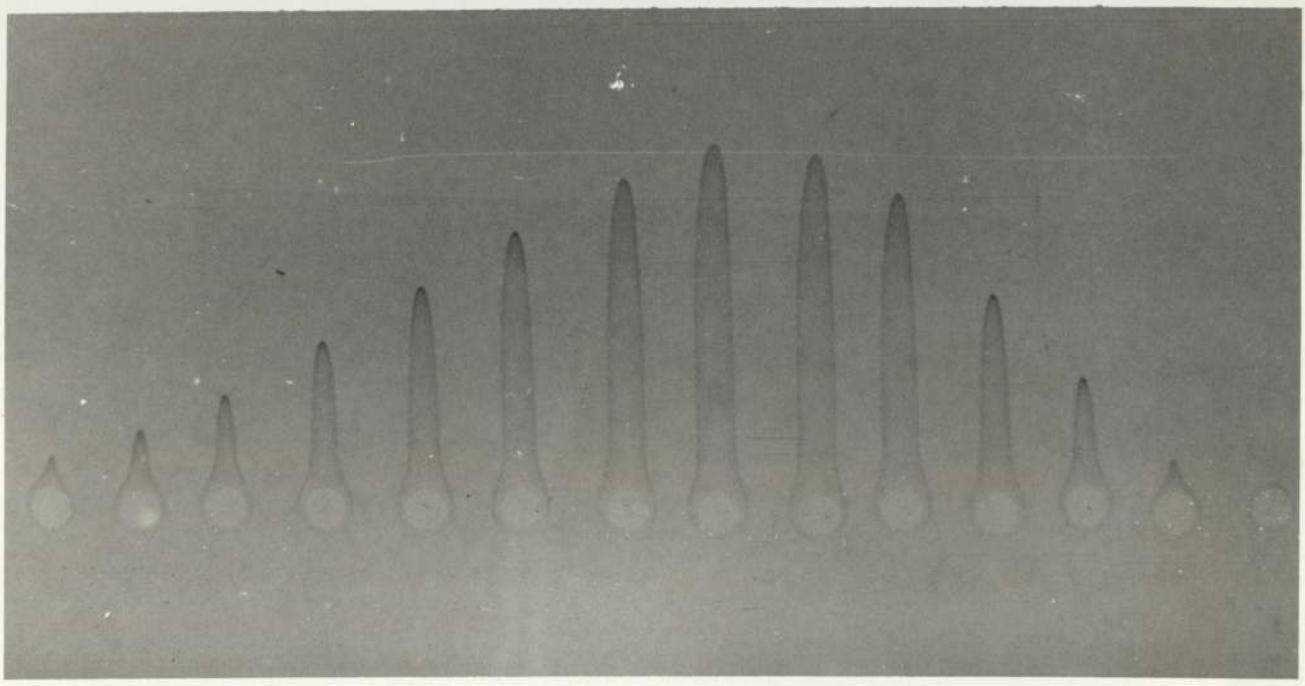
Fig.4. The protein elution profile of 5ml rat serum through a Bio-Gel A-1.5m column in 50mM sodium barbitone - sodium acetate buffer, pH 8.6. The absorbance at 280nm ——— , the trypsin-protein esterase activity ■———■ and the alpha-1-macroglobulin concentration (using "rocket" immunoassay) ●———● of the eluted protein were measured.

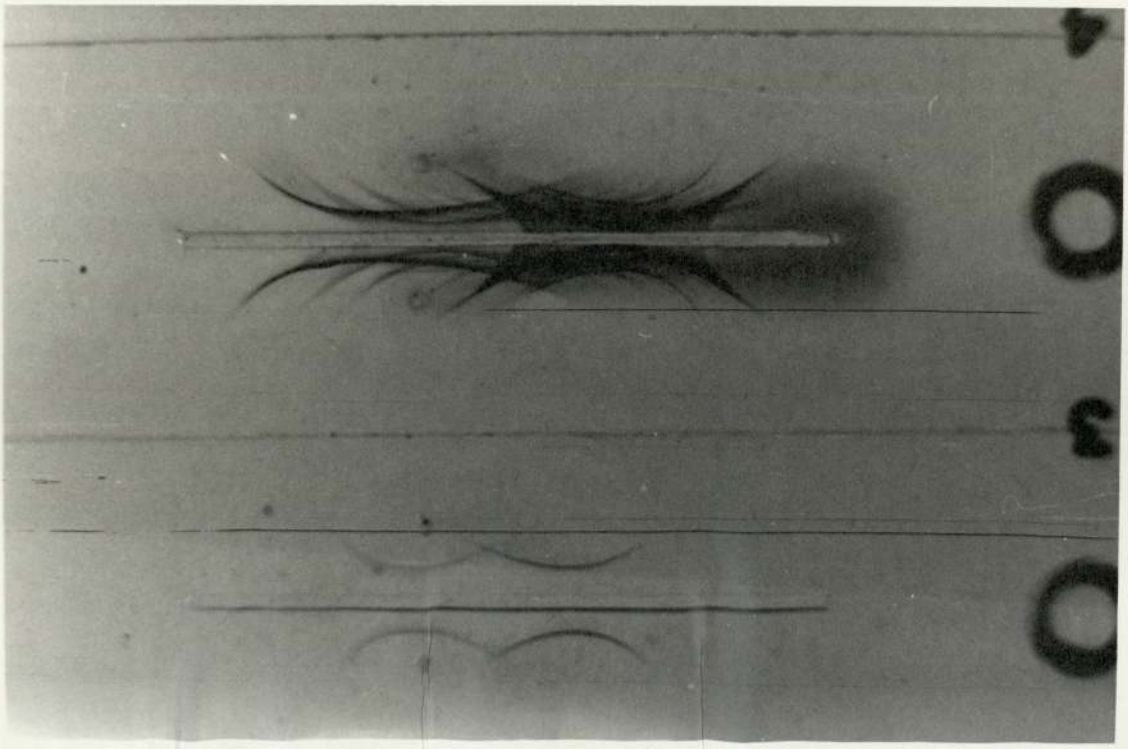
Fig.3. Immunoelectrophoresis of whole rat serum against rabbit antisera to rat alpha-1-macroglobulin.

Fig.5. "Rocket" immunoassay of rat serum protein fractions (with TPE activity, see Fig.4.) against rabbit antisera to rat alpha-1-macroglobulin. The rat serum protein fractions were obtained by gel filtration of whole rat serum through Bio-Gel A-1.5m in 50mM sodium barbitone - sodium acetate buffer, pH 8.6.

Fig.6. Immunoelectrophoresis of injured rat serum against rabbit antisera to rat alpha-2-acute phase globulin.







The rat plasma protein fraction that had been circulated through the disulphide-exchange column still retained 92% of its original TIC activity. These results may indicate that rat alpha-1-antitrypsin has fewer cysteine residues available for disulphide-exchange than human alpha-1-antitrypsin. This could account for the poor yield of rat alpha-1-antitrypsin obtained by this method.

of whole injured rat serum with the rabbit antisera gave one strong precipitin line in the alpha-2-globulin region (Fig. 6). The line was not present after immunoelectrophoresis of whole normal rat serum, therefore the antisera was assumed to be specific for rat alpha-2-acute phase globulin.

3.1.3. Alpha-1-Antitrypsin.

The alpha-1-antitrypsin fraction prepared from human plasma, by ammonium sulphate precipitation and disulphide exchange chromatography, had a TIC activity of 0.48 μmole/min. per mg. protein. 180 mg Protein fraction were isolated from 500 ml plasma. Immunoelectrophoresis of the protein fraction with rabbit antisera against whole human serum demonstrated that the fraction contained two or three other protein contaminants (Fig. 7(a)). Immunoelectrophoresis of the protein fraction with rabbit antisera against human alpha-1-antitrypsin showed that the major precipitin line was due to alpha-1-antitrypsin (Fig. 7(b)).

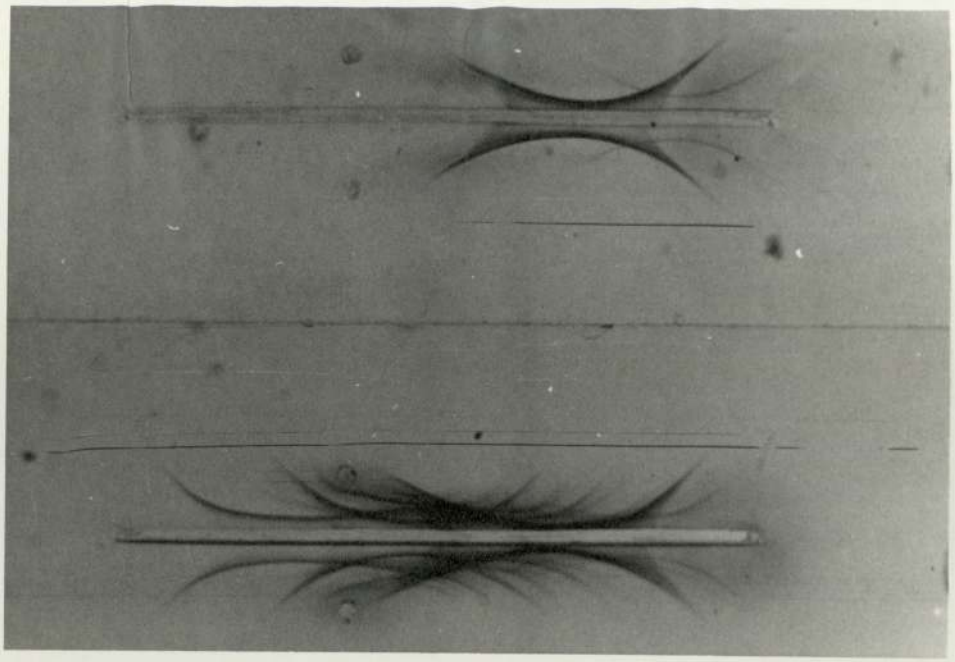
The alpha-1-antitrypsin fraction, prepared from rat serum by disulphide exchange chromatography had a TIC activity of only 0.023 μ mole/min. per mg protein. 16 mg Protein fraction were isolated from 400 ml rat serum. (cont. on opposite page)

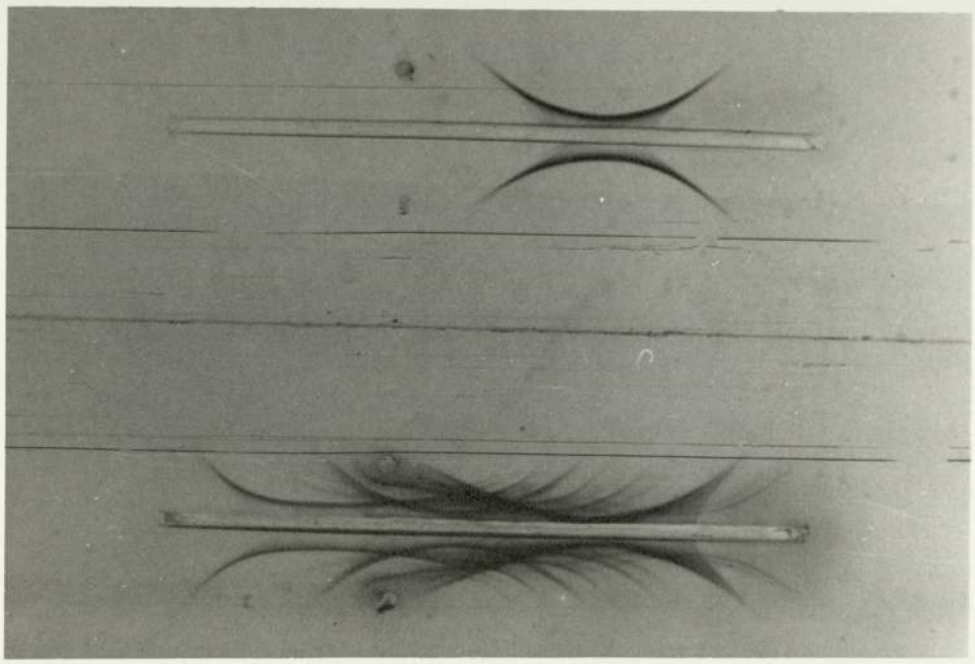
The initial elution profile of rat serum after ammonium sulphate precipitation and ion-exchange chromatography on DEAE-Sephadex A-50-120 is shown in Fig. 8. After rechromatography of the fractions with high TIC activity, the freeze-dried protein fraction produced had a TIC activity of 1.23 μ mole/min per mg. 60 mg Protein fraction were recovered from 400 ml serum. Immunoelectrophoresis of this protein fraction with rabbit antisera against whole rat serum produced a major precipitin line in the alpha-1-globulin region and several minor lines

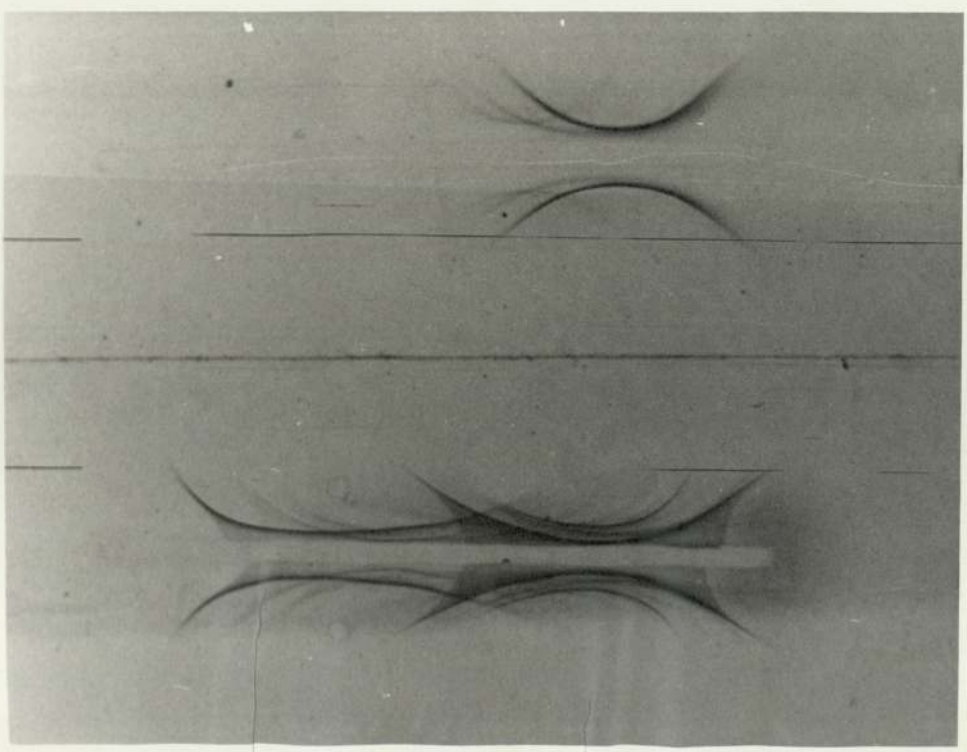
Fig.7(a). Immunoelectrophoresis of the human alpha-1-antitrypsin fraction against rabbit antisera to whole human serum.

Fig.7(b). Immunoelectrophoresis of the human alpha-1-antitrypsin fraction against rabbit antisera to human alpha-1-antitrypsin.

Fig.9. Immunoelectrophoresis of rat alpha-1-antitrypsin fraction against rabbit antisera to whole rat serum.







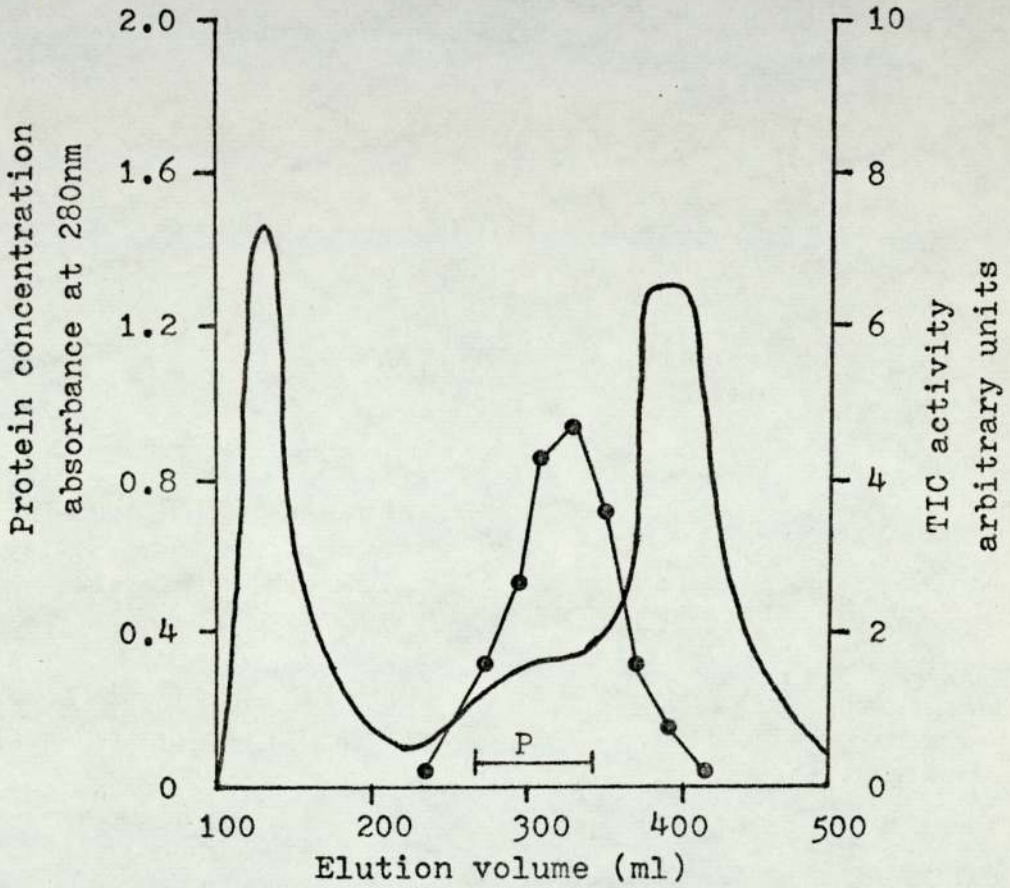


Fig.8. The elution profile of a rat serum protein fraction, previously prepared by ammonium sulphate precipitation, through a DEAE-Sephadex A-50-120 column in Tris-HCl buffer pH 8.6 of increasing ionic strength. The absorbance at 280nm ——— and the trypsin inhibitory capacity ●———● of the eluted protein were measured. Fractions pooled are indicated by P.

(Fig. 9). The major precipitin line was assumed to be due to alpha-1-antitrypsin, but an attempt to raise antisera against this protein fraction, in two rabbits, proved unsuccessful.

3.2. Adjuvant-Induced Arthritis in the Rat.

3.2.1. The Assessment and Development of Adjuvant Arthritis.

The development of adjuvant-induced arthritis in the feet of male and female Wistar rats is shown in Fig. 10, Fig. 11 and Fig. 12. The intensity of the arthritis was assessed either as the increase in joint diameter of the hind feet (Fig. 10) or as the increase in volume of the hind feet (Fig. 11 and Fig. 12). Both methods of assessment gave similar results. The acute or primary inflammation of the injected foot increased over the first four or five days after the injection of adjuvant. Chronic or secondary inflammation started to develop in both hind feet from day seven onwards, with the greatest increase between days twelve and twenty one. There was no difference between the intensity of arthritis in male and female rats (Fig. 12). Daily subcutaneous injections of cortisol-acetate clearly reduced the arthritis by up to 50% in the injected feet and by an even greater amount in the non-injected feet (Fig. 10 and Fig. 11).

3.2.2. Serum Protein Patterns of Adjuvant Arthritic Rats.

Fig. 13 shows the micro-densitometer scans of normal rat serum protein after separation by electrophoresis on polyacrylamide gels. The serum protein patterns of normal rats treated daily with cortisol-acetate for twenty four days were similar (Fig. 14). The serum protein patterns of rats injected four days earlier with adjuvant demonstrate

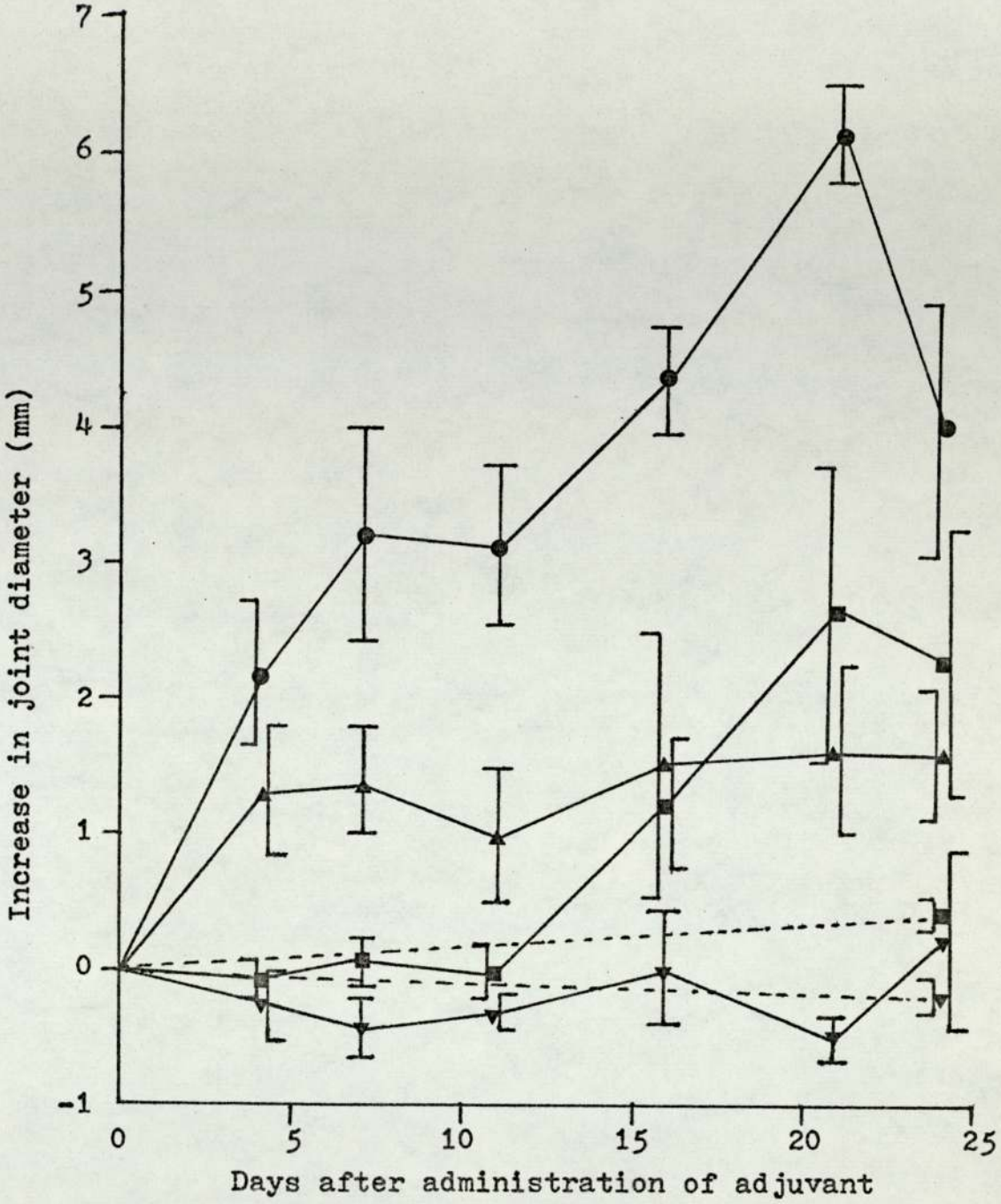


Fig.10. Increase in joint diameter of left ●——● and right ■——■ hind feet of rats after injection of mycobacterial adjuvant into the left hind feet. Increase in joint diameter of left ▲——▲ and right ▼——▼ hind feet of rats treated daily with cortisol-acetate (5mg/kg) after injection of mycobacterial adjuvant into the left hind feet. Dotted lines represent non-arthritic control rats. Each result represents the mean of five animals and vertical lines indicate S.E.M.

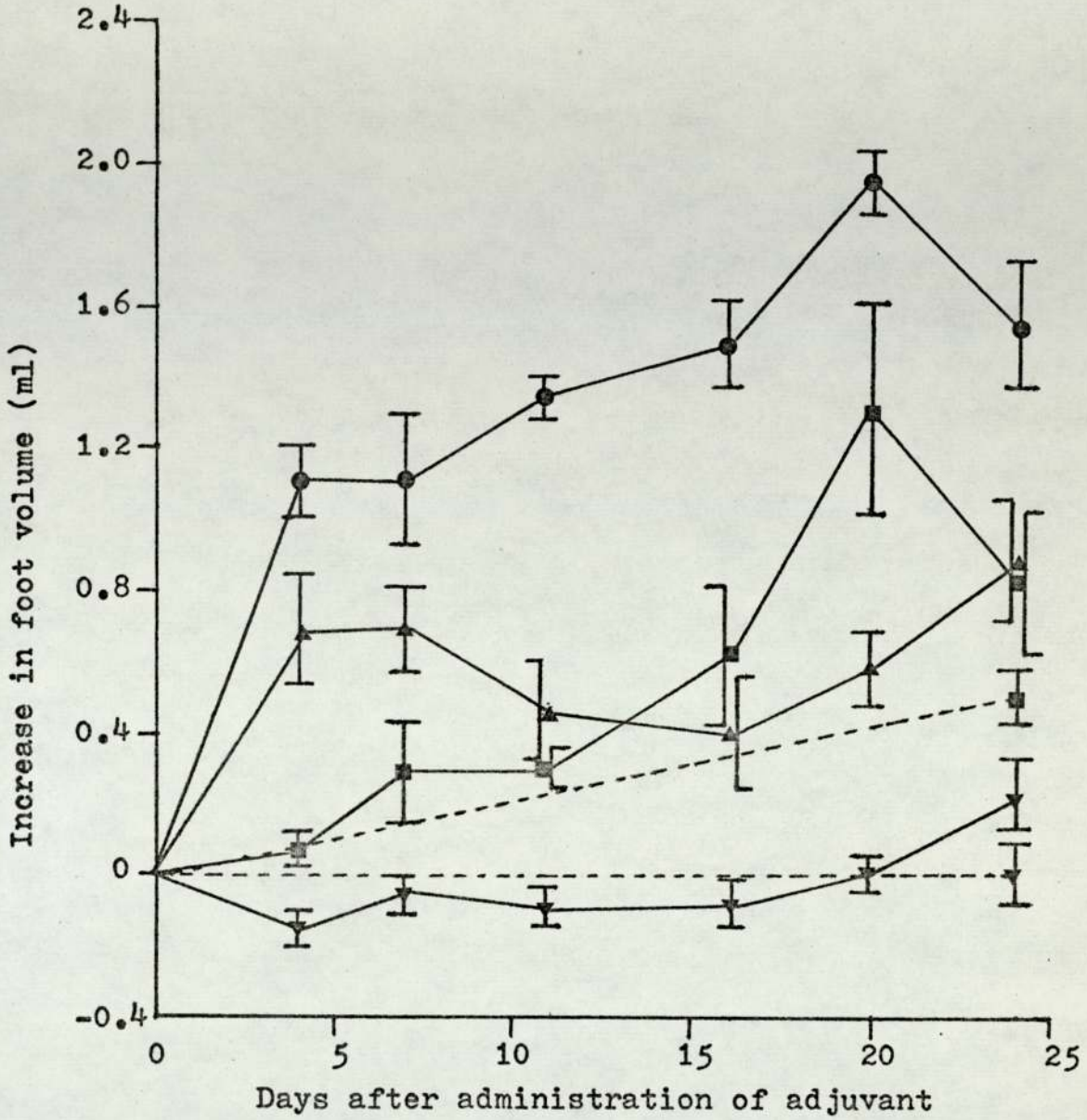


Fig.11. Increase in foot volume of left ●——● and right ■——■ hind feet of rats after injection of mycobacterial adjuvant into the left hind feet. Increase in foot volume of left ▲——▲ and right ▼——▼ hind feet of rats treated daily with cortisol-acetate (5mg/kg) after injection of mycobacterial adjuvant into the left hind feet. Dotted lines represent non-arthritic control rats. Each result represents the mean of five animals and vertical lines indicate S.E.M.

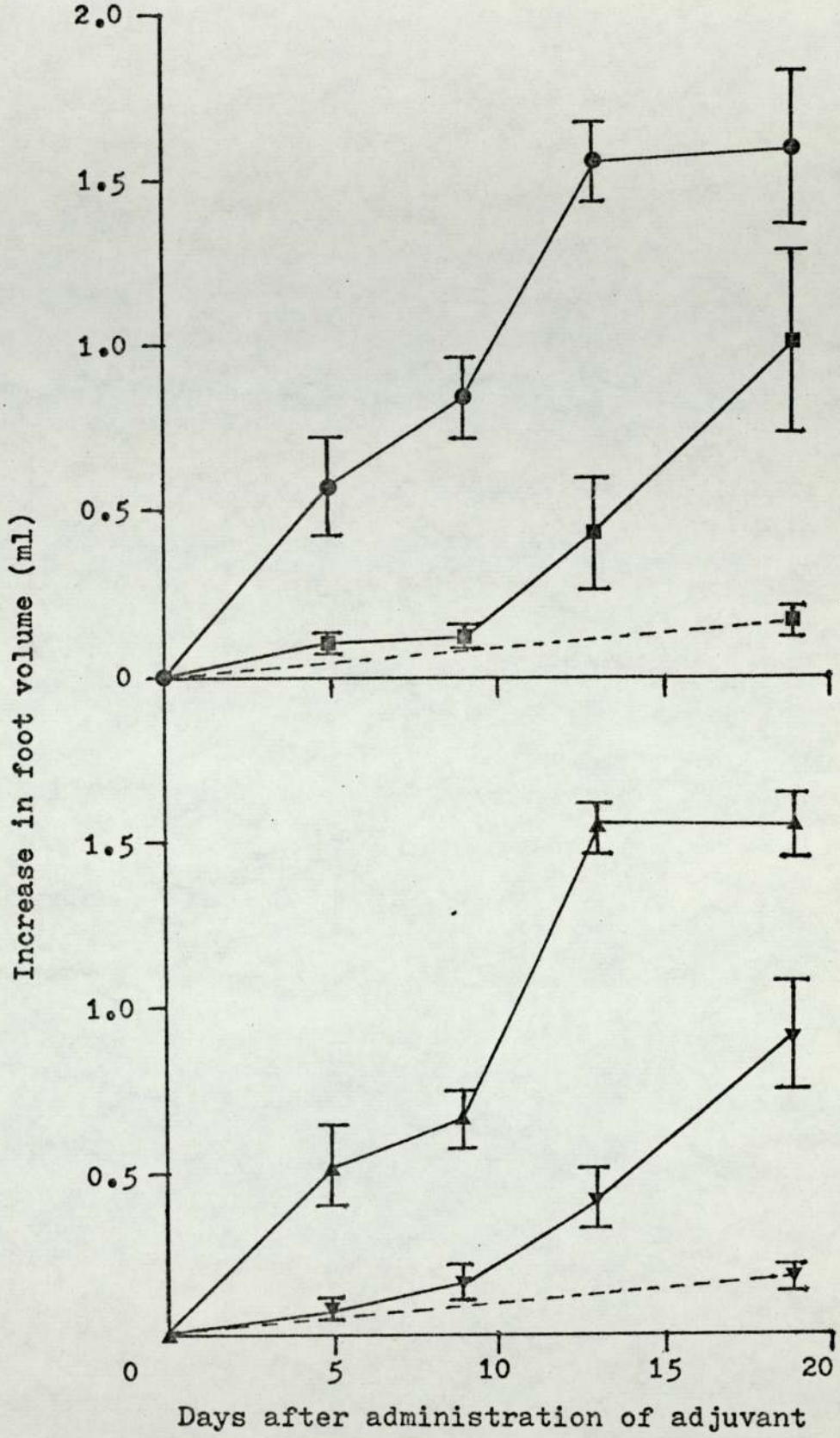


Fig.12. Increase in size of left ●——● and right ■——■ hind feet of female rats, and left ▲——▲ and right ▼——▼ hind feet of male rats, after injection of mycobacterial adjuvant into the left hind feet. Dotted lines represent non-arthritic control rats. Each result represents the mean of five animals and vertical lines indicate S.E.M.

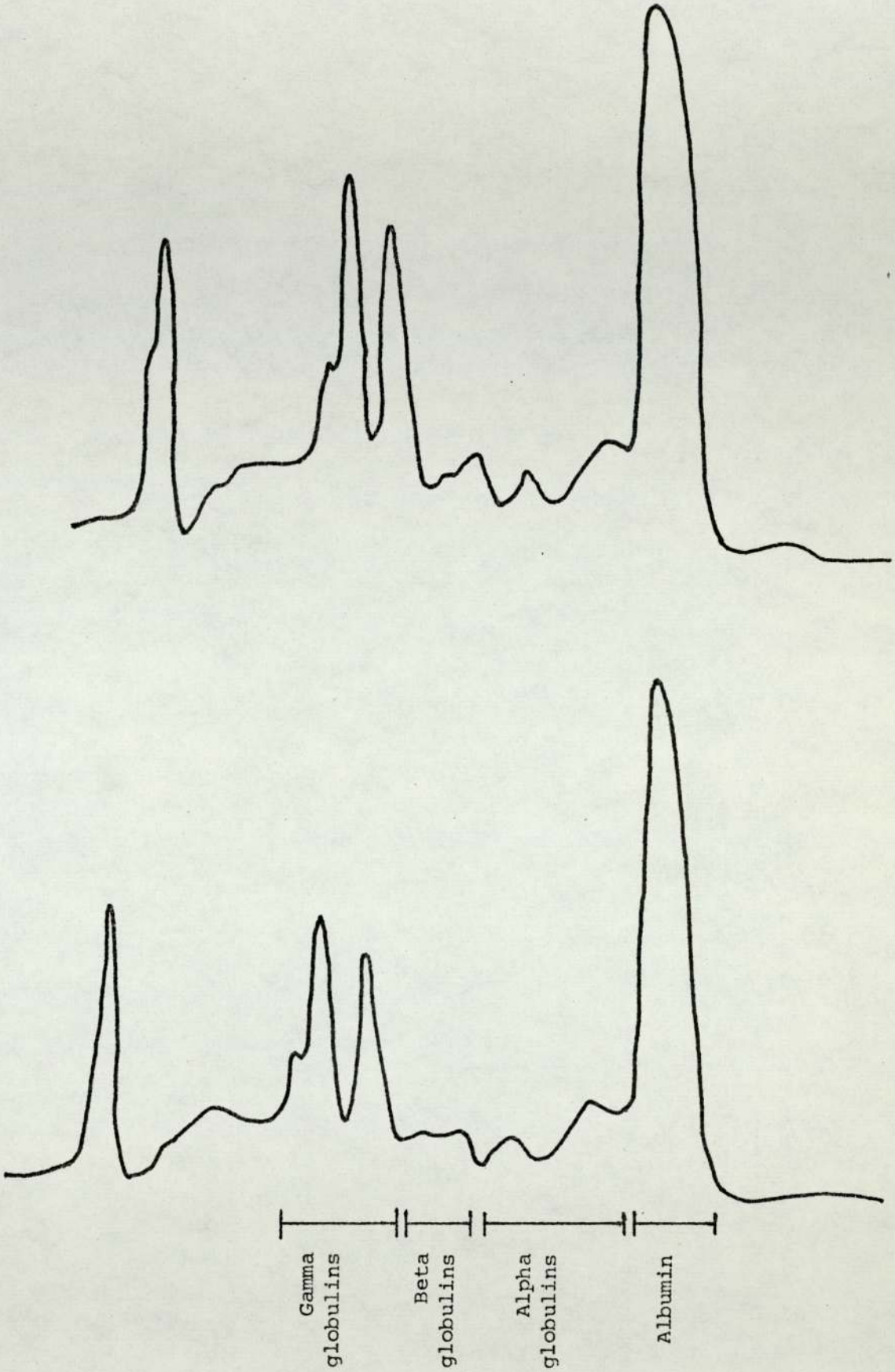


Fig.13. Microdensitometer scans of rat serum proteins after separation by polyacrylamide gel electrophoresis. Sera from control animals.

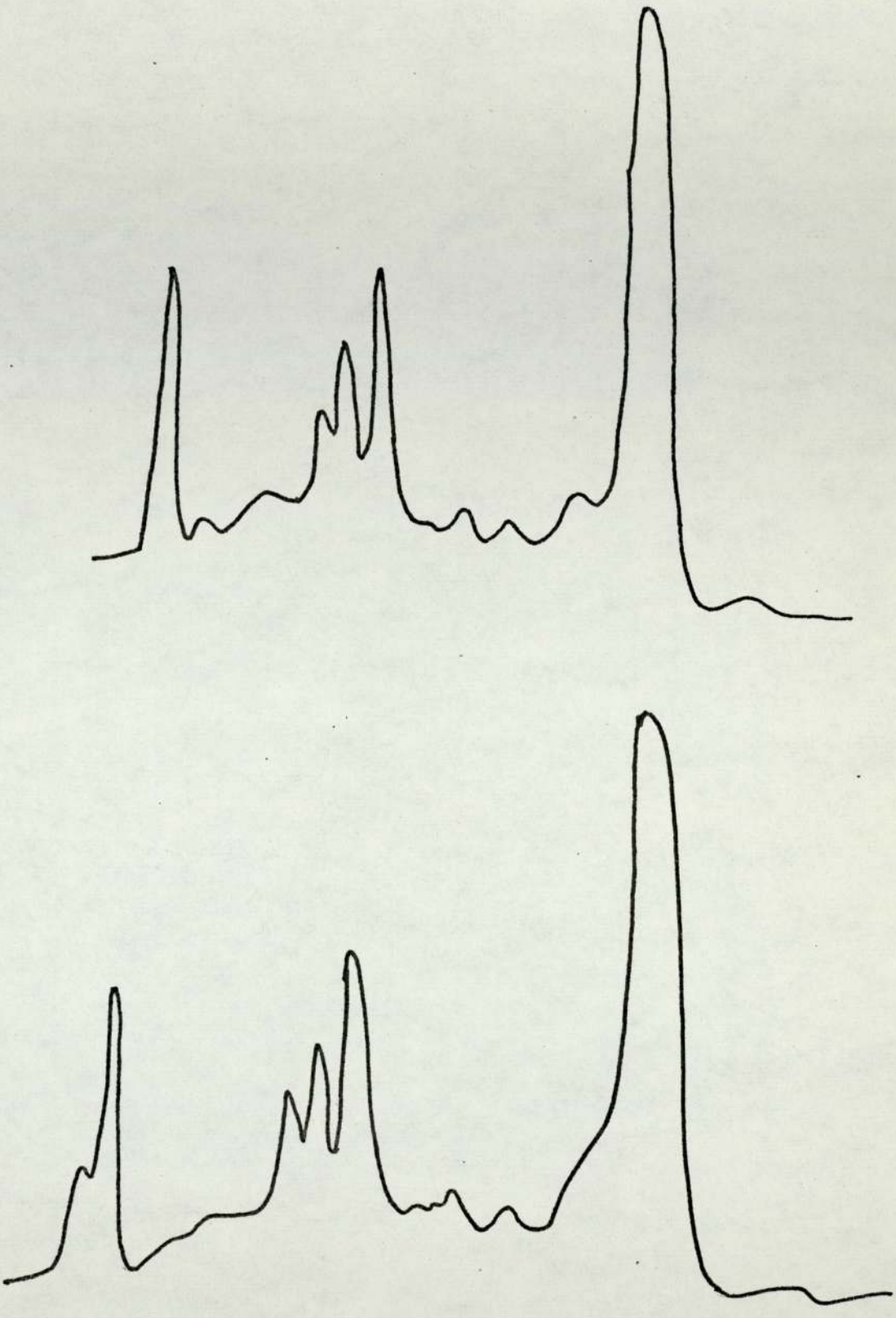


Fig.14. Microdensitometer scans of rat serum proteins after separation by polyacrylamide gel electrophoresis. Sera from non-arthritic rats treated daily with cortisol-acetate (5mg/kg) for twenty four days.

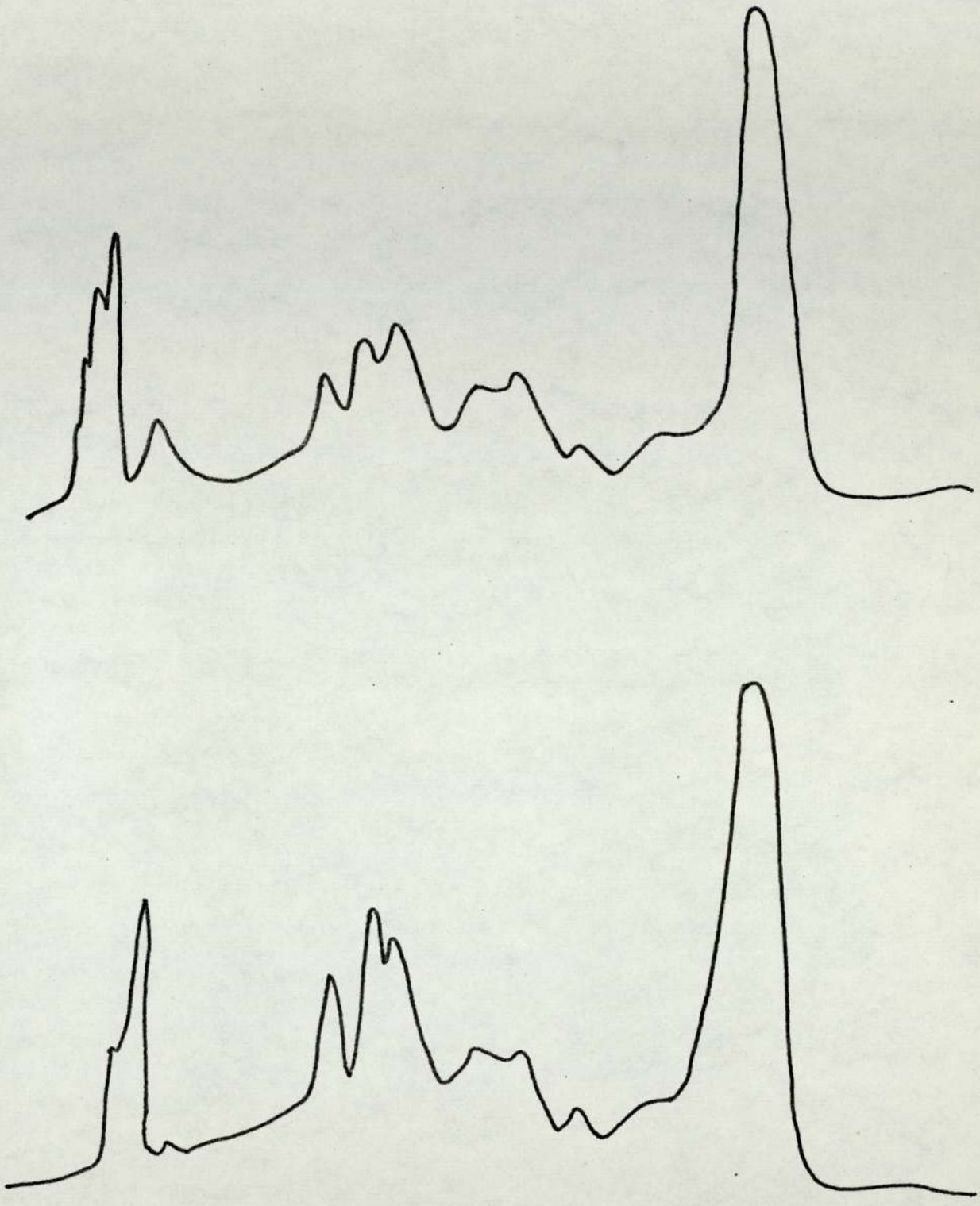


Fig.15. Microdensitometer scans of rat serum proteins after separation by polyacrylamide gel electrophoresis. Sera from rats injected with mycobacterial adjuvant four days earlier.

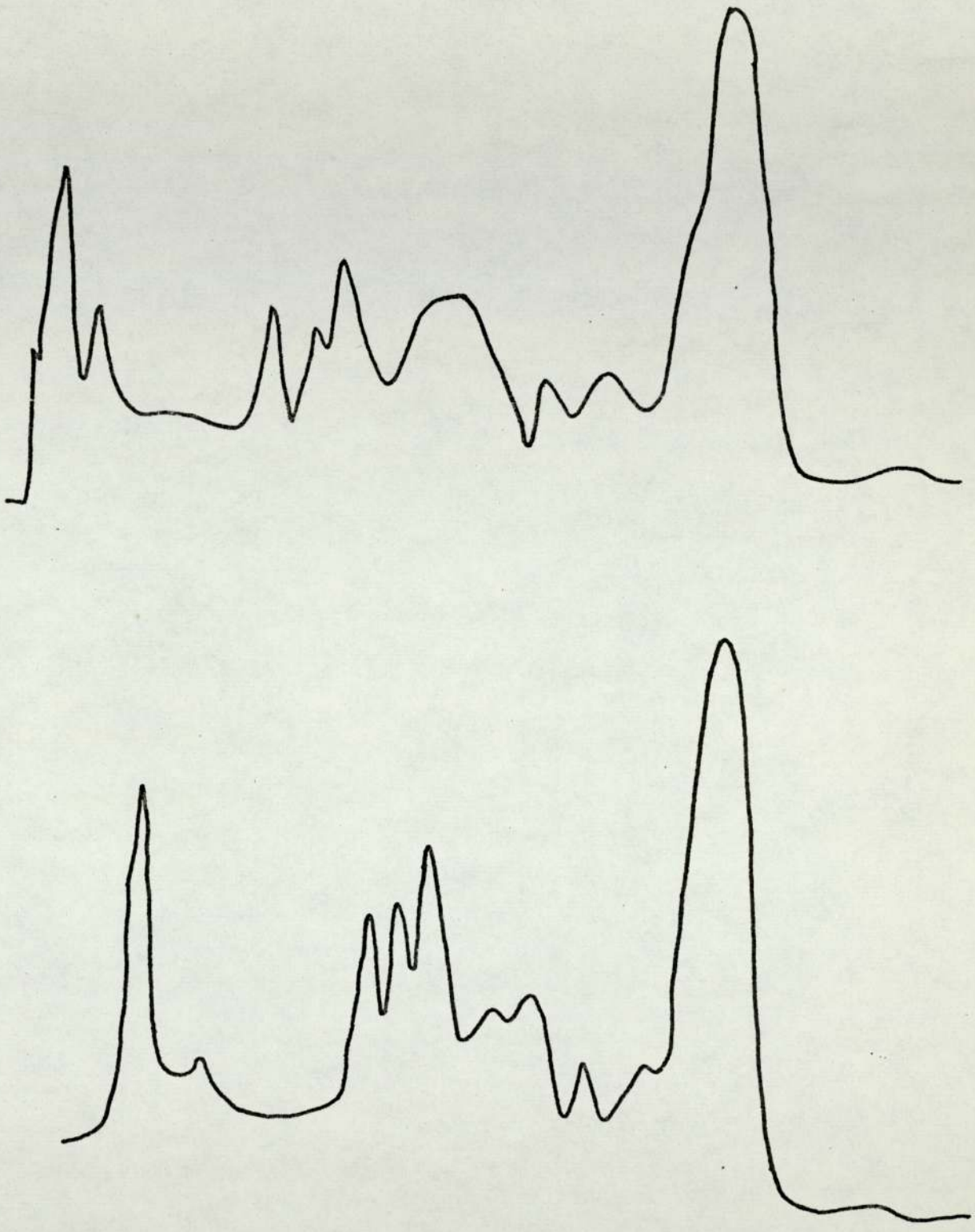


Fig.16. Microdensitometer scans of rat serum proteins after separation by polyacrylamide gel electrophoresis. Sera from rats injected with mycobacterial adjuvant four days earlier and treated daily with cortisol-acetate (5mg/kg).

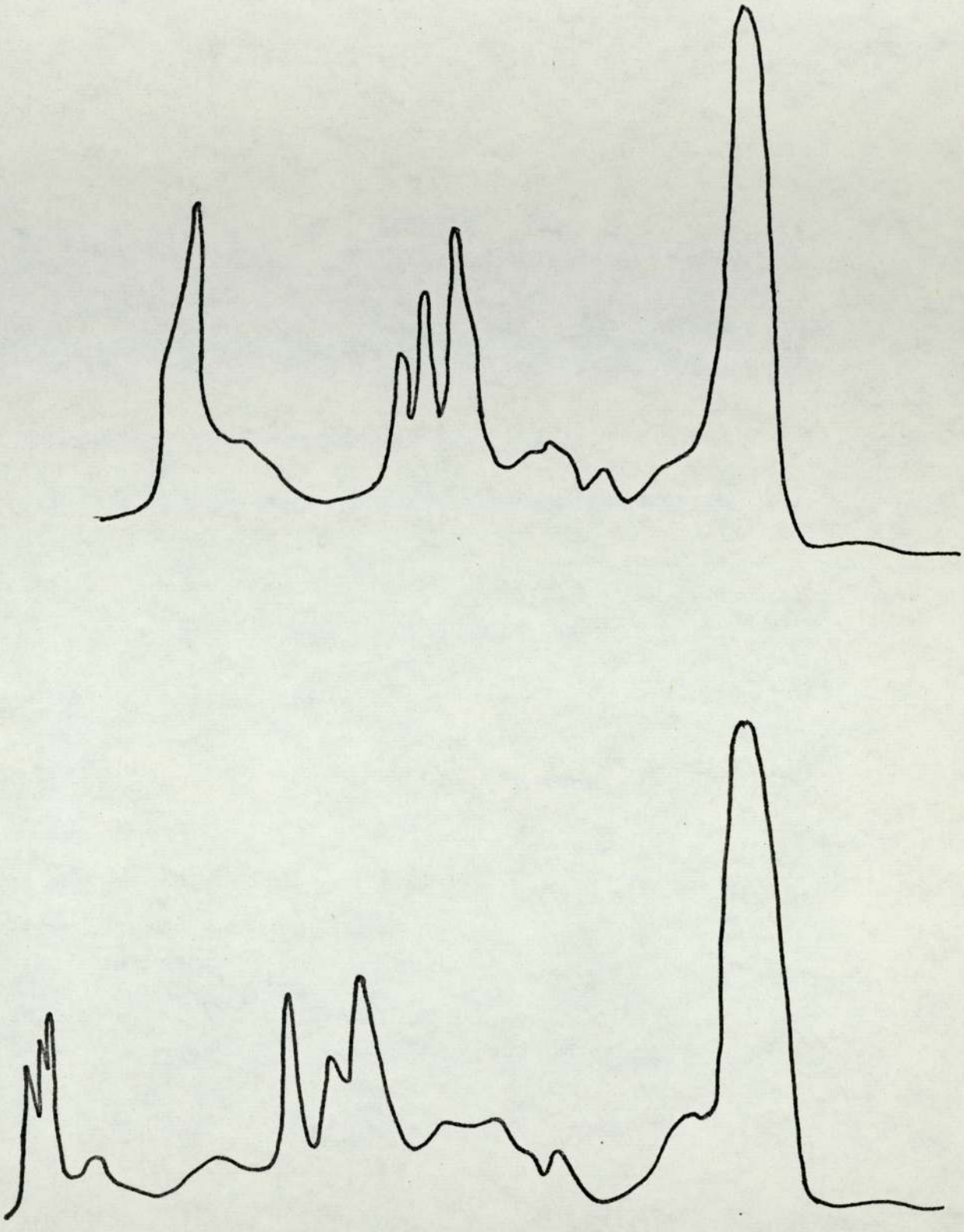


Fig.17. Microdensitometer scans of rat serum proteins after separation by polyacrylamide gel electrophoresis.Sera from rats injected with mycobacterial adjuvant seven days earlier.

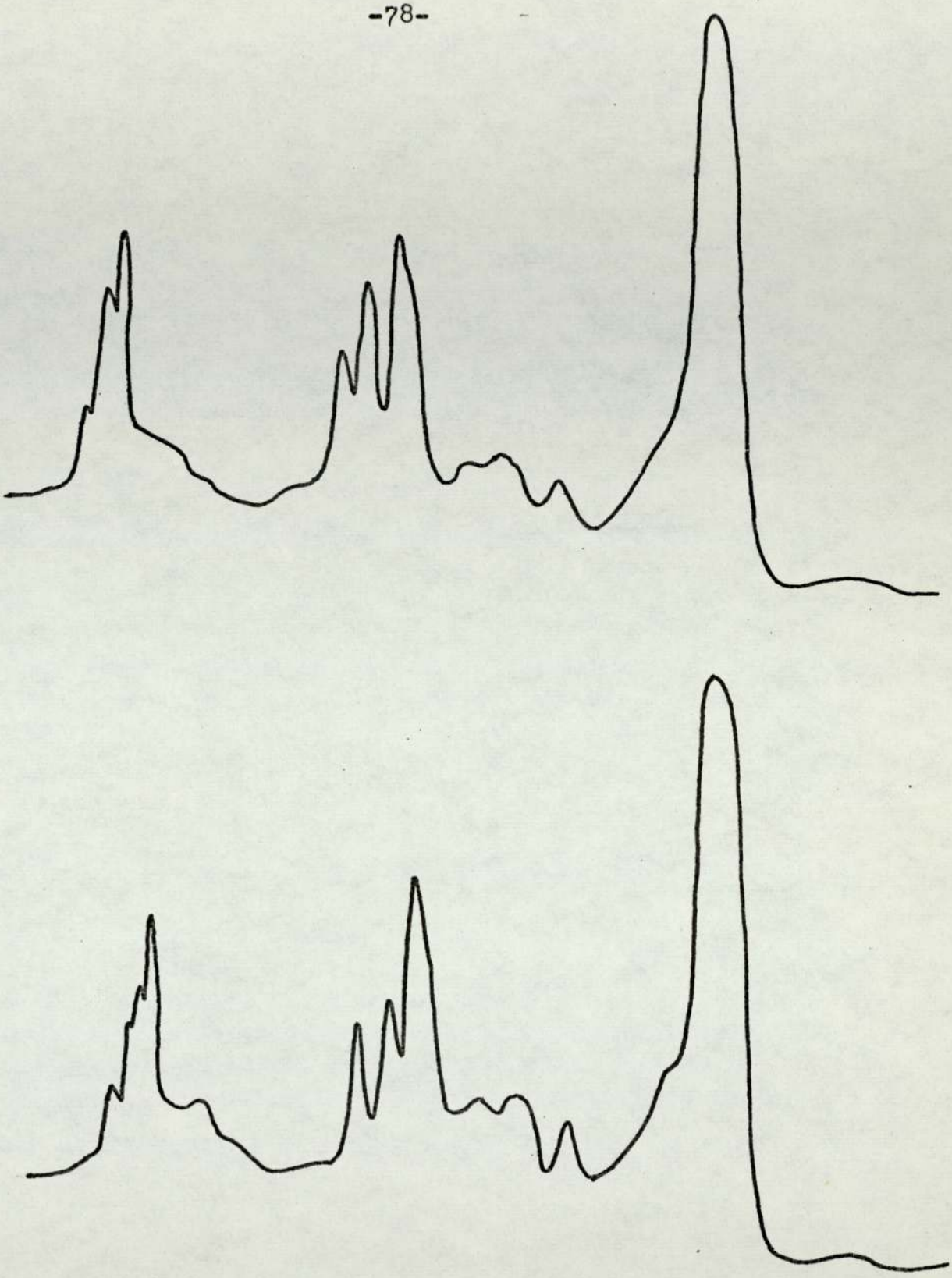


Fig.18. Microdensitometer scans of rat serum proteins after separation by polyacrylamide gel electrophoresis.Sera from rats injected with mycobacterial adjuvant seven days earlier and treated daily with cortisol-acetate(5mg/kg).

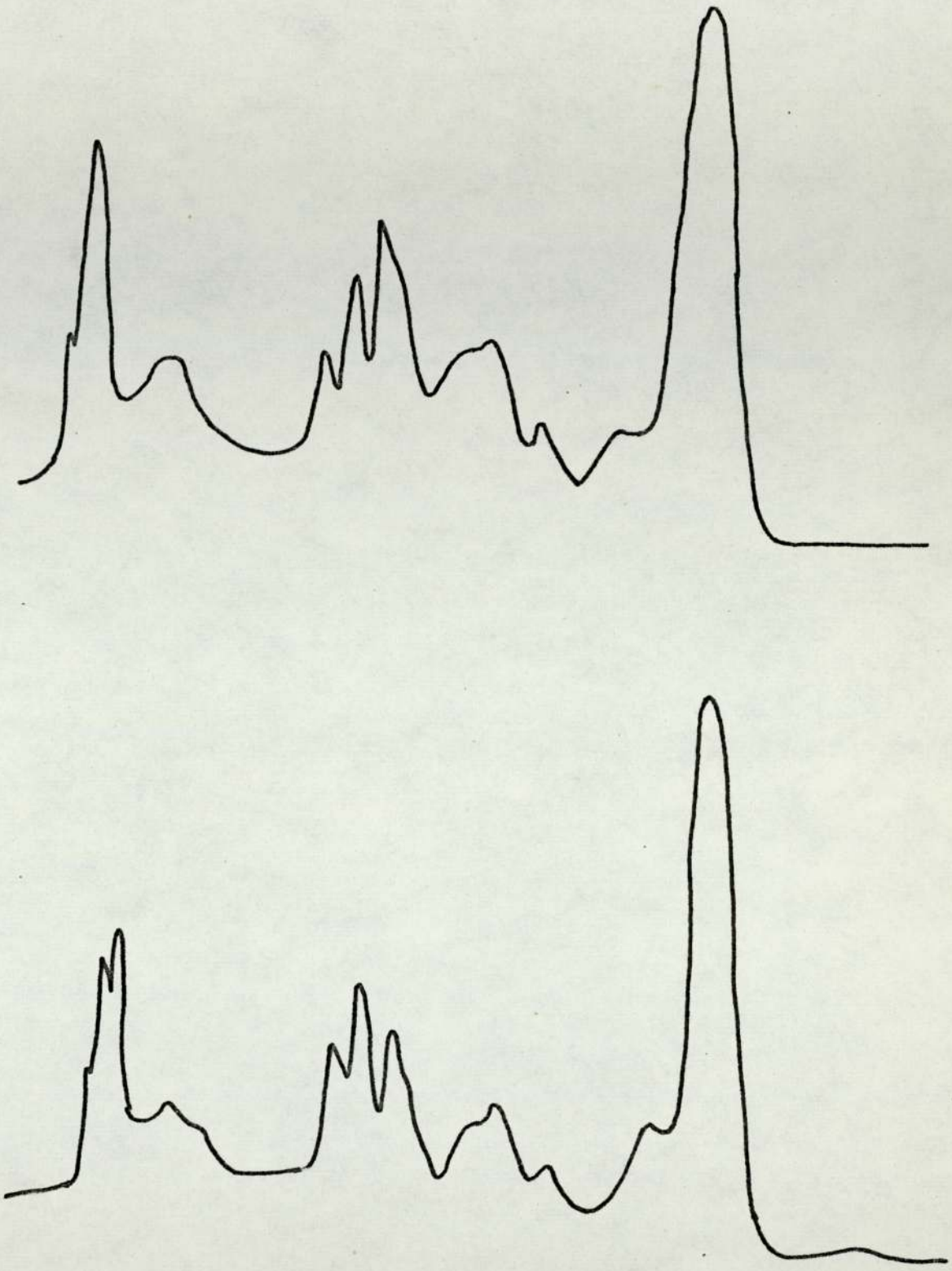


Fig.19. Microdensitometer scans of rat serum proteins after separation by polyacrylamide gel electrophoresis. Sera from rats injected with mycobacterial adjuvant eleven days earlier.

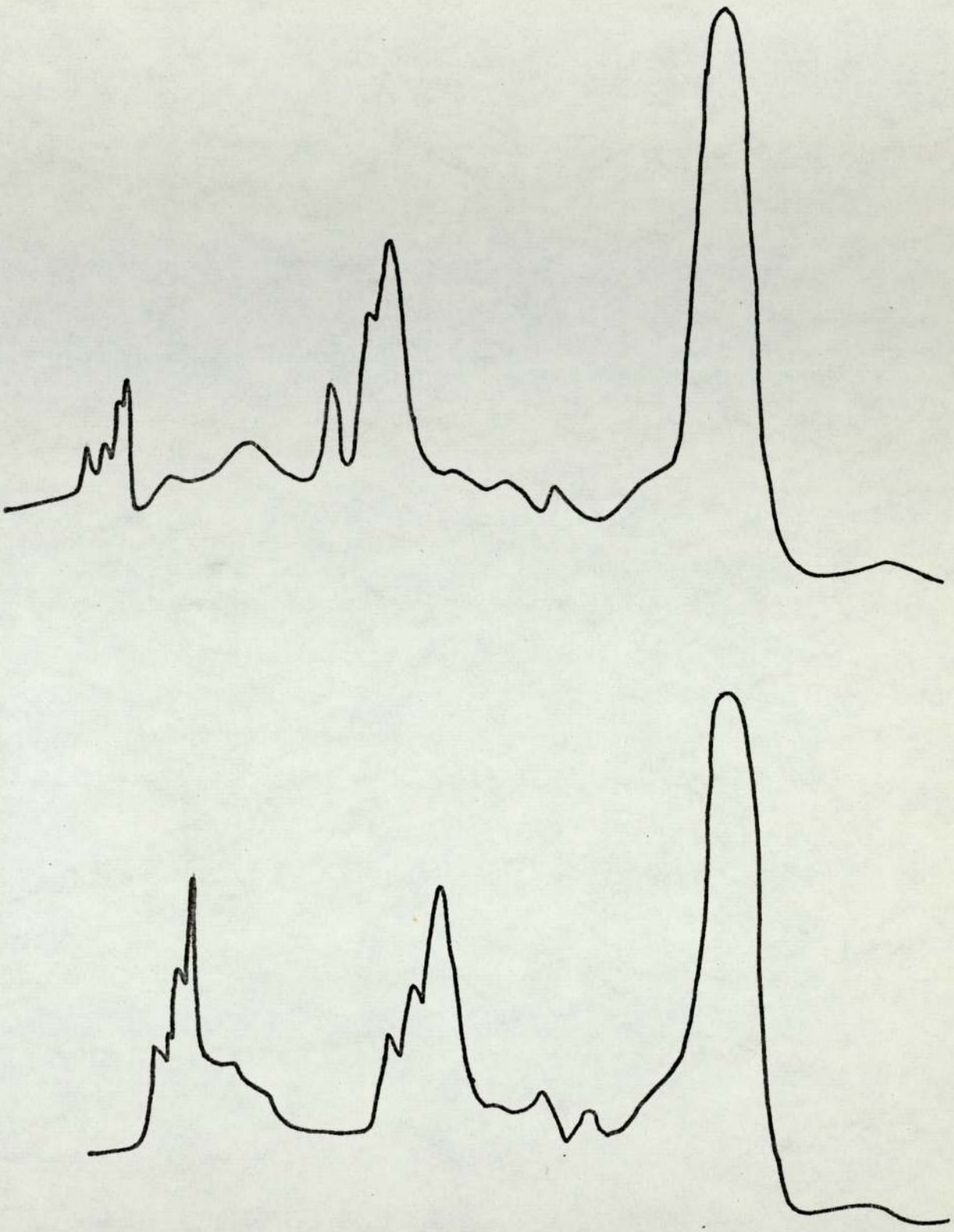


Fig.20. Microdensitometer scans of rat serum proteins after separation by polyacrylamide gel electrophoresis. Sera from rats injected with mycobacterial adjuvant eleven days earlier and treated daily with cortisol-acetate(5mg/kg).

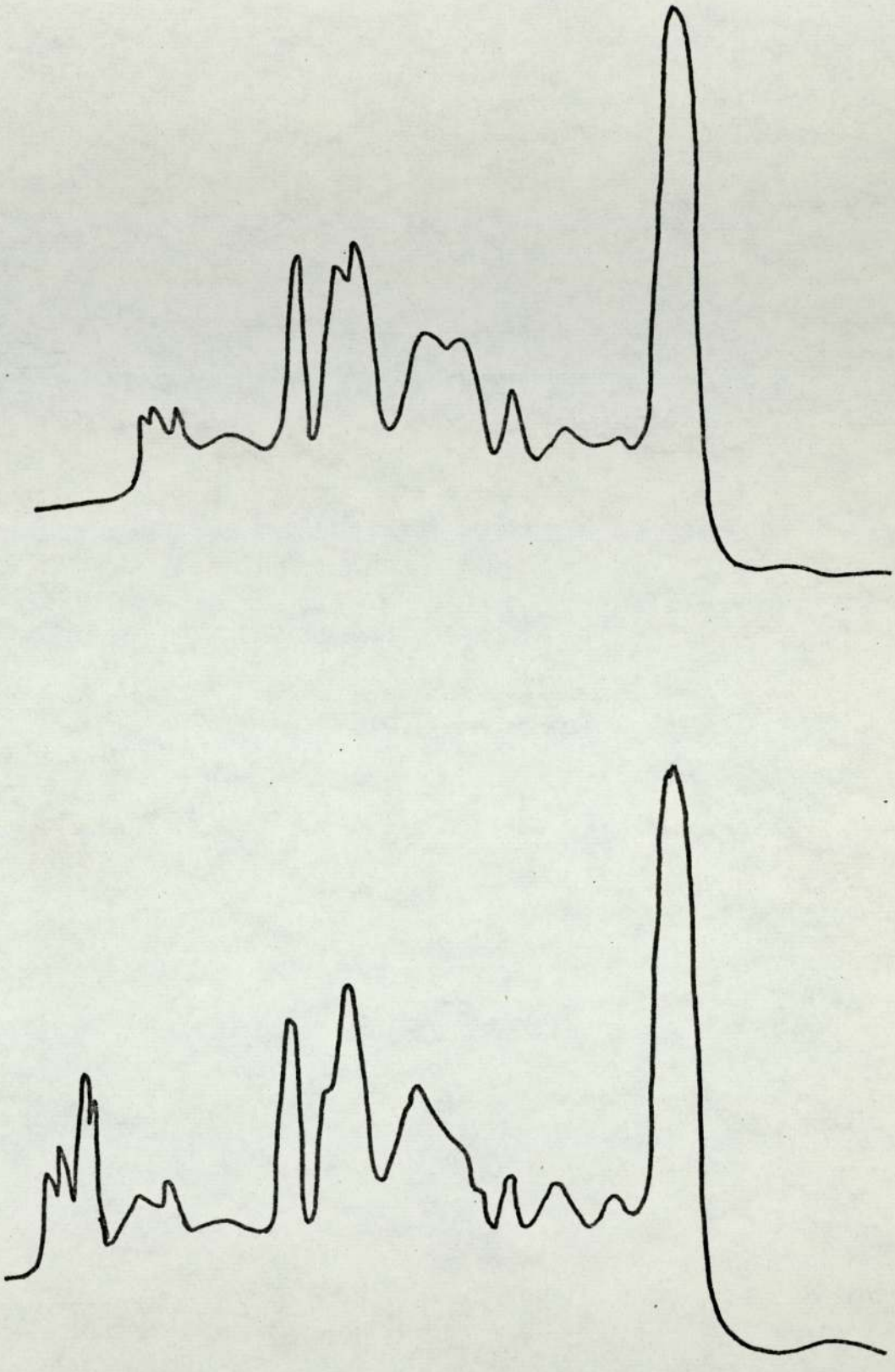


Fig.21. Microdensitometer scans of rat serum protein after separation by polyacrylamide gel electrophoresis. Sera from rats injected with mycobacterial adjuvant sixteen days earlier.

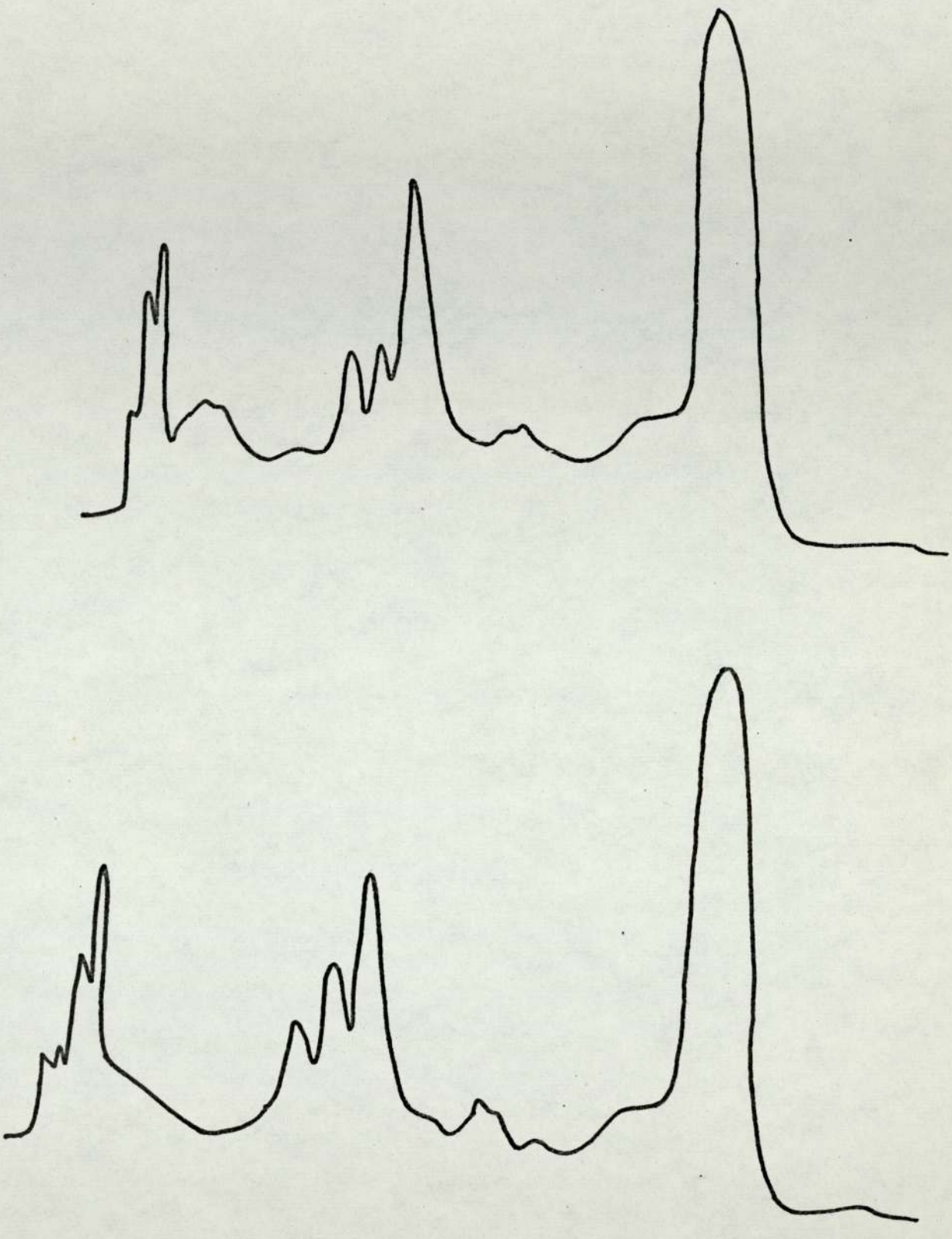


Fig.22. Microdensitometer scans of rat serum protein after separation by polyacrylamide gel electrophoresis. Sera from rats injected with mycobacterial adjuvant sixteen days earlier and treated daily with cortisol-acetate (5mg/kg).

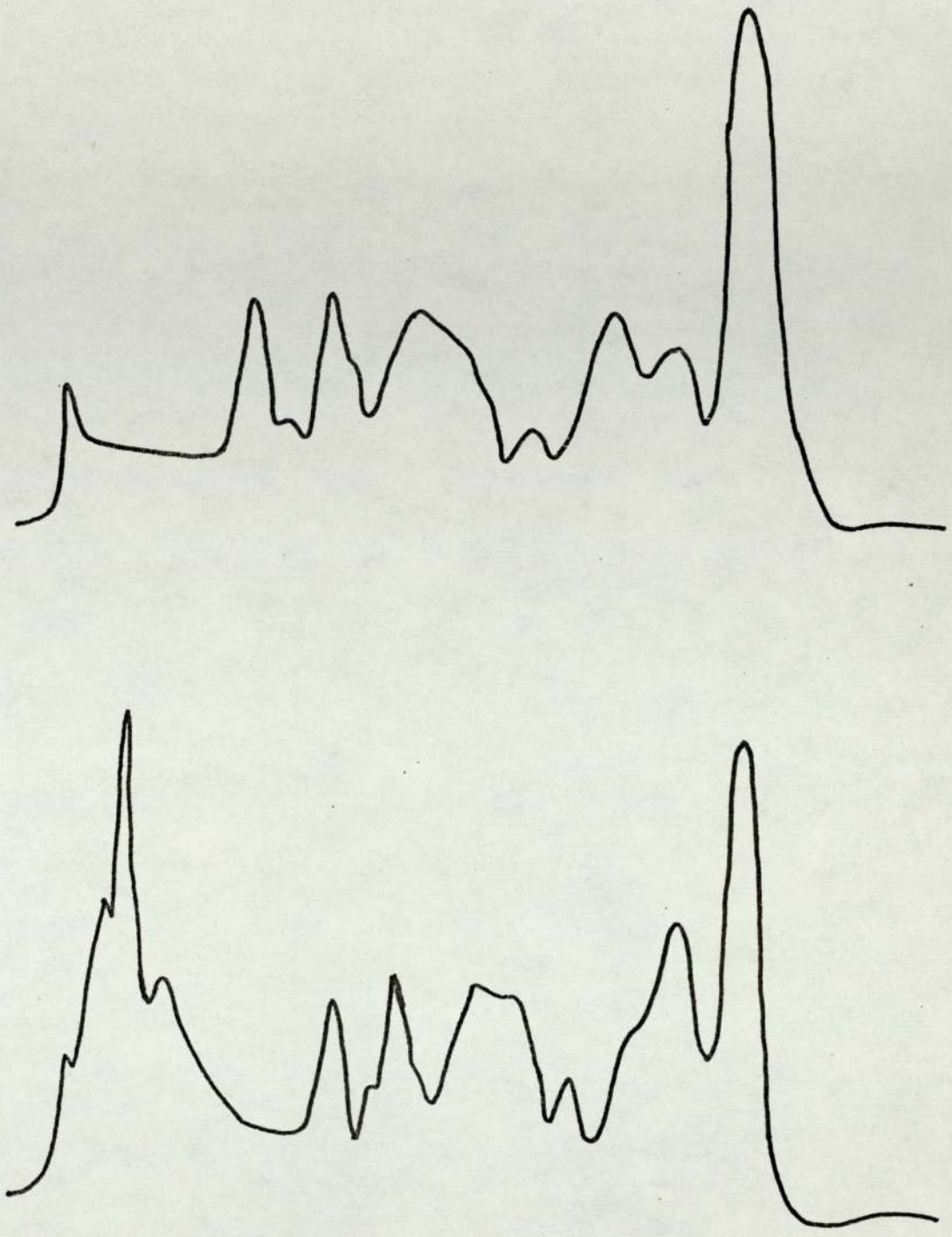


Fig.23. Microdensitometer scans of rat serum proteins after separation by polyacrylamide gel electrophoresis. Sera from rats injected with mycobacterial adjuvant twenty one days earlier.

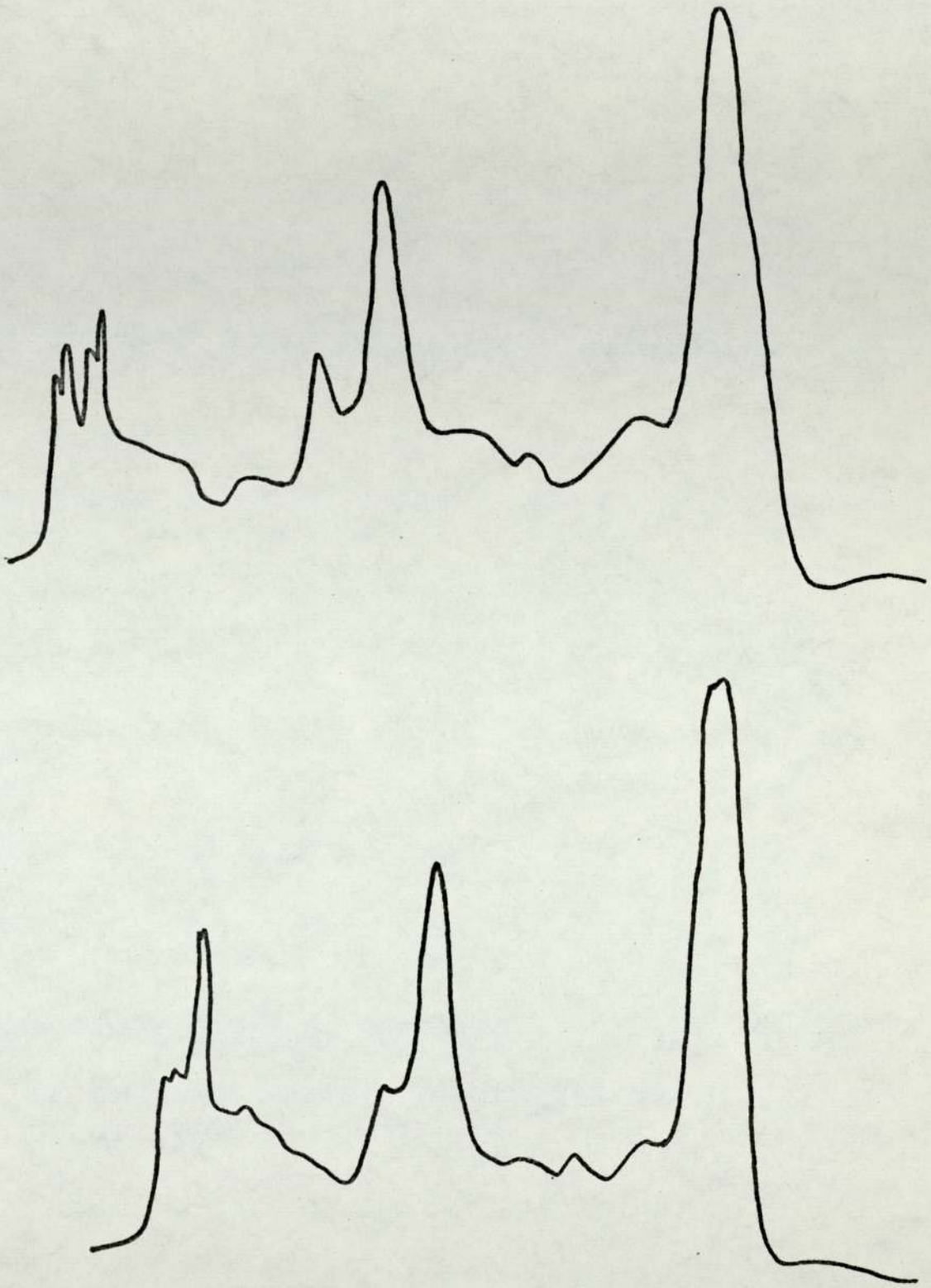


Fig.24. Microdensitometer scans of rat serum proteins after separation by polyacrylamide gel electrophoresis. Sera from rats injected with mycobacterial adjuvant twenty one days earlier and treated daily with cortisol-acetate (5mg/kg).

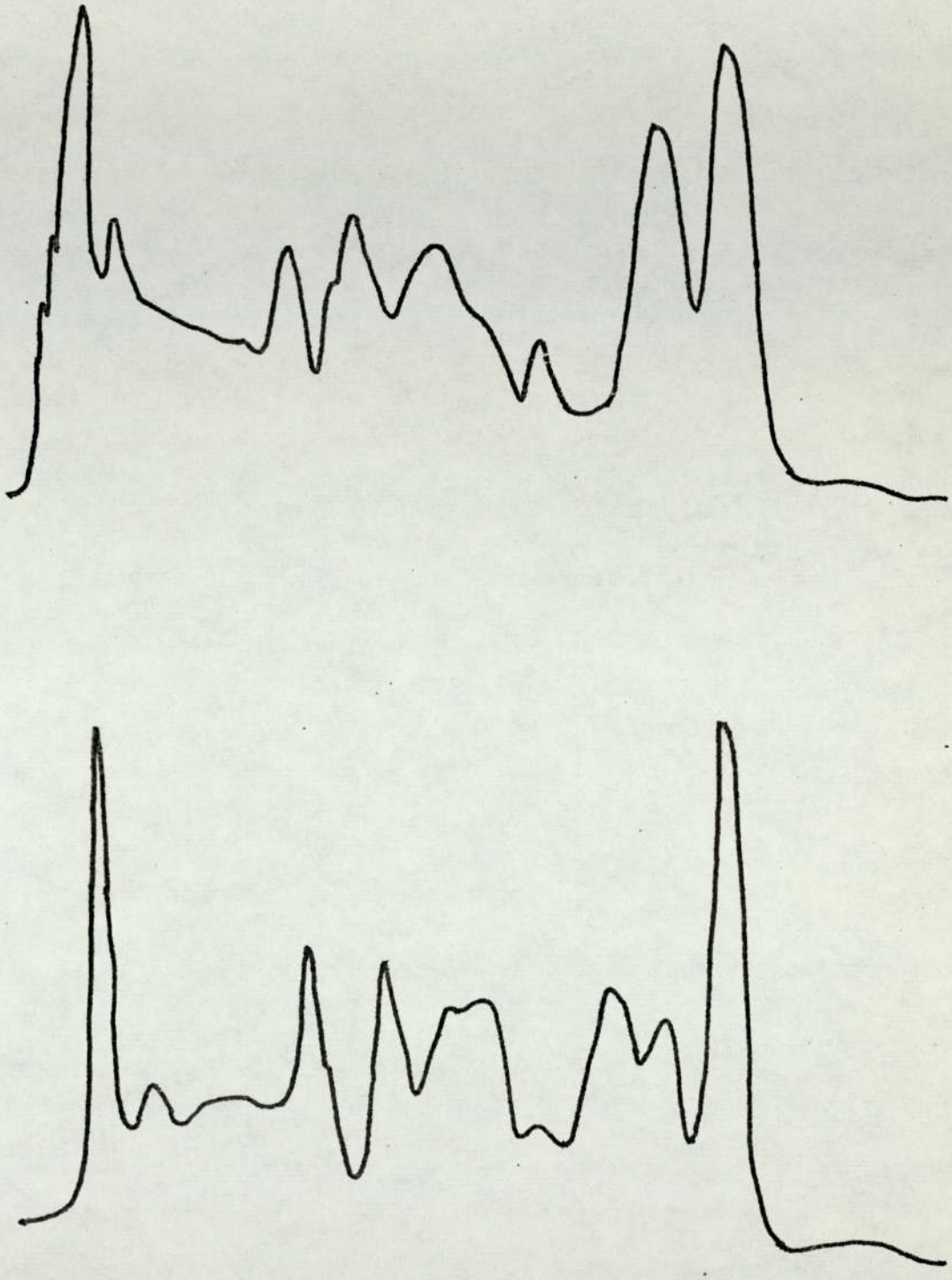


Fig.25. Microdensitometer scans of rat serum proteins after separation by polyacrylamide gel electrophoresis. Sera from rats injected with mycobacterial adjuvant twenty four days earlier.

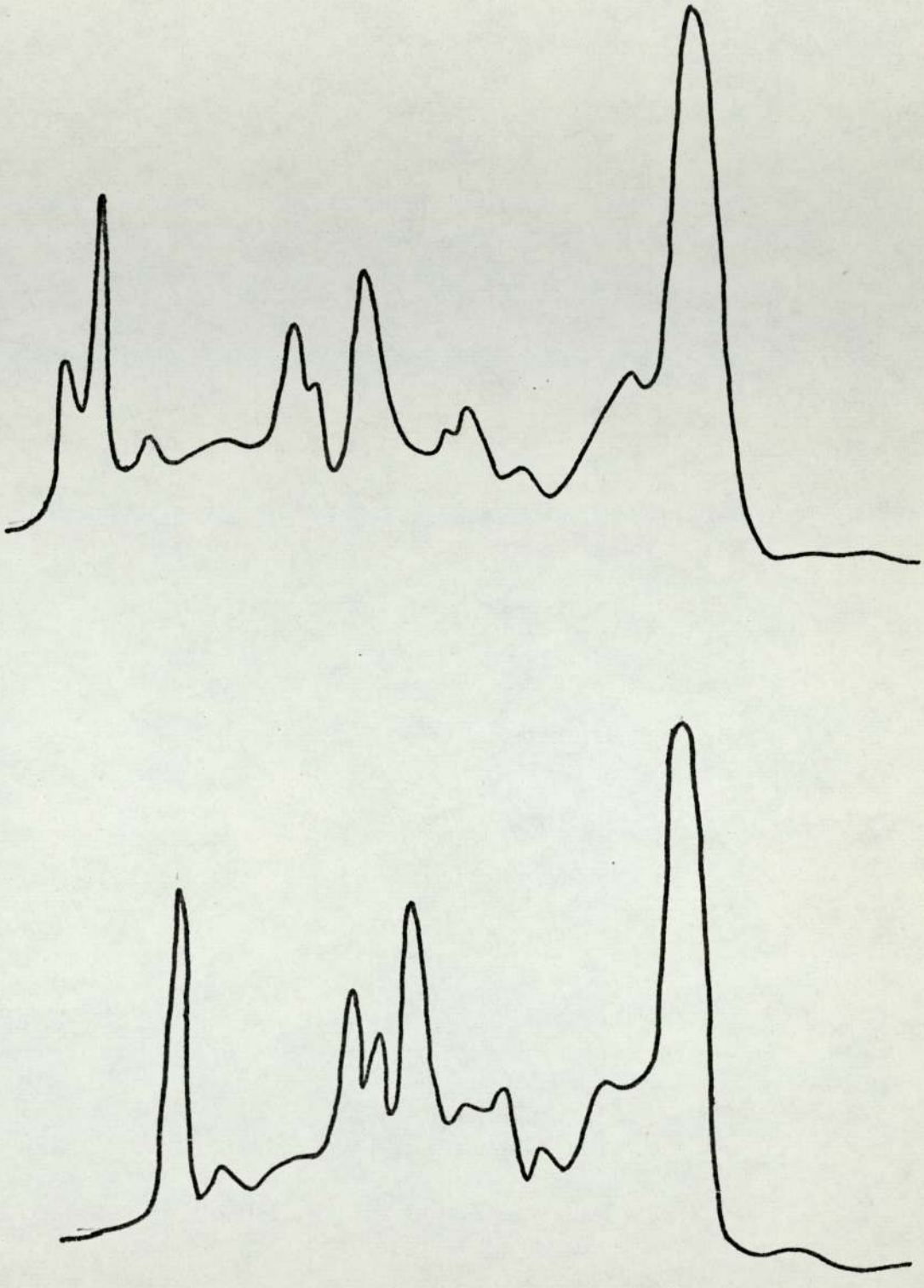


Fig.26. Microdensitometer scans of rat serum proteins after separation by polyacrylamide gel electrophoresis. Sera from rats injected with mycobacterial adjuvant twenty four days earlier and treated daily with cortisol-acetate (5mg/kg).

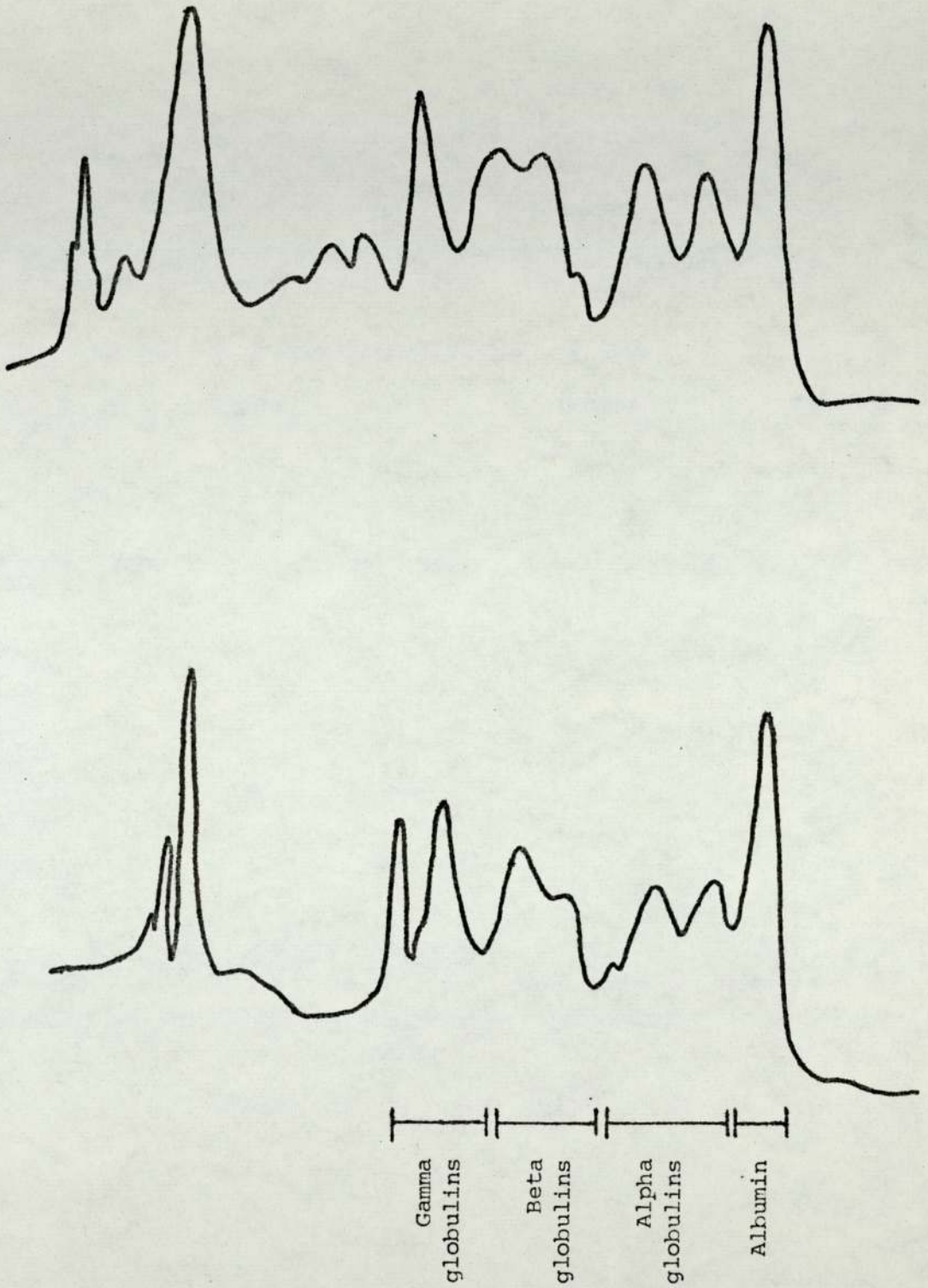


Fig.27. Microdensitometer scans of rat serum proteins after separation by polyacrylamide gel electrophoresis. Sera from rats injected with mycobacterial adjuvant ninety days earlier.

Figs.28. and Figs.29. Photographs of polyacrylamide gels containing rat serum proteins separated by electrophoresis.

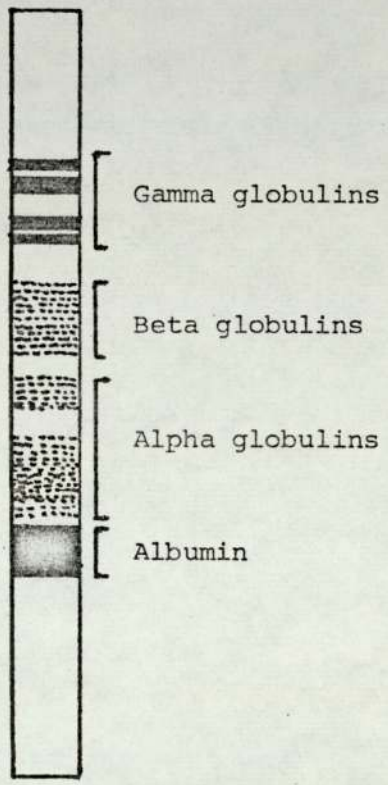
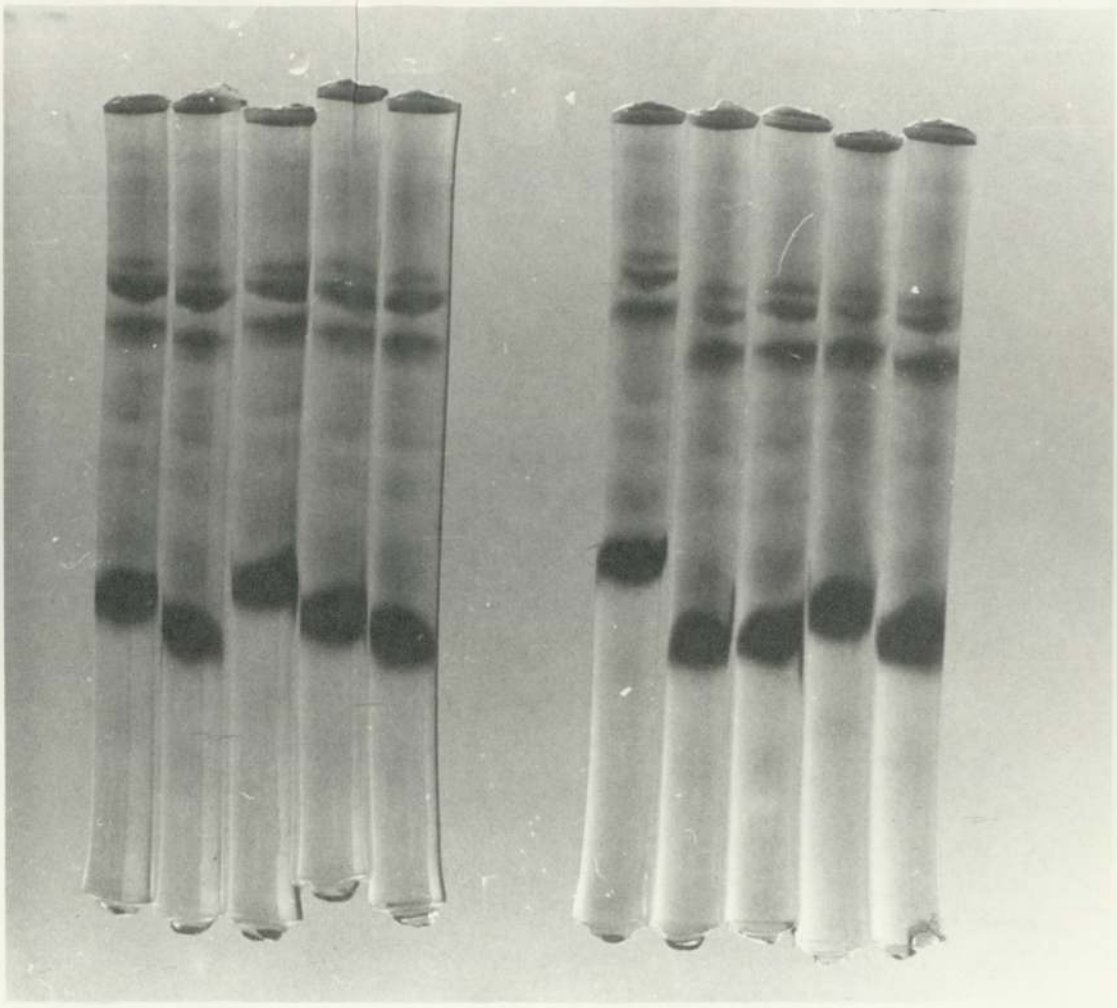


Fig.28(a). Top left: Sera from normal control rats.
Top right: Sera from control rats treated for twenty four days with cortisol-acetate (5mg/kg).

Fig.28(b). Bottom left: Sera from rats injected with mycobacterial adjuvant four days earlier.
Bottom right: Sera from rats injected with mycobacterial adjuvant four days earlier and treated daily with cortisol-acetate (5mg/kg).



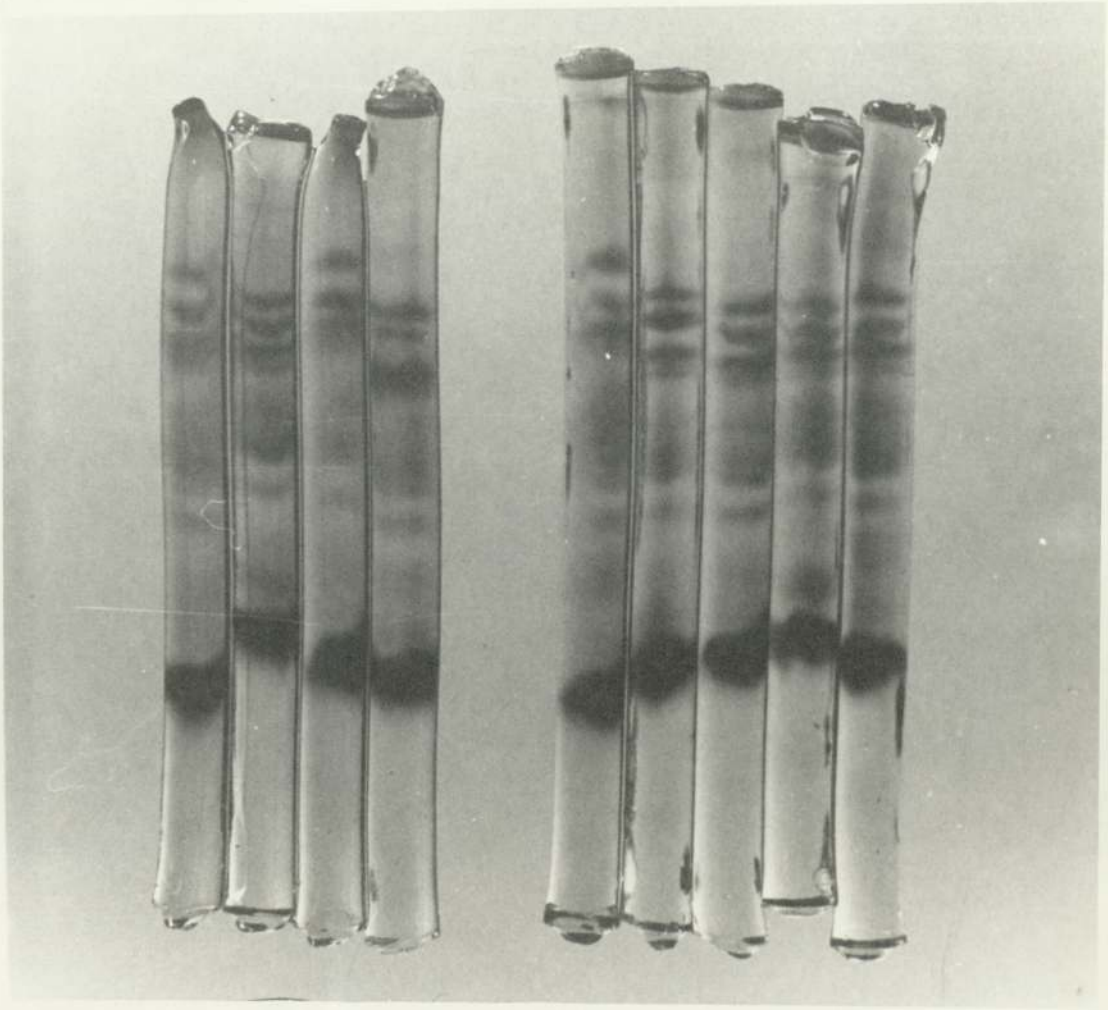
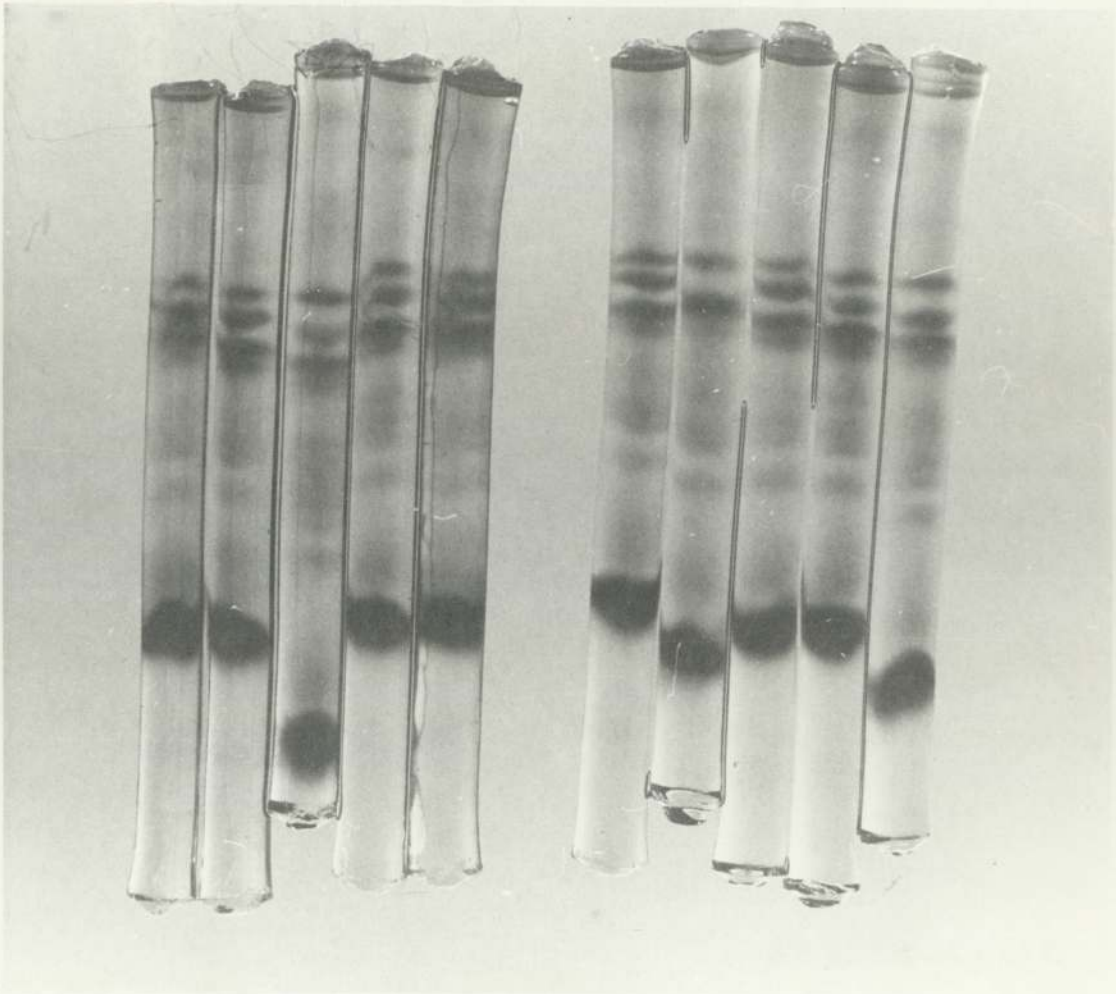


Fig.28(c). Top left: Sera from rats injected with mycobacterial adjuvant seven days earlier.
Top right: Sera from rats injected with mycobacterial adjuvant seven days earlier and treated daily with cortisol-acetate (5mg/kg).

Fig.28(d). Bottom left: Sera from rats injected with mycobacterial adjuvant eleven days earlier.
Bottom right: Sera from rats injected with mycobacterial adjuvant eleven days earlier and treated daily with cortisol-acetate (5mg/kg).



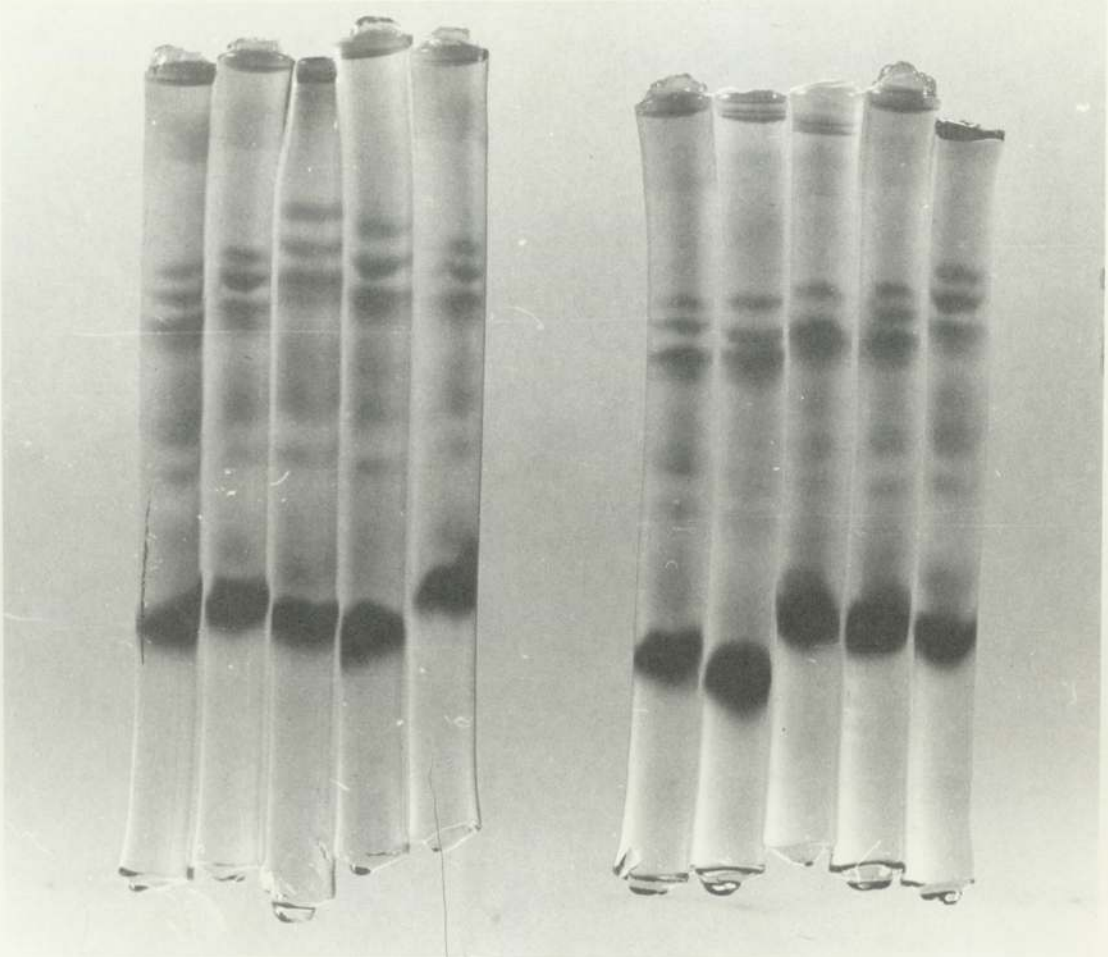
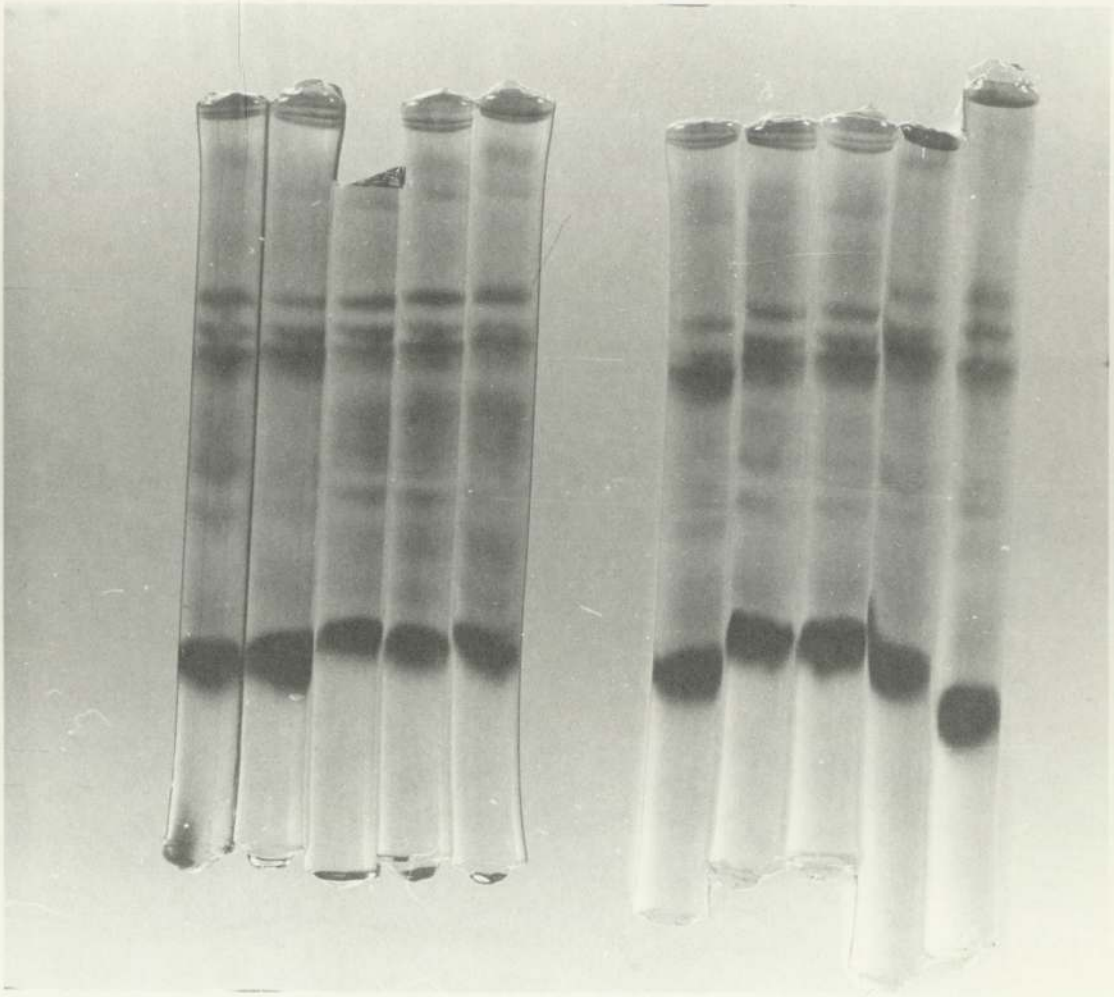


Fig.29(a). Top left: Sera from rats injected with mycobacterial adjuvant sixteen days earlier.
Top right: Sera from rats injected with mycobacterial adjuvant sixteen days earlier and treated daily with cortisol-acetate (5mg/kg).

Fig.29(b). Bottom left: Sera from rats injected with mycobacterial adjuvant twenty one days earlier.
Bottom right: Sera from rats injected with mycobacterial adjuvant twenty one days earlier and treated daily with cortisol-acetate (5mg/kg).



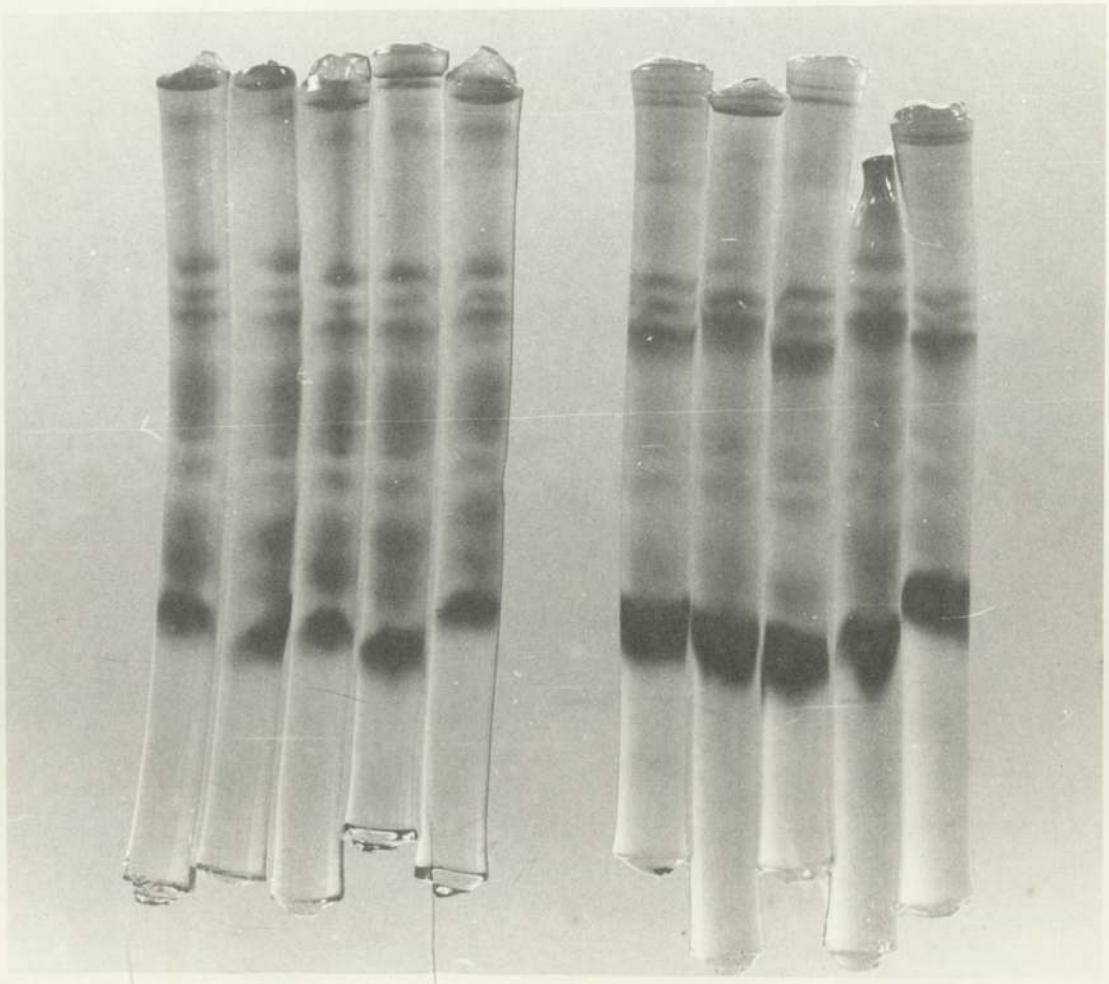
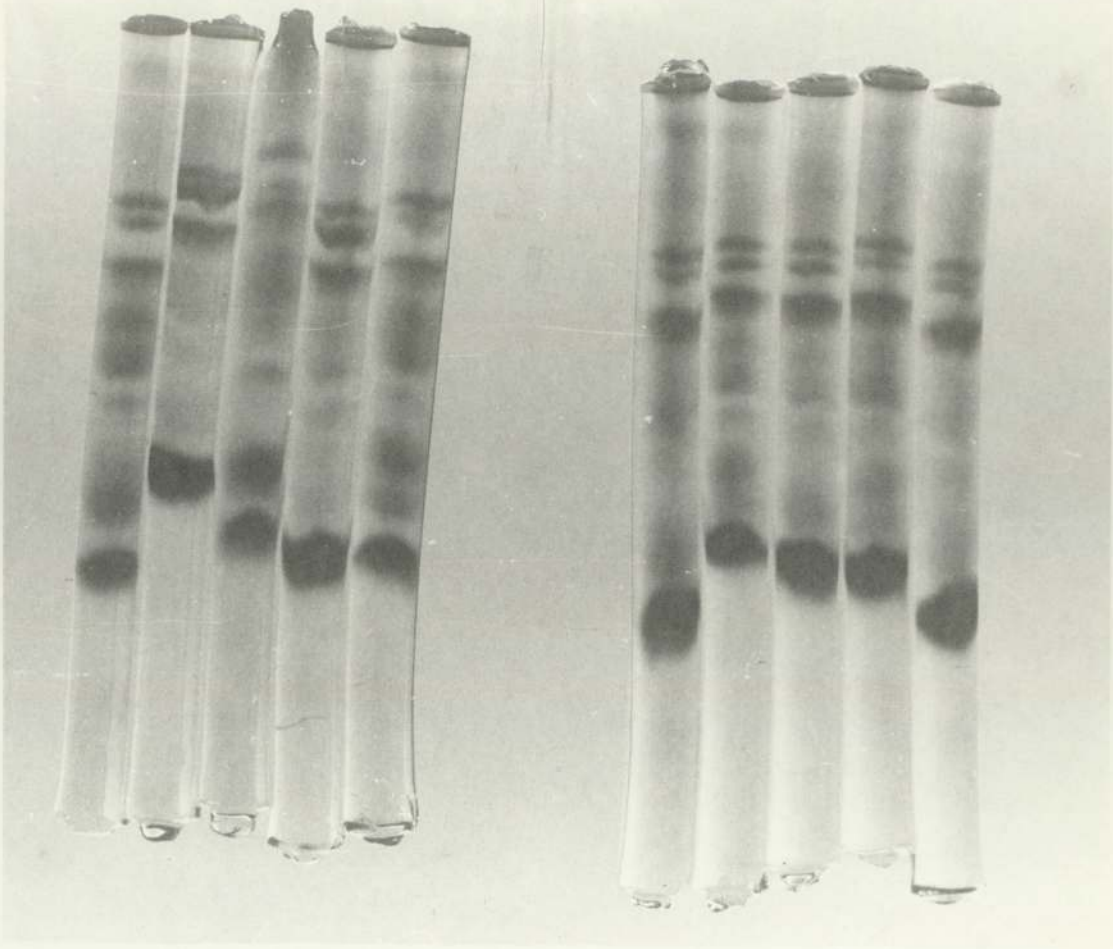
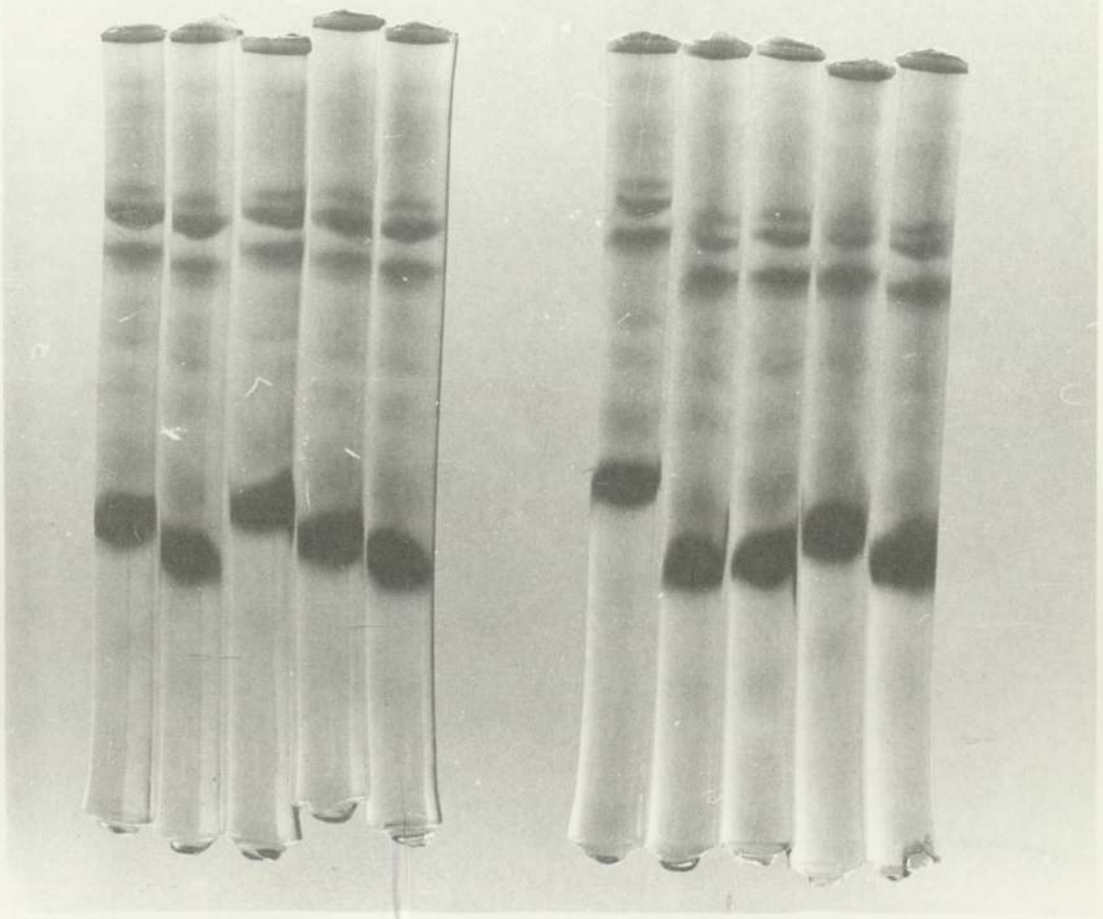


Fig.29(c). Top left: Sera from rats injected with mycobacterial adjuvant twenty four days earlier.
Top right: Sera from rats injected with mycobacterial adjuvant twenty four days earlier and treated daily with cortisol-acetate (5mg/kg).

Fig.29(d). Bottom left: Sera from normal control rats.
Bottom right: Sera from control rats treated for twenty four days with cortisol-acetate (5mg/kg).





that there was an increase in beta-globulin levels in these rats (Fig. 15). This was also the case for four day adjuvant arthritic rats treated with cortisol-acetate (Fig. 16). Apart from increased levels of beta-globulins there appeared to be no further changes in the serum protein patterns of rats on days seven and eleven after adjuvant (Fig. 17 and Fig. 19). On day seven the beta-globulin levels of the cortisol-acetate treated arthritic rats were slightly elevated (Fig. 18), but on day eleven the serum protein patterns were back to normal (Fig. 20). The serum of arthritic rats on day sixteen had increased beta-globulin levels and decreased albumin levels (Fig. 21), but the serum from cortisol-acetate treated arthritic rats still appeared normal after sixteen days (Fig. 22). On days twenty one and twenty four the arthritic rat serum had increased levels of beta-globulins and alpha-globulins, with decreased levels of albumin (Fig. 23 and Fig. 25). The serum of cortisol-acetate treated arthritic rats appeared normal after twenty one days (Fig. 24), but there were small increases in beta-globulins and alpha-globulins along with a decrease in albumin after twenty four days (Fig. 26). After ninety days the albumin levels in the serum of arthritic rats were greatly reduced and there were large compensating increases in beta-globulin and alpha-globulin levels (Fig. 27).

The changes in albumin, alpha-globulin and beta-globulin levels during the development of adjuvant arthritis, and the effect of cortisol treatment, can be seen in the photographs of the individual polyacrylamide gels (Fig. 28 and Fig. 29).

3.2.3. Acid Phosphatase and Protease Levels of Adjuvant Arthritic Rat Serum.

The acid phosphatase and protease levels in adjuvant arthritic rat serum are shown in Fig. 30. There was a large increase in the activities

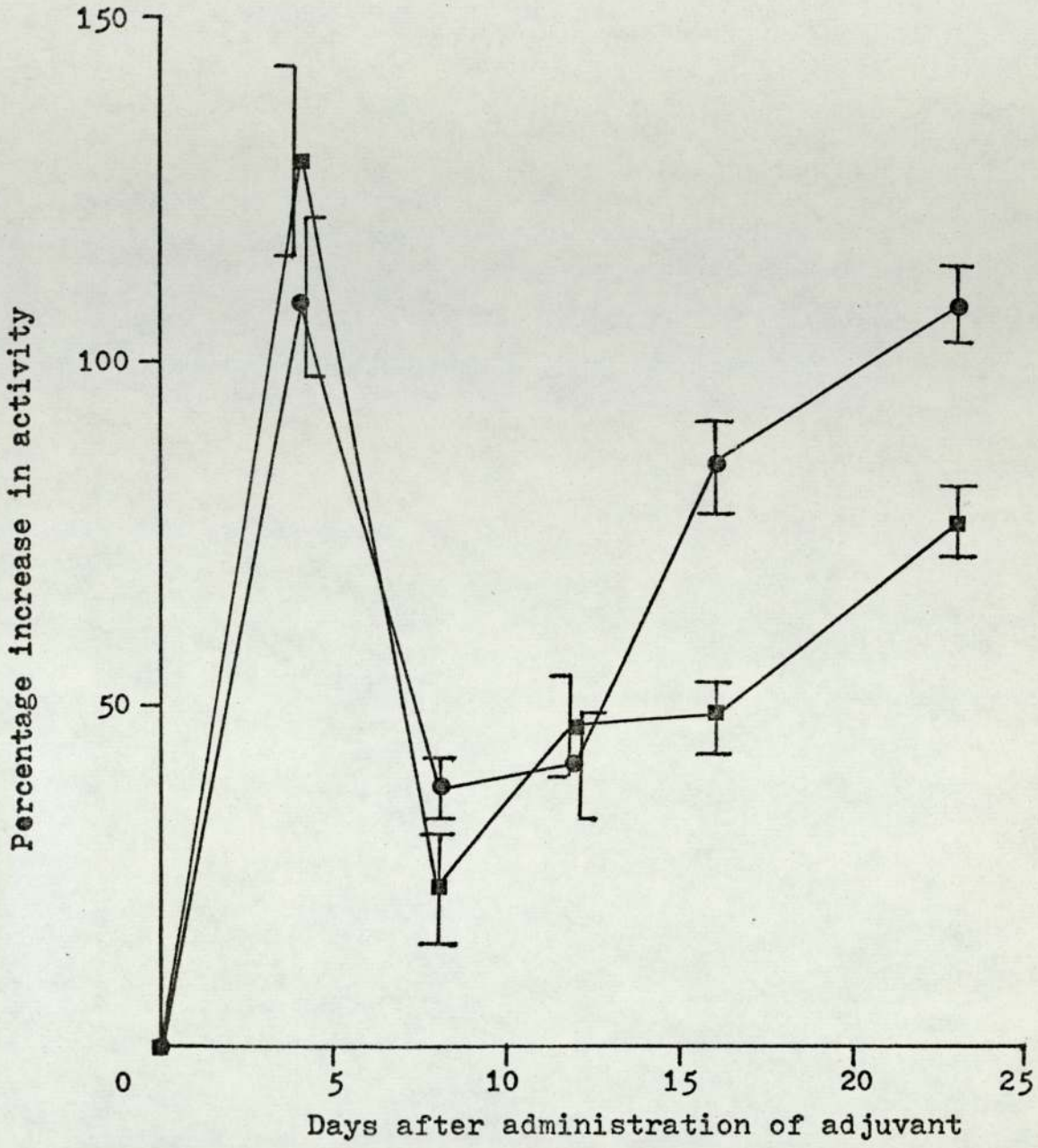


Fig.30. Increase in acid phosphatase ●——● and protease ■——■ levels of serum of rats after injection of mycobacterial adjuvant. Each result represents the mean of five animals and vertical lines indicate S.E.M.

of both during the acute inflammation with the activities almost returning to normal eight days after the administration of adjuvant. Both acid phosphatase and protease levels again increased during the chronic inflammation.

3.2.4. The Trypsin Inhibitory Capacity (TIC) of Adjuvant Arthritic Rat Serum.

The effects of adjuvant arthritis on the TIC activity of rat serum are shown in Fig. 31. The TIC activity was depressed during the acute and chronic inflammation, although it was increased above normal in both normal and adjuvant arthritic rats that were treated daily with cortisol-acetate.

3.2.5. The Trypsin-Protein Esterase (TPE) Activity of Adjuvant Arthritic Rat Serum.

The TPE activity, as shown in Fig. 32, was greatly increased during both acute and chronic inflammation. The TPE activity of adjuvant arthritic rats treated daily with cortisol-acetate was also increased, but to a lesser extent during the chronic inflammation. The TPE activities of normal rats appeared to be slightly decreased by treatment with cortisol-acetate.

3.2.6. Alpha-1-Macroglobulin Levels in Adjuvant Arthritic Rat Serum.

The concentration of alpha-1-macroglobulin in arthritic rat serum was found to increase during acute and chronic inflammation (Fig. 33). However, adjuvant arthritic rats that were treated with cortisol had increased serum levels of alpha-1-macroglobulin only during acute inflammation, during secondary inflammation the levels were slightly depressed. Cortisol-acetate was also found to reduce the alpha-1-macroglobulin levels in normal rats.

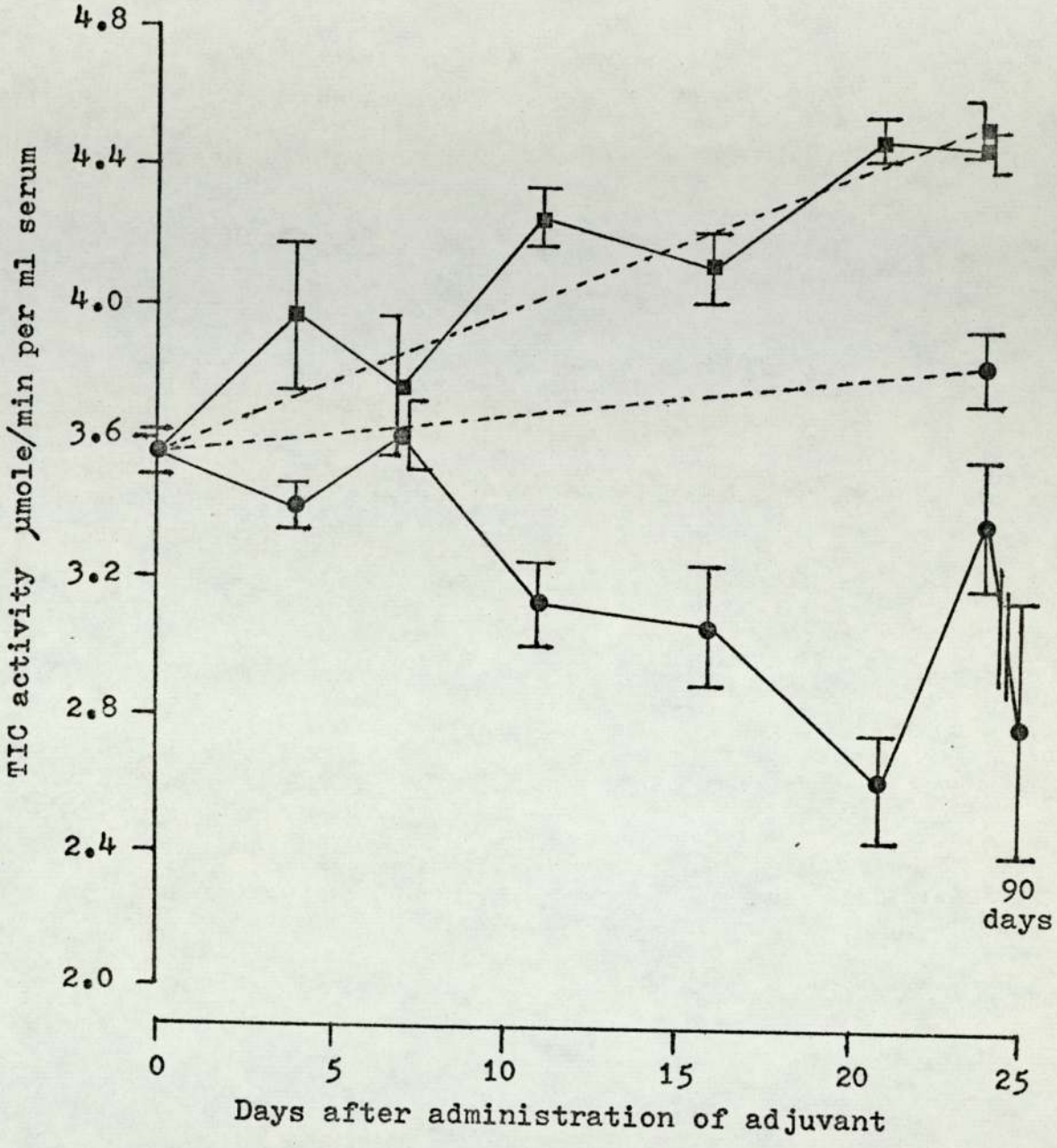


Fig.31. Variation of the trypsin inhibitory capacity of serum of rats injected with mycobacterial adjuvant ●——● ,and rats injected with mycobacterial adjuvant and treated daily with cortisol-acetate(5mg/kg) ■——■ . Dotted lines represent non-arthritic control rats. Each result represents the mean of five animals and vertical lines indicate S.E.M.

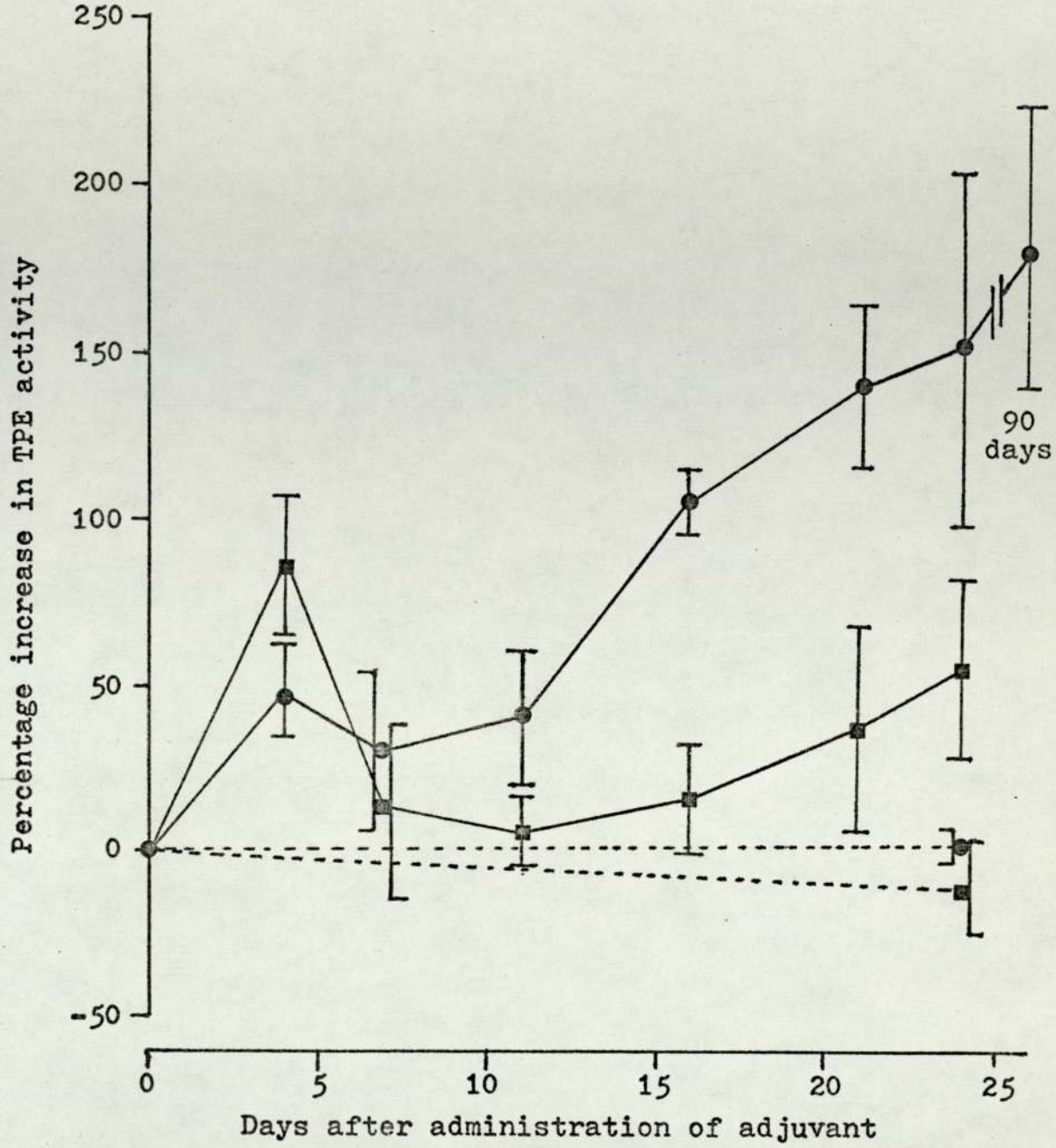


Fig.32.Variation of the trypsin- protein esterase activity of serum of rats injected with mycobacterial adjuvant ●——● ,and rats injected with mycobacterial adjuvant and treated daily with cortisol-acetate (5mg/kg) ■——■.Dotted lines represent non-arthritic control rats. Each result represents the mean of five animals and vertical lines indicate S.E.M.

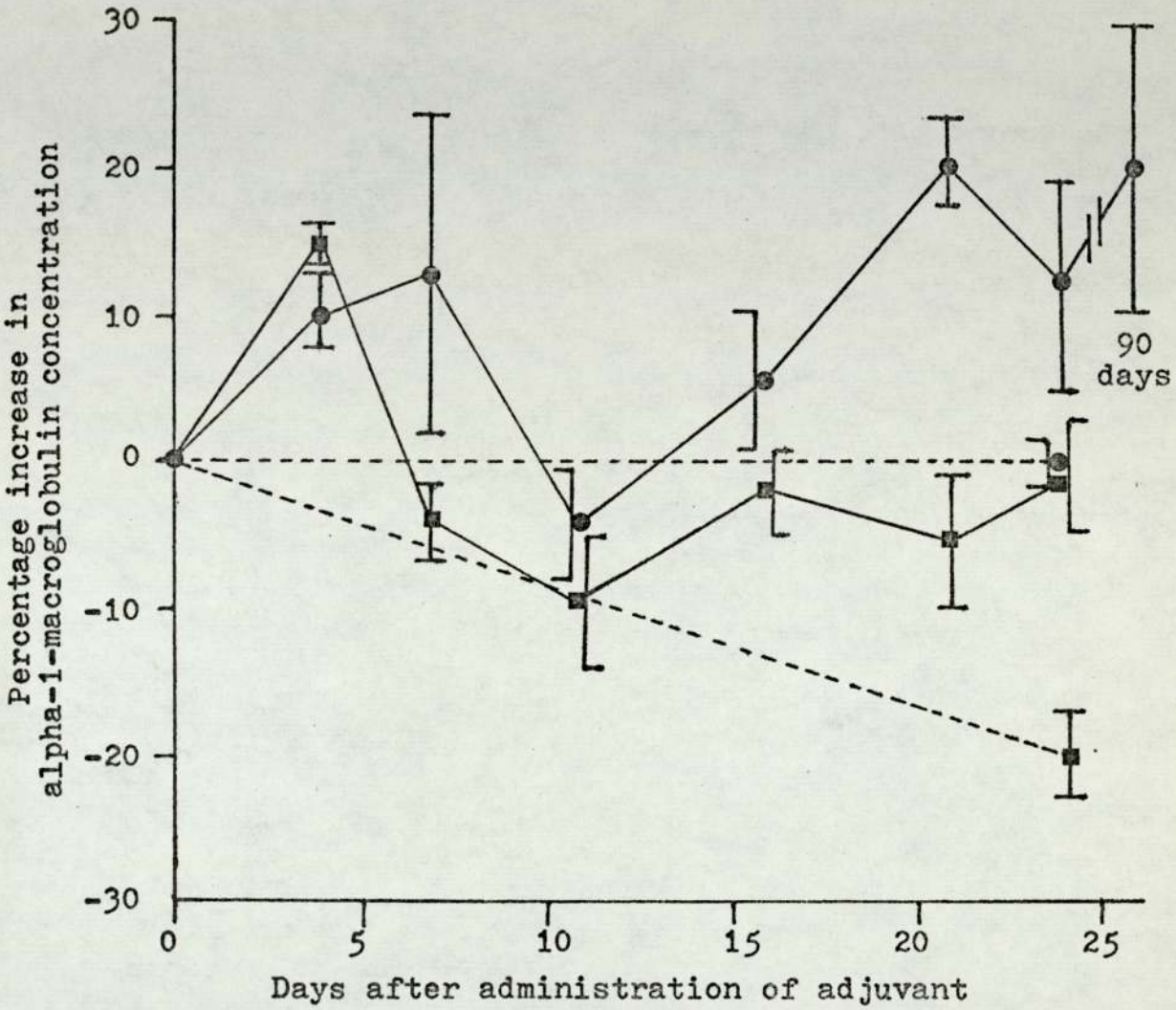


Fig.33. Variation of the alpha-1-macroglobulin concentration of serum of rats injected with mycobacterial adjuvant ●——●, and rats injected with mycobacterial adjuvant and treated daily with cortisol-acetate (5mg/kg) ■——■. Dotted lines represent non-arthritic control rats. Each result represents the mean of five animals and vertical lines indicate S.E.M.

3.2.7. Alpha-2-Acute Phase Globulin Levels in Adjuvant Arthritic Rat Serum.

The alpha-2-acute phase globulin serum concentration increased during both acute and chronic inflammation (Fig. 34). The levels also increased in arthritic rats treated with cortisol-acetate, although during the chronic inflammatory stage the increase was relatively modest. No alpha-2-acute phase globulin was detected in the serum of normal rats or normal rats treated with cortisol-acetate.

3.2.8. Transcortin Levels in the Serum of Adjuvant Arthritic Male and Female Rats.

The rates of release of transcortin-bound 1,2-^{3}H-cortisol in different serum samples, during incubation with dextran-coated charcoal suspension, were plotted as graphs of log counts per min. against time, as shown in Fig. 35. The straight line portion of the graphs were extrapolated back to zero time to give the initial amount of transcortin-bound 1,2-^{3}H-cortisol. The number of moles of transcortin were calculated assuming a binding ratio of cortisol to transcortin of 1:1.

The transcortin levels of normal female rats were found to be considerably higher than those of males (Fig. 36). The transcortin levels of both male and female rats, with adjuvant arthritis, fell below the levels of normal male rats during the onset of chronic inflammation. However, the transcortin concentration increased and approached normal levels during the later stages of the chronic inflammation.

3.3. The Anti-Inflammatory Effects of Dimethylnitrosamine.

3.3.1. Adjuvant-Induced Arthritis in the Rat.

Rats that were given an intra-peritoneal (i.p.) injection of

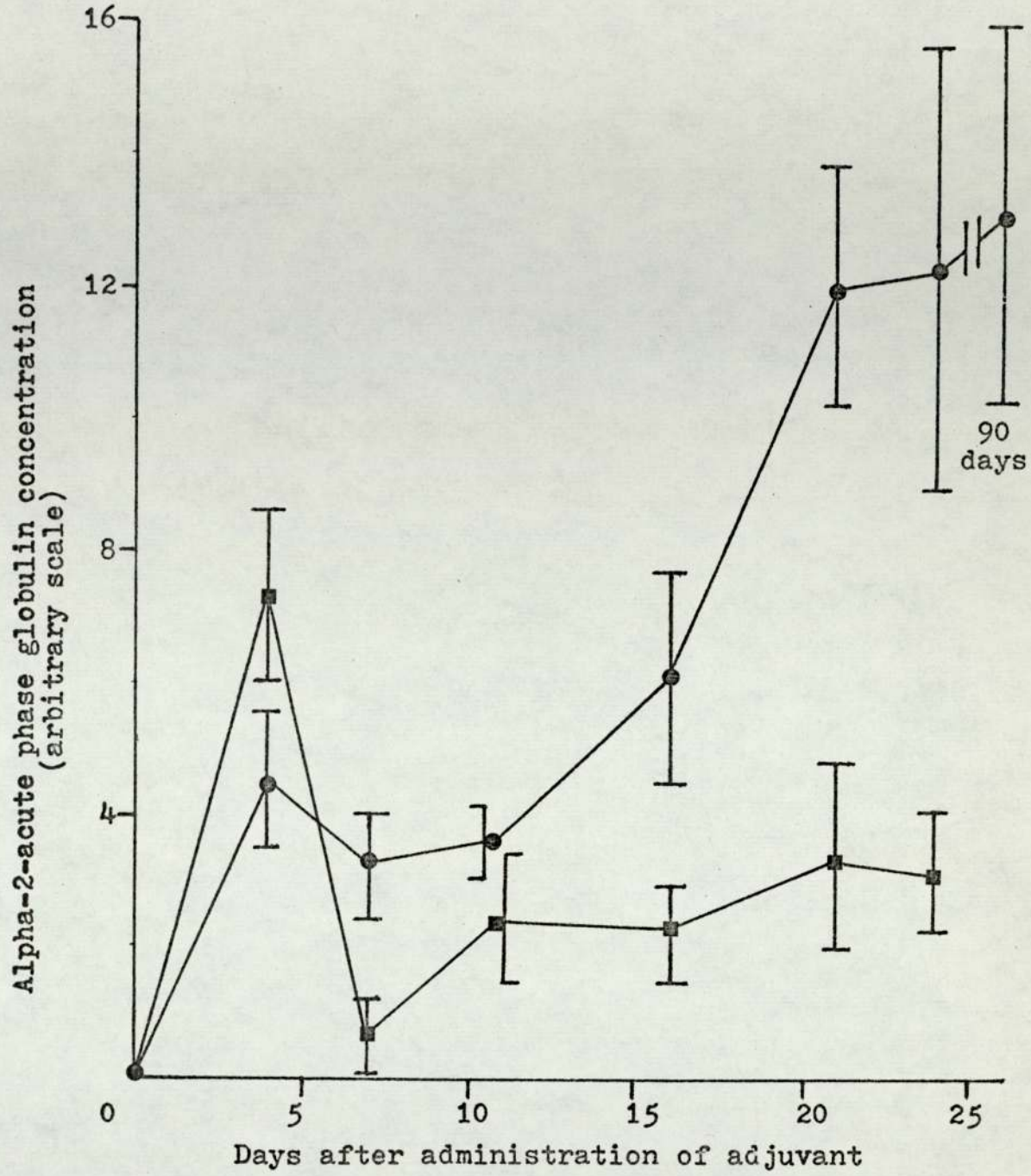


Fig.34. Variation of the alpha-2-acute phase globulin concentration of serum of rats injected with mycobacterial adjuvant ●——● ,and rats injected with mycobacterial adjuvant and treated daily with cortisol-acetate (5mg/kg) ■——■ .Each result represents the mean of five animals and vertical lines indicate S.E.M.

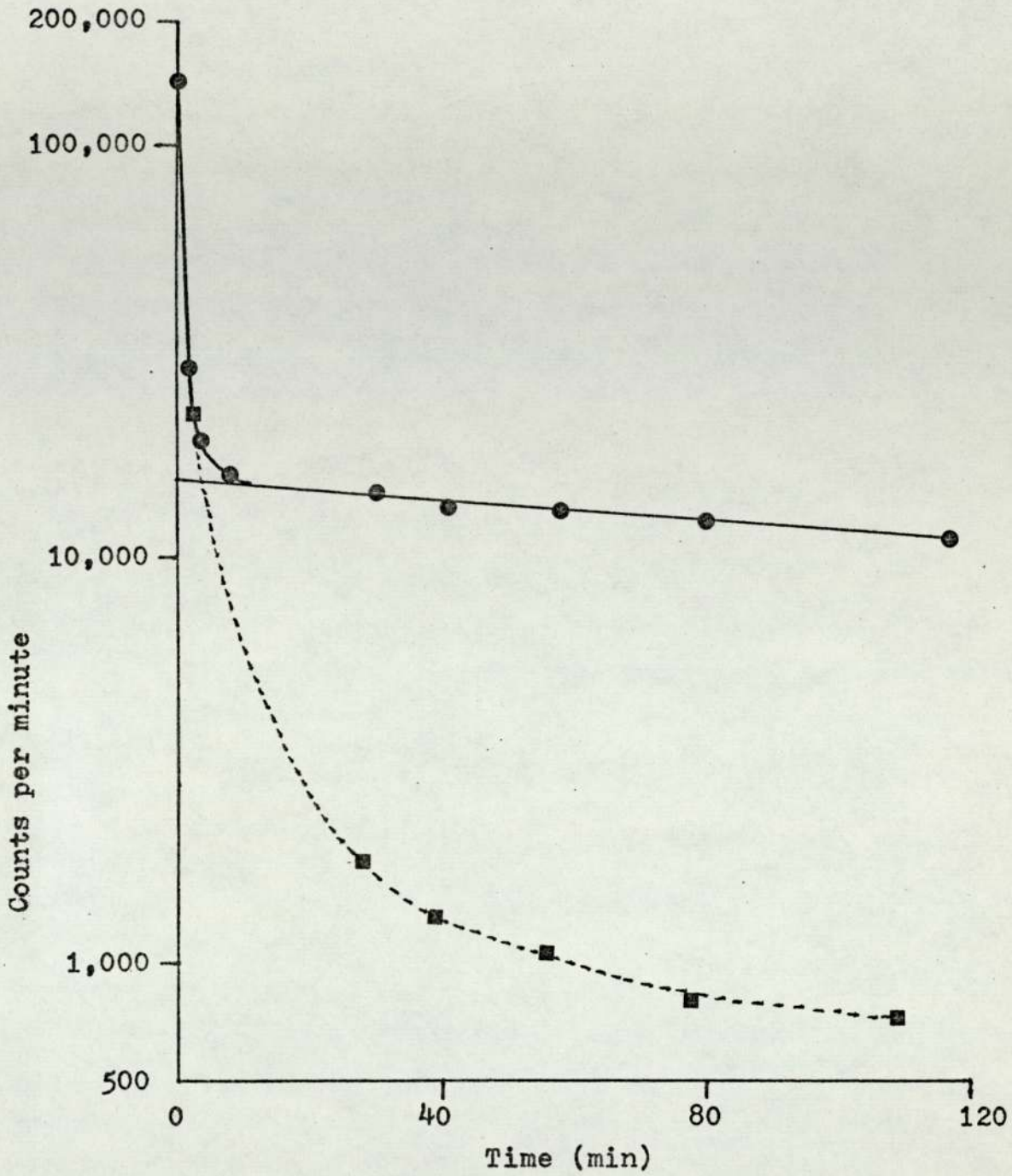


Fig.35. The adsorption of 1,2-^{3}H-cortisol from rat serum by dextran-coated charcoal suspension ●——● and from 0.9% saline ■- - - -■.

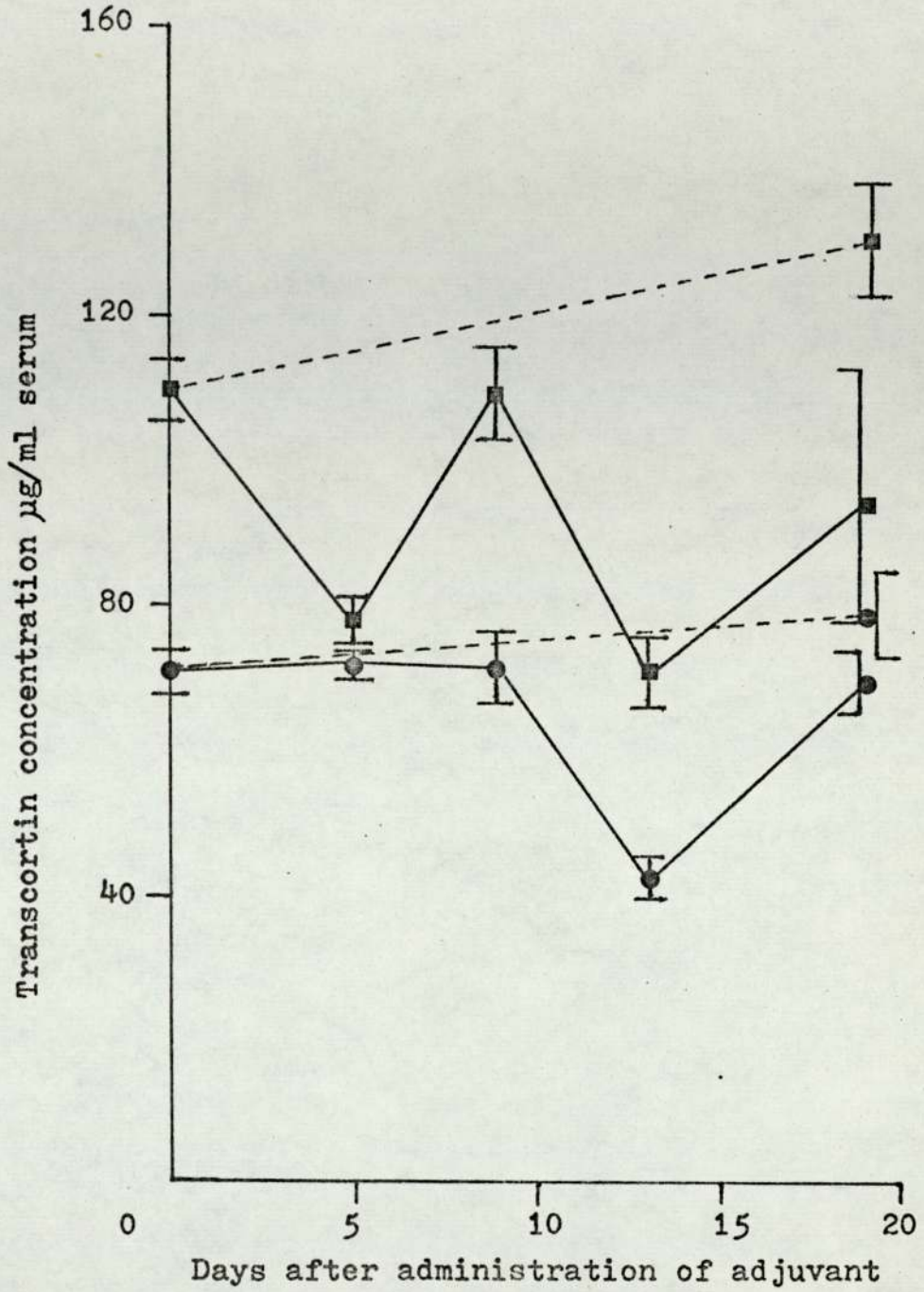


Fig.36. Variation of serum transcortin levels of female ■——■ and male ●——● rats after injection of mycobacterial adjuvant. Dotted lines represent non-arthritic control rats. Each result represents the mean of five animals and vertical lines indicate S.E.M.

dimethylnitrosamine (25 mg/Kg body weight) five days after the injection of adjuvant developed a much less severe form of arthritis (Fig. 37). The acute inflammation was unaffected, but the dimethylnitrosamine significantly reduced chronic inflammation by 40% to 50% in both the adjuvant-injected and non-injected hind feet.

3.3.2. Carrageenan-Induced Oedema in the Rat.

Rats that were given an i.p. injection of dimethylnitrosamine (25 mg/Kg body weight) three days prior to treatment with carrageenan, had significantly reduced carrageenan-induced inflammation (Fig. 38). The oedema was reduced by 50% to 60% compared to control rats.

3.3.3. The Effect of Liver Extracts from Dimethylnitrosamine Treated Rats on Carrageenan-Induced Oedema.

Fig. 39 shows the effect on carrageenan-induced inflammation in the rat of i.p. injections of liver extracts prepared from rats that had been dosed three days earlier with dimethylnitrosamine. The liver extracts of dimethylnitrosamine treated rats significantly reduced the carrageenan-induced oedema, when compared with i.p. injections of normal liver extracts and saline alone. Dialysed dimethylnitrosamine liver extracts were almost as effective as undialysed dimethylnitrosamine liver extracts in controlling the inflammation. The significance of these results is given in Table 1.

3.3.4. The TIC, TPE, Alpha-1-Macroglobulin and Alpha-2-Acute Phase Globulin Levels in Liver Extracts of Dimethylnitrosamine Treated Rats.

The TIC activity of the pooled liver extracts from rats treated with dimethylnitrosamine (25 mg/Kg body weight) three days earlier was

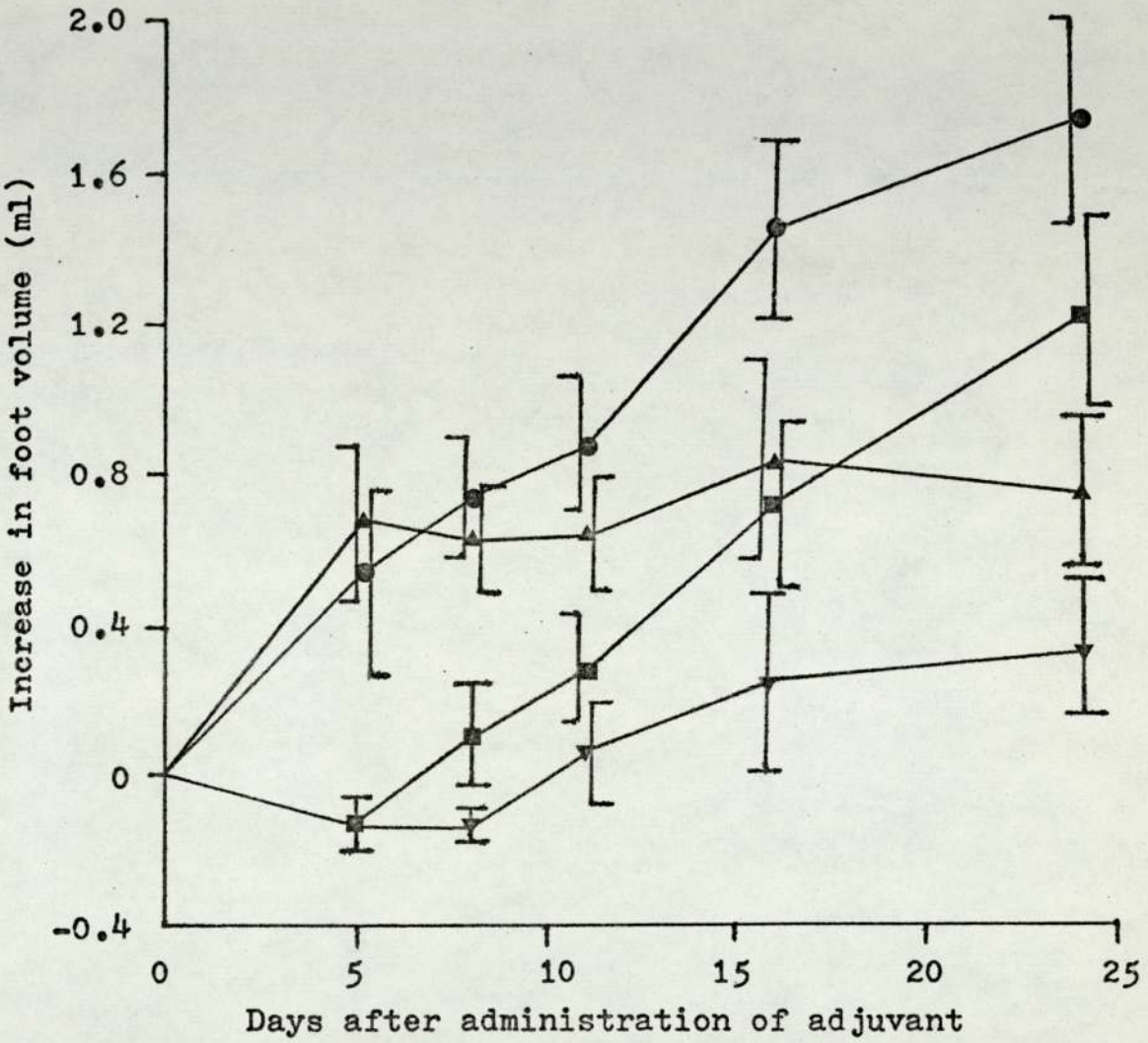


Fig.37. Increase in size of left ●——● and right ■——■ hind feet of rats after injection of mycobacterial adjuvant into the left hind feet. Increase in size of left ▲——▲ and right ▼——▼ hind feet of rats treated with dimethylnitrosamine (25mg/kg) five days after injection of mycobacterial adjuvant into the left hind feet. Each result represents the mean of five animals and vertical lines indicate S.E.M.

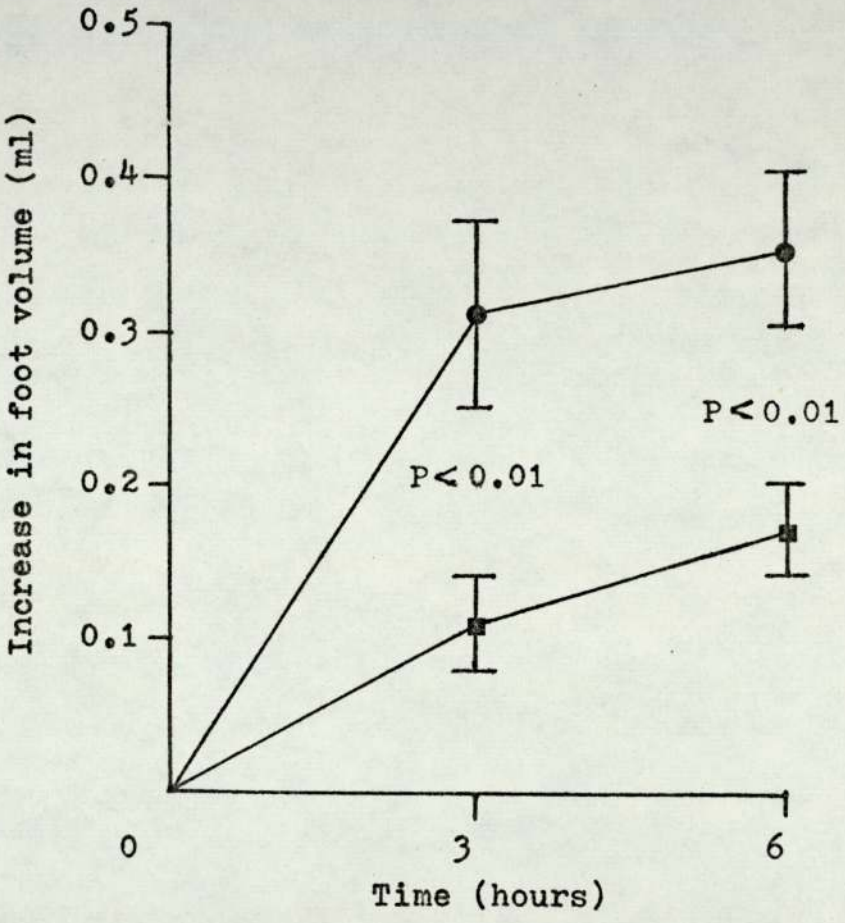


Fig.38. Increase in foot volume of rats injected with carrageenan three days after treatment with dimethylnitrosamine (25mg/kg) ■——■, and increase in foot volume of control rats injected with carrageenan ●——● .

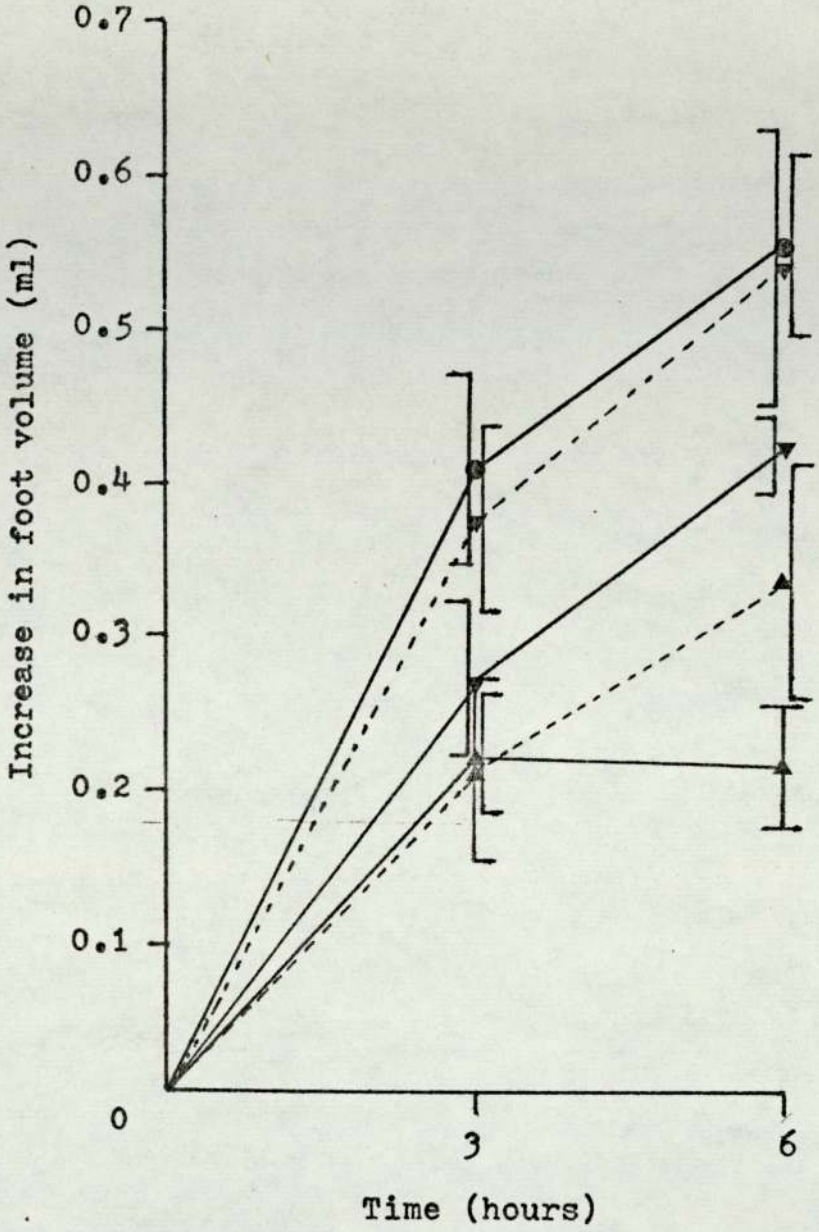


Fig.39. Increase in foot volume of rats injected with carrageenan and given i.p. injections of saline liver extract from dimethylnitrosamine treated rats ▲——▲ ,dialysed saline liver extract from dimethylnitrosamine treated rats ▲-----▲ , saline liver extract from normal rats ▼——▼ , dialysed saline liver extract from normal rats ▼-----▼ and 0.9% saline alone ●——● .

TABLE 1.

The effect of intra-peritoneal injections of saline liver extracts prepared from normal rats and rats treated three days earlier with dimethylnitrosamine (DMN), on carrageenan-induced oedema in the rat.

Extract	Increase in joint volume (ml)		Percentage of inhibition of oedema.	
	3 h	6 h	3 h	6 h
Saline alone	0.40 ± 0.06	0.55 ± 0.06	0	0
Normal liver	0.27 ± 0.05	0.42 ± 0.03	32	24
Normal liver dialysed	0.37 ± 0.06	0.54 ± 0.08	7	2
DMN liver	0.22 ± 0.04	0.21 ± 0.04	45	62
DMN liver dialysed	0.21 ± 0.06	0.33 ± 0.08	47	40

Extract	Normal liver		DMN liver		DMN liver dialysed	
	3 h	6 h	3 h	6 h	3 h	6 h
Saline alone	Not significant	$P<0.05$	$P<0.05$	$P<0.0025$	$P<0.05$	$P<0.05$
Normal liver	-	-	Not significant	$P<0.005$	Not significant	Not significant
Normal liver dialysed	Not significant	Not significant	$P<0.05$	$P<0.05$	$P<0.05$	$P<0.05$
DMN liver	$P<0.05$	$P<0.005$	-	-	Not significant	Not significant
DMN liver dialysed	Not significant	Not significant	Not significant	Not significant	-	-

Each result represents the mean of five animals ± SEM.

Extract	Percentage of inhibition of oedema	
	3 h	6 h
DMN liver with respect to normal liver	19	50
Dialysed DMN liver with respect to dialysed normal liver	43	39

45% above that of the corresponding levels in normal rat liver extracts. There was no measureable difference between the TPE activities or the alpha-1-macroglobulin levels in the liver extracts of normal and dimethylnitrosamine treated rats. Alpha-2-Acute phase globulin was not detected in either of the liver extracts.

3.3.5. The TIC, TPE, Alpha-1-Macroglobulin, Alpha-2-Acute Phase Globulin Levels and the Total Protein Concentration in the Serum of Dimethylnitrosamine Treated Rats.

Three days after treatment with dimethylnitrosamine (25 mg/Kg body weight) there was a small but significant increase in the TIC activity of the rat serum ($\sim$3%), compared to that of control rats. However, after six days there was no significant difference in the serum TIC activities (Table 2).

The serum TPE activities of dimethylnitrosamine treated rats were slightly reduced after three days, when compared to control rats, although after six days the levels were almost back to normal (Table 2). The alpha-1-macroglobulin levels gave a similar picture (Table 2). Trace amounts of alpha-2-acute phase globulin were found in the sera of only 50% of the rats treated with dimethylnitrosamine, after both three and six days (Table 2). No alpha-2-acute phase globulin was detected in the sera of control rats. The total protein concentration of the serum was significantly reduced three days after injection of dimethylnitrosamine, although after six days the serum protein concentration was back to the normal levels of the control rats (Table 2).

TABLE 2.

The effect of dimethylnitrosamine administration on the levels of certain serum proteins in the rat.

	Days after administration of dimethylnitrosamine (DMN)			
	Three days		Six days	
	Control	DMN-treated	Control	DMN-treated
TIC activity μ mole/min per ml.	3.63 ± 0.01	3.78 ± 0.05	3.69 ± 0.03	3.66 ± 0.03
	P<0.0125		Not significant	
TPE activity % control	100 ± 6	83 ± 10	95 ± 5	89 ± 9
	P<0.01		Not significant	
Total protein concentration. mg/ml.	64.7 ± 1.0	56.7 ± 2.0	67.1 ± 0.5	67.0 ± 1.2
	P<0.005		Not significant	
Alpha-1-macroglobulin concentration. % control	100.0 ± 6.0	97.2 ± 3.6	102.8 ± 2.0	102.0 ± 3.6
	Not significant		Not significant	
Alpha-2-acute phase globulin concentration % control	Zero	Trace in 50%	Zero	Trace in 50%
	Not significant		Not significant	

Each result represents the mean of five animals ± SEM.

3.4. The Effect of a Human Alpha-1-Antitrypsin Fraction on Carrageenan-Induced Oedema in the Rat.

The human alpha-1-antitrypsin fraction when mixed and injected with carrageenan into the hind feet of rats caused a small increase in the inflammatory response, compared to control rats given carrageenan alone (Fig. 40). The human alpha-1-antitrypsin fraction when given to rats in saline alone, however, was not inflammatory.

3.5. The Effect of Non-Steroidal Anti-Inflammatory Drugs on the Stability of Transcortin-Bound 1,2-^{3}H-Cortisol.

The rates of release of transcortin-bound 1,2-^{3}H-cortisol, in the presence of therapeutic concentrations of various drugs, were plotted as graphs of log counts per min. against time (as shown in Fig. 41). Extrapolation of the straight line portion of the graph to zero time therefore gave a direct measure of the initial amount of transcortin-bound 1,2-^{3}H-cortisol, in the presence of the appropriate drug.

As shown in Table 3, none of the non-steroidal drugs assayed appeared to affect the stability of transcortin-bound 1,2-^{3}H-cortisol. However, prednisolone, which is a synthetic glucocorticoid known to compete with cortisol for binding sites, decreased the binding of cortisol by 25% and 50% at concentrations of 0.2 µg/ml plasma and 0.4 µg/ml plasma respectively.

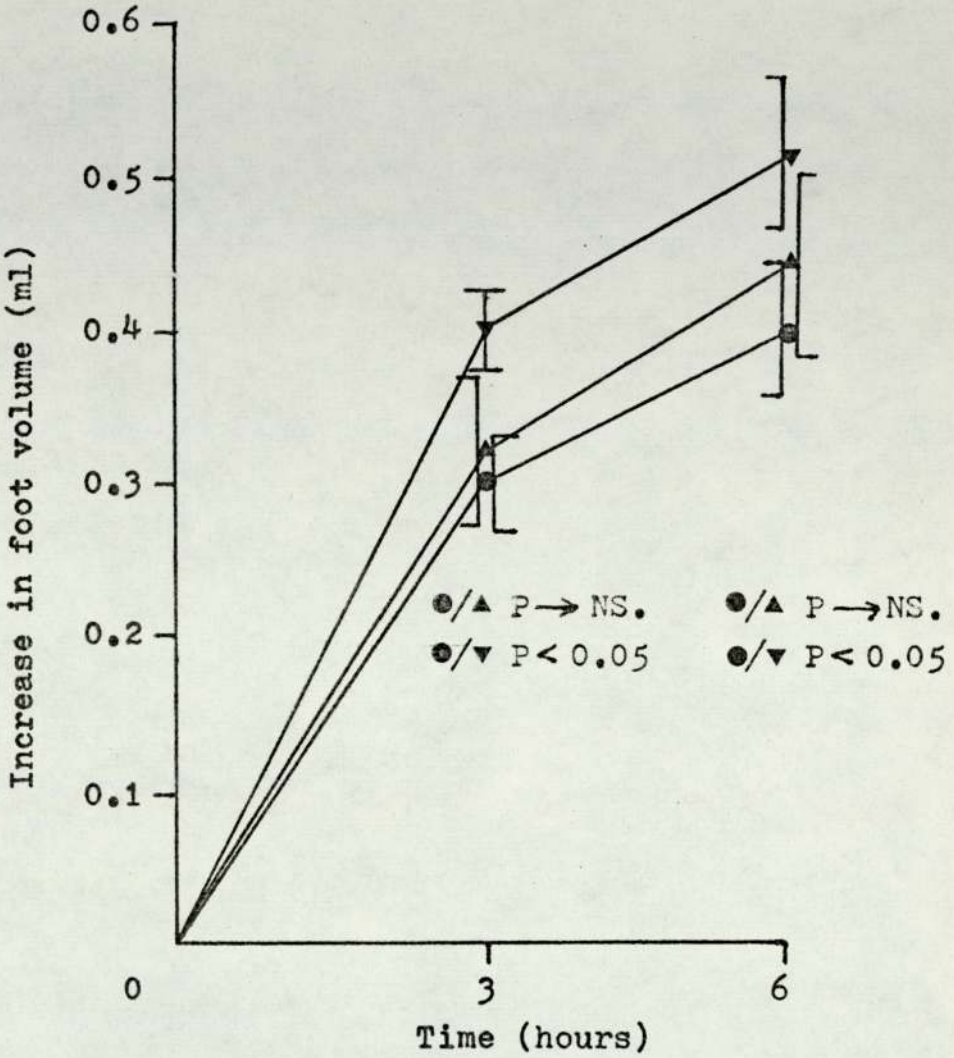


Fig.40. Increase in foot volume of rats injected with carrageenan containing human alpha-1-antitrypsin fraction (60mg/ml) ▼——▼, carrageenan containing human alpha-1-antitrypsin fraction (30mg/ml) ▲——▲ and carrageenan alone ●——●.

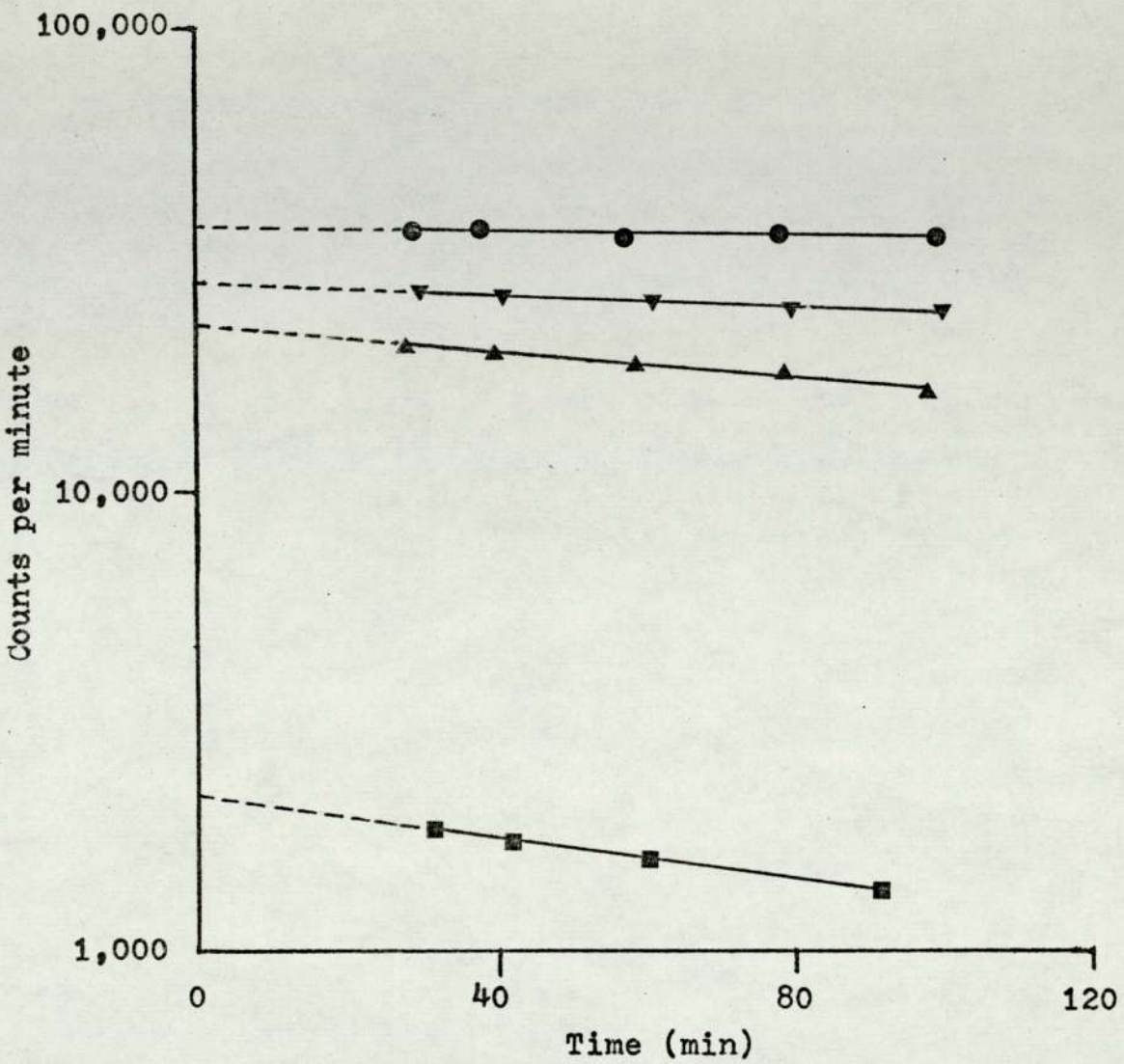


Fig.41. The adsorption of 1,2-^{3}H-cortisol from human plasma in the absence of any drug ●——● , in the presence of prednisolone (0.2µg/ml) ▼——▼ and in the presence of prednisolone (0.4µg/ml) by dextran-coated charcoal suspension. The adsorption of 1,2-^{3}H-cortisol from heated human plasma (transcortin inactivated) is represented by ■——■ . Dotted lines represent extrapolation of the straight line portions of the graphs to zero time.

TABLE 3.

The stability of transcortin-bound 1,2-^{3}H-cortisol to non-steroidal anti-inflammatory drugs *in vitro*.

Drug	Drug concentration (µg/ml plasma)	Percentage of 1,2-^{3}H-cortisol transcortin-bound compared to control
Aspirin	250	99, 102
Sodium salicylate	250	100, 97
Phenylbutazone	50	97, 97
Oxyphenbutazone	50	94, 97
Indomethacin	5	98, 98
Ibuprofen	20	97, 97
D-Penicillamine	500	98, 97
Dapsone	30	97, 97
Prednisolone	0.2	72, 72
Prednisolone	0.4	53, 54

4. DISCUSSION.

4.1. Changes in the Serum Protein Patterns of Adjuvant Arthritic Rats and the Effects of Cortisol Administration.

These results have confirmed earlier findings (Lowe, 1964; Glenn, Bowman and Koslowske, 1968) that there are significant changes in the levels of certain plasma proteins, or acute phase reactants (APR), in rats with adjuvant arthritis. Alpha-globulin and beta-globulin levels were found to increase, whilst the levels of albumin decreased. These changes appeared to be more pronounced as the intensity of the inflammation increased.

It is not known whether APR are simply an end result of inflammation or whether some APR have an anti-inflammatory role. It is possible that inflammation at any site in the body might release factors that could influence the synthesis of plasma proteins in the liver. These might have the effect of slowing down the synthesis of proteins such as albumin and inducing the synthesis of other proteins in the liver that have an anti-inflammatory or repair function. Indeed a "humoral initiator substance" (HIS) that induces the synthesis of alpha-2-glycoprotein (alpha-2-acute phase globulin) has been demonstrated in the sera of traumatized rats (Bogden, Gray and Fuss, 1966).

Cortisol treatment greatly reduced the chronic inflammation and tended to return the serum protein patterns of arthritic rats towards normality. It is possible that an anti-inflammatory action of cortisol would be to increase or induce the synthesis of anti-inflammatory proteins. However, it was not possible to tell whether cortisol

treatment had directly or indirectly raised the levels of any serum proteins, other than albumin, in either normal or arthritic rats.

4.2. Acid Phosphatase and Protease Levels in Adjuvant Arthritic Rat Serum.

The increase in the levels of lysosomal acid phosphatase in the serum of arthritic rats during both acute and chronic inflammation confirms the previous results of Collins and Lewis (1971). Since the protease levels in the serum of arthritic rats were found to increase in a similar fashion it may well be that these were lysosomal in origin.

4.3. Antiprotease Levels in the Serum of Adjuvant Arthritic Rats and the Effect of Cortisol Administration.

4.3.1. The Trypsin Inhibitory Capacity (TIC).

The fall in the TIC activity of the serum of adjuvant arthritic rats, during acute and chronic inflammation, probably reflects a fall in the alpha-1-antitrypsin levels, since alpha-1-antitrypsin is a major contributor to the TIC activity in the rat (Huttenen and Korhonen, 1973). In humans alpha-1-antitrypsin is an APR (Werner and Odenthal, 1967) and its levels have been found to rise, when assayed by immunological methods, in patients with rheumatoid

arthritis (Swedlund, Hunder and Gleich, 1974). This apparent difference between the increase in the levels of alpha-1-antitrypsin in humans and the fall in the levels in rats with arthritis may be simply due to species differences. However, adjuvant-induced arthritis in rats appears to be a much more severe disease than human rheumatoid arthritis, and increased levels of lysosomal enzymes are found in arthritic rat serum (Collins and Lewis, 1971). High serum protease levels may lead to the build up of a pool of alpha-1-antitrypsin-protease complexes in the circulation, which may inhibit further synthesis of active antiprotease by a feedback mechanism. Even if, the complexes are rapidly removed from the circulation, it is possible that the rate of removal may be greater than the rate of synthesis, increased or otherwise, of active alpha-1-antitrypsin. It should be possible to establish whether there is a build up of protease-antiprotease complexes by assaying the concentration of alpha-1-antitrypsin both immunologically and enzymatically (TIC) and comparing the results.

The increase in the TIC levels of control and adjuvant arthritic rats treated with cortisol is probably due to an increase in the rate of antiprotease synthesis in the liver, although it could be due to a decreased rate of catabolism. Since the result of cortisol administration in the rat is an increase in TIC levels, well above normal levels, it may well reflect an anti-inflammatory action of the drug. In humans alpha-1-antitrypsin has been reported to increase during pregnancy and oestragen-progestin therapy (Laurell, Kullander and Thorell, 1968), but there appears to be no mention of the effects of glucocorticoids on human alpha-1-antitrypsin levels, in the literature.

4.3.2. The Trypsin-Protein Esterase (TPE) Activity, Alpha-1-Macroglobulin and Alpha-2-Acute Phase Globulin Levels.

Ganrot, K. (1973) has demonstrated that there is a direct correlation between the TPE activity of normal rat serum and the levels of alpha-1-macroglobulin. He has also shown that in the serum of rats with inflammation there is a direct correlation between the additional TPE activity (that is not due to alpha-1-macroglobulin) and the levels of alpha-2- acute phase globulin.

The increase in the TPE activity of adjuvant arthritic rats during acute and chronic inflammation is partly due to an increase in the levels of alpha-1-macroglobulin. However, since the increase in alpha-1-macroglobulin levels is relatively small (approximately 10-15%) and the increase in TPE activity is relatively large (approximately 50-150%), the majority of the increase in TPE activity must be due to alpha-2-acute phase globulin.

Compared to the untreated arthritic rats, the cortisol treated arthritic rats have higher TPE values during acute inflammation, due to increased alpha-2-acute phase globulin levels. During chronic inflammation the TPE levels are lower in cortisol treated arthritic rats than in untreated arthritic rats, due to falls in both alpha-2-acute phase globulin and alpha-1-macroglobulin levels. Indeed the alpha-1-macroglobin levels fall below those of normal rats. Therefore, except for acute inflammation, cortisol did not appear to elevate either the alpha-2-acute phase globulin or the alpha-1-macroglobulin levels in normal or arthritic rats.

The fact that cortisol reduced the increase in alpha-2-acute phase globulin levels during chronic inflammation was probably due to the anti-inflammatory action of cortisol reducing the severity of the inflammation

and hence indirectly reducing the levels of alpha-2-acute phase globulin. The alpha-1-macroglobulin levels appear to be depressed by cortisol in both arthritic rats with chronic inflammation and normal rats. Therefore it is likely that relatively high levels of cortisol slightly depress alpha-1-macroglobulin synthesis.

The increase in the levels of alpha-2-acute phase globulin during acute inflammation in cortisol treated arthritic rats is more difficult to understand. It may be due to an initial intolerance of the administered cortisol leading to some form of trauma that causes an elevation of the alpha-2-acute phase globulin levels. For instance it has been demonstrated that high concentrations of cortisol, above 10^{-6}M, labilize rat liver lysosomes in vitro (Lewis, Symons and Ancill, 1970). Indeed intra-articular injections of steroids have occasionally produced short-lived inflammation and give rise to temporary increases in lysosomal enzyme levels (Collins and Cosh, 1970).

Weiner and Benjamin (1966), using adrenalectomised rats traumatized with turpentine, demonstrated that both trauma and cortisol were necessary to induce the synthesis of alpha-2-acute phase globulin. They also found that for a standard trauma cortisol up to a concentration of 3 mg/Kg would increase the alpha-2-acute phase globulin levels in adrenalectomised rats, but concentrations above this depressed the synthesis of alpha-2-acute phase globulin. Bogden, Gray and Fuss (1966) have designated the substance, in the sera of traumatized rats, that induces the synthesis of alpha-2-acute phase globulin as "HIS". Both inflammation and cortisol therefore play an important role in the induction of alpha-2-acute phase globulin. However, it appears that above a certain threshold the anti-inflammatory action of cortisol reduces the inflammatory response, causing a fall in the alpha-2-acute phase globulin levels. Therefore,

although the presence of low levels of cortisol is important for the induction of alpha-2-acute phase globulin, high levels of cortisol that are normally anti-inflammatory do not increase its synthesis any further. Thus it is unlikely that alpha-2-acute phase globulin mediates an anti-inflammatory action of cortisol.

It is not known whether alpha-1-macroglobulin synthesis is under similar control to alpha-2-acute phase globulin, but from these results it would appear that its levels increase during inflammation and that it is an APR. It seems unlikely that cortisol mediates any anti-inflammatory action through the increased synthesis of alpha-1-macroglobulin, since high concentrations of cortisol depressed the levels of alpha-1-macroglobulin in both normal and adjuvant arthritic rats.

4.4. Transcortin Levels in the Serum of Adjuvant Arthritic Male and Female Rats.

The results of the transcortin levels in normal male and female rats confirm the earlier findings of Gala and Westphal (1965) that there is a sex difference in the levels. However, there did not appear to be any sex difference in the response to adjuvant, since both male and female animals developed arthritis of similar severity. It seems unlikely therefore that the higher transcortin levels in the female rats had any anti-inflammatory effect. One can speculate that female rats would be more susceptible than male rats to adjuvant arthritis were it not for their increased levels of transcortin. There is no sex difference in the transcortin levels in humans and yet two thirds of rheumatoid patients are female (Walter and Israel, 1970). It is also possible that the fall in transcortin levels in both male and female rats, during the onset of chronic inflammation, leads to transcortin levels that are too low to have any anti-inflammatory significance.

It has been demonstrated that increased levels of transcortin in humans lead to increased levels of total and "free" cortisol (Rosenthal, Slaunwhite and Sandberg, 1969). It is possible therefore that if the levels of transcortin were high enough they would have an anti-inflammatory action. Further work could assess the effect of high transcortin levels in adjuvant arthritic rats on the severity of the arthritis. High transcortin levels could probably be maintained either by oestragen therapy or by injections of purified rat transcortin fractions.

4.5. Effect of a Human Alpha-1-Antitrypsin Fraction on Carrageenan-Induced Oedema in the Rat.

The human alpha-1-antitrypsin fraction administered with the carrageenan at the site of inflammation was equivalent to about 0.5 ml serum when assayed by TIC activity. Although this amount of the fraction was not anti-inflammatory, it is possible that higher concentrations might have been, but it is more likely that alpha-1-antitrypsin has little effect on this type of inflammatory oedema. It was expected that the alpha-1-antitrypsin would reduce the production of kinins by inhibiting kallikrein and therefore reduce the inflammatory oedema. However, the major function of alpha-1-antitrypsin is probably the inhibition of degradative enzymes and hence the inhibition of tissue destruction and joint erosion. Therefore with hindsight it appears that the antigen-induced arthritis model in rabbits (Dumonde and Glynn, 1962) would be more useful for investigating the effects of antiproteases. Since it would be possible to give intra-articular injections of antiproteases and directly assess any reduction in joint or cartilage destruction.

4.6. The Anti-Inflammatory Effects of Dimethylnitrosamine.

4.6.1. Adjuvant-Induced Arthritis and Carrageenan-Induced Oedema in the Rat.

The finding that dimethylnitrosamine could delay the onset and reduce the severity of chronic inflammation in rats injected with adjuvant five days earlier confirmed some of the previous work of Pinals (1973). It was also found that dimethylnitrosamine given to normal rats, three days before challenge with carrageenan, also reduced the inflammatory response in these animals. It is possible that this anti-inflammatory effect of dimethylnitrosamine could be due to its toxicity, perhaps crippling the inflammatory response of the plasma cells. Another possibility is that the hepatic inflammation, due to the action of dimethylnitrosamine, acts as a "counter irritant" (Atkinson and Hicks, 1975) and reduces inflammation at other sites in the body, by competing for inflammatory mediators. A third possibility is that the dimethylnitrosamine induces the rat to produce an endogenous anti-inflammatory substance.

4.6.2. The Effect of Liver Extracts from Dimethylnitrosamine Treated Rats on Carrageenan-Induced Oedema in the Rat.

Pinals (1973) demonstrated that saline extracts of livers from rats, treated with dimethylnitrosamine two to six days earlier, could delay the onset and severity of chronic inflammation in rats with adjuvant arthritis. Saline extracts of normal rat livers were ineffective. The results presented in this thesis also demonstrate that intra-peritoneal injections of saline liver extracts, taken from rats treated three days earlier with dimethylnitrosamine, inhibit carrageenan inflammation in rats to a greater extent than saline extracts from normal rat liver. The anti-inflammatory activity of the liver

extract, taken from dimethylnitrosamine treated rats, is not due to the toxicity of the dimethylnitrosamine itself, since it is rapidly metabolised and is not present in the liver extracts (Pinals, 1973). It is possible that the intra-peritoneal injections of the liver extracts could act as "counter irritants" (Atkinson and Hicks, 1975), if the dimethylnitrosamine liver extract is more irritant than the normal liver extract. Otherwise it appears that the dimethylnitrosamine liver extracts must contain some anti-inflammatory substances. These anti-inflammatory substances are likely to be of large molecular weight, since dialysis of the dimethylnitrosamine liver extract against saline did not greatly effect its anti-inflammatory activity.

Further work could involve an attempt to separate possible inflammatory and anti-inflammatory factors, with a view to establishing the existence of anti-inflammatory factors and their possible identity.

4.6.3. Antiprotease Levels in the Serum and Liver of Dimethylnitrosamine Treated Rats.

The finding that both the serum and liver of dimethylnitrosamine treated rats contained significantly increased concentrations of alpha-1-antitrypsin after three days, suggests that alpha-1-antitrypsin might be an early anti-inflammatory factor. However, the anti-inflammatory effect of dimethylnitrosamine does not appear to be due to alpha-1-antitrypsin alone, because the increase in alpha-1-antitrypsin concentration in serum after three days is only relatively modest and does not last for as long as six days. The alpha-1-macroglobulin levels in the liver and serum of dimethylnitrosamine treated rats were decreased after three days and back to normal after six, therefore alpha-1-macroglobulin could not have had an anti-inflammatory effect.
Neither could alpha-2-acute phase globulin, since its presence was only detected in 50% of the sera of dimethylnitrosamine rats in very low quantities.

4.7. The Stability of Transcortin-Bound 1,2-^{3}H-Cortisol to Non-Steroidal Anti-Inflammatory Drugs.

Stenlake, Williams, Davison and Downie (1971) reported that non-steroidal anti-inflammatory drugs did not significantly alter the "free" levels of cortisol in plasma. It is possible that non-steroidal anti-inflammatory drugs could adversely affect the binding of cortisol to transcortin, causing a decrease in transcortin-bound cortisol and an increase in albumin-bound cortisol. The association constant for the binding of cortisol by albumin is much less than that of transcortin and it is possible that albumin-bound cortisol is more available and has some anti-inflammatory function. However, the finding, that none of the tested non-steroidal anti-inflammatory drugs affected the binding of 1,2-^{3}H-cortisol to transcortin, eliminates any possibility that these drugs act by increasing the levels of active cortisol.

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