Proteinase Activity in Rheumatoid Arthritis

A thesis submitted by

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SUMMARY

1. Collagenase and elastase act together in causing joint damage in rheumatoid arthritis, and they may be in part of plasma origin as there was significant correlation in synovial fluids, between proteinases and total protein.

2. It was confirmed that alpha-1-antitrypsin plays a major role as antiproteinase as there was significant correlation between total alpha-1-antitrypsin and proteinase.

3. Collagenase and elastase are present in a pro-enzyme form in the human rheumatic synovial fluid, which could be activated by trypsin.

4. Human rheumatic synovial fluid can inhibit collagenase and elastase, and this inhibitory capacity diminishes after addition of trypsin.

5. Collagenase and elastase levels were depressed in the serum of adjuvant arthritic rats which corresponded with a decrease in trypsin inhibitory capacity (TIC) and an increased level of alpha-1-macroglobulin.

Key Words: Rheumatoid arthritis, Collagenase, Elastase, Alpha-1-antitrypsin.

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TO

MY HUSBAND AND SON

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1. INTRODUCTION

1.1. Aetiology of Rheumatic Diseases

The aetiology of rheumatic diseases is diverse and in many cases unknown. It is conceivable that a wide variety of different initiating factors, either infective or traumatic, may initiate an immune reaction within the joint which then becomes perverted and self perpetuating, perhaps after the initiating factors have ceased to operate (Barnes, 1970).

Many aetiological hypotheses have been tested with mainly negative results, thus there is no evidence that a specific disease such as rheumatoid arthritis is due to nutritional factors, metabolic errors, endocrine abnormalities or an imbalance in the autonomic nervous system; nor that occupational factors, cold, damp or injury play a major role (Lawrance, 1970).

Rheumatoid arthritis and allied inflammatory rheumatic diseases have been thought to be due to infective processes almost since the time of Pasteur, yet intensive investigation has failed to reveal classical microbiological evidence for defined infective agents at sites of rheumatic inflammation, L-phase bacteria which are organisms with little or no cell walls have been reported in some rheumatoid tissue (Benedek, 1955). Pease (1974) claimed finding the same organisms in human rheumatoid samples.

There have been a number of attempts to isolate mycoplasma (organisms slightly larger than viruses) from human rheumatoid material with little success (Pearson, Sharp and Rawls, 1973; Stewart, Duthie, Mackay, Marmion and Alexander, 1974).

A viral infection could initiate rheumatoid disease by producing an immune response aginst its antigenic components. Rubella virus has been

shown to produce mild arthritis in human (Weibel, Stokes, Buyank and Hilleman, 1969) but no viral type particles have been isolated from rheumatoid samples (Smith and Hamerman, 1969; Wilkes, Simsarion, Hopps, Roth, Decker, Apetekar and Meyer, 1973).

In recent studies more attention has been paid to the role of the immunological processes in the pathogenis of rheumatoid lessions. The mechanism is probably more complex than a straight forward autoimmune situation. Proponents of autoimmune hypothesis have long sought evidence for either an inappropriate immune response directed against a normal tissue or an appropriate response directed against an altered tissue component (or product of inflammation) (Glynn,1968).

It was shown by Zvaifler (1974) that rheumatoid synovitis is associated with the presence in the joint of (antigen/antibody) complexes which activate the complement system thus causing polymorphonuclear leucocytes to migrate into the joint. These polymorphonuclear leucocytes engulf the immune complexes by phagocytosis and in doing so release lysosomal enzymes which act as chemical mediators of inflammation, the immediate cause of synovitis. It was reported that cell mediated immunity with the release of lymphokines from lymphocytes within the joint are also able to mediate synovitis (Yu and Peter, 1974).

The presence of an immunological activity implies the presence of an antigen to sustain it and the identification of the antigen may well be the key to the aetiology of rheumatoid arthritis.

1.2 Pathology of Some Rheumatic Diseases

The term rheumatic diseases comprises a large number of illnesses with the common feature of pain and loss or difficulty in the movement of joints.

Rheumatic diseases can be divided into two groups: The diseases which affect the joints are called arthropathies with the most common of this group being rheumatoid arthritis and osteoarthritis. The second group affects the connective tissues of other parts of the body such as skin, heart, blood vessels, etc., for example systemic lupus erythematosus and Reiter's syndrome.

1.2.1 The Arthropathies

Rheumatoid Arthritis

Is the most commonest and destructive of joint diseases and may attack at any age. A variant in childhood is called juvenile rheumatoid arthritis (Still's disease). The first noticeable change in the joint is the inflammatory condition of the synovial lining tissue in which small blood vessels become congested, resulting in swelling and hyperaemia (Gardner, 1970). The membrane forms enlarged highly vascular villi which often lie on the edge of the cartilage (Caygill, 1968). Fibrin collects on the synovial surfaces and may be deposited at any of the synovial or surrounding connective tissue as fibrinoid (Gardner, 1970).
The synovial membrane grows over the cartilage which may already be softened in the central areas replacing it by rough granulation tissue called pannus (Gardner, 1970). The villi may fuse with the pannus and occupy most of the joint, eventually total replacement of the cartilage by granulation tissue may take place. Ultimately fusion of the granulation

tissue with the opposed bones and its subsequent ossification may lead to complete loss of movement of the joint. The tendons and ligaments of the capsule may become affected and they or their attachment to the bone may weaken and rupture (Gardner, 1970).

Systemic lessions also occur in rheumatoid arthritis including; subcutaneous nodules usually over affected joints and a much enlarged lymphoarticular system. In extreme cases the lungs, skin and other organs may be affected, the disease is often characterised by the occurance of unusual macroglobulin in the plasma (Gardner, 1970).

Osteoarthritis

Osteoarthritis is an abnormality of synovial joints characterised by splitting and fragmentation of articular cartilage which is not directly attributable to an inflammatory process. Osteoarthritis is a degenerative joint disease, early changes may occur in areas of mechanical stress and trauma may exacerbate the damage leading to accelerated loss of cartilage matrix. Collagen fibres at the articular surface become loosened resulting in a characteristic fibrillation.

Eventually the gradual flaking of the cartilage and further release of matrix components leads to the exposure of bone (Sharp, 1978). There is no regeneration of the cartilage or replacement by granulation tissue as in rheumatoid arthritis. New bone synthesis, particularly at the edges of the articulating surfaces, may form outgrowths called osteophytes (Swinson, and Swinburn, 1980).

Calcification may involve the associated tendons and ligaments. Although causing severe pain and loss of mobility of the joints, the disease is rarely crippling as with rheumatoid arthritis.

Osteoarthritis is common in older people and is sometimes described

as "Wear and Tear" arthritis.

Ankylosing Spondylitis

Is an erosive arthropathy with a marked tendancy to bony ankylosis always involving the sacroiliac joints and the spine and is less common in the peripheral joints.

Ankylosing spondylitis occurs more commonly in males than females. The precise cause of the disease is unknown, but there is a strong familial and inherited tendancy and the genetic component of the disease is illustrated by the fact that 95% of ankylosis spondylitis patients have the tissue type HLA-B27 (Swinson and Swinburn, 1980).

Gout

Gout is an inflammatory arthritis due to the deposition of urate crystals within the soft tissue of the joint. Gout is a disease with strong familial associations seen predominantly in adult men (Scott, 1978). It is characterised by acute arthritis and later by chronic damage to the joint. The main cause for gout is hyperuricaemia (an excess urate in the blood and tissue).

1.2.2 Other Arthritic Diseases

Systemic Lupus Erythematosus (SLE)

This disease is characterised by widespread organ involvment, a variety of clinical manifestations and by a tendancy to exacerbation and remission. It is also associated with the prescence of anti-nuclear antibodies.

Systemic lupus erythematosus is rare in males and it mainly affects females. In systemic lupus erythematosus, like other auto-immune diseases, the body behaves as though it is allergic to its own tissue and becomes self-destructive. Damage may occur in the joints, muscles, nerves, skin, kidney, lungs, etc. Associated with this damage are many different types of antibodies in the patient's blood, for example anti-nuclear factor (ANF), anti-DNA and anti-lymphocyte antibody (Graham, 1978).

Rheumatic Fever

Rheumatic fever is caused by group A beta-haemolytic streptococci and the antibodies formed against the organism give an immune reaction with connective tissue components, notably the heart. Damage to articular cartilage also occurs but is usually completely resolved if the patient survives the cardiovascular attack (Bywater, 1978).

Reiter's Disease

Is a seronegative arthropathy of unknown causes affecting males predominantly in the third decades. It is characterised by recurrent attacks of polyarthritis with a pattern of joint involvement showing preference for the lower limbs, the sacroiliac joints and the spine. It is also associated with non-gonococcal inflammation of the genital tract, dysentry or non-specific diarrhoea (Wright, 1978).

1.3 Pathogensis of Rheumatoid Arthritis

To facilitate description of some important features of arthritic diseases it is necessary to outline the main anatomical features of synovial joints. The non-moving joints or synarthroses have no joint cavities or synovia whilst the moving or articulating joints, known as diarthroses, have joint cavities and synovia, (Davies, 1970). Both types are affected in arthritic disease but generally the diseased synovial joints give rise to greater discomfort. Figure 1.1 shows diagrammatically a typical normal joint, osteoarthritic joint and a rheumatoid joint.

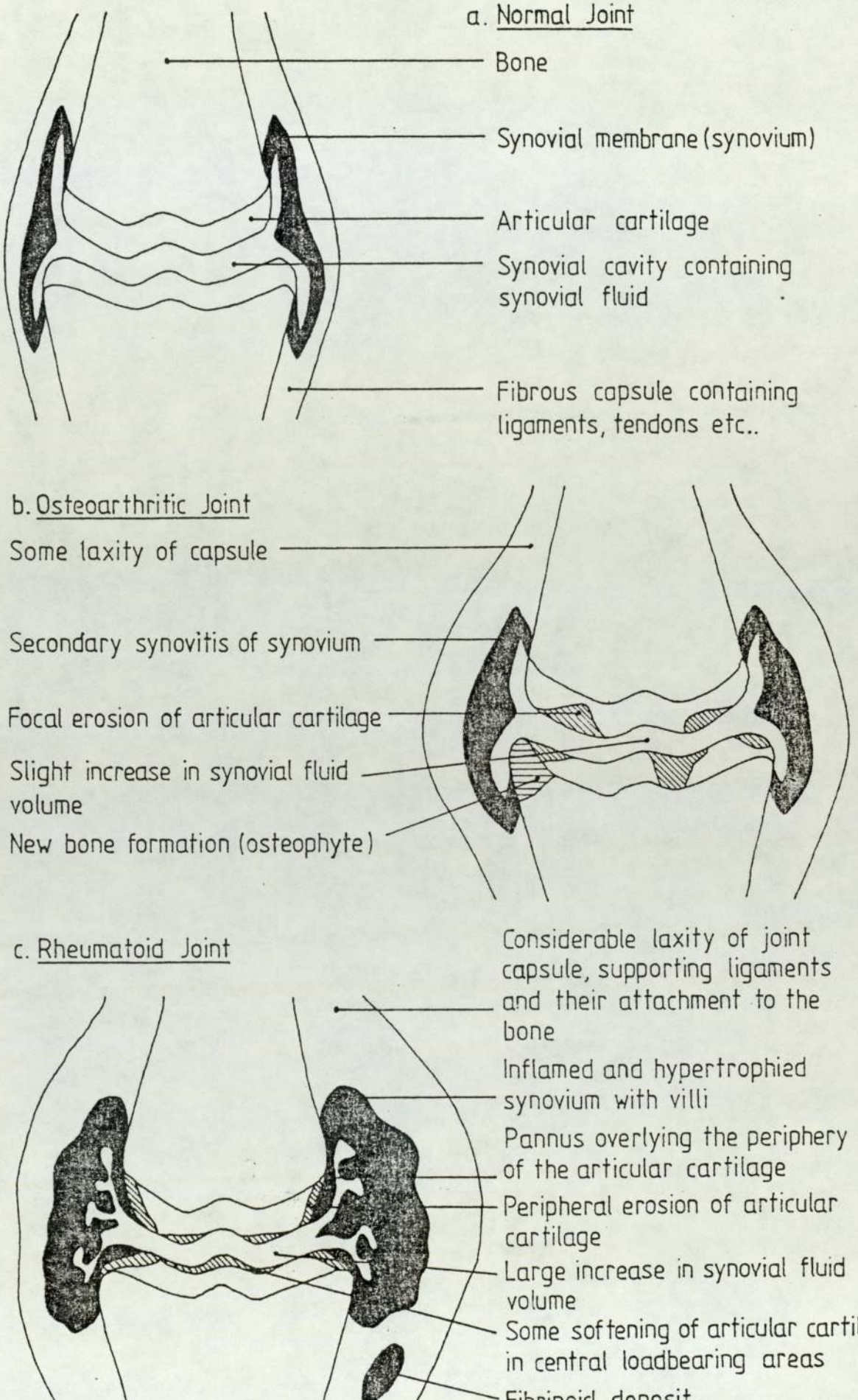
1.3.1 Normal Joints

A simple diarthrose joint consists of two bone ends covered by articular cartilage, and held together by a sleeve of white fibrous connective tissue - the joint capsule. The inner layer of this capsule consists of specialized connective tissue cells called the synovium. Within the joint space is a small amount of synovial fluid (Bole, 1979).

Articular cartilage is tough resilient connective tissue, consisting of cells embedded in a firm extracellur matrix which is made up of a sponge-like network of type II collagen fibres into which proteoglycans are tightly packed (Bole, 1979). Collagen fibres give the cartilage its strength and proteoglycans give its elasticity.

Collagen accounts for 50% of the dry weight of cartilage although it is not uniformly distributed (Anderson, Ludoweig, Harper and Engelman, 1964). The structure of collagen has been reviewed by Kuhn (1969). Its primary structure consists of a polypeptide chain composed of 33% glycine residues, 25% proline and hydroxyproline residues with the remainder consisting of the other amino-acids, including hydroxylysine. There are

Fig 1.1


a. Normal Joint
Bone
Synovial membrane (synovium)
Articular cartilage
Synovial cavity containing synovial fluid
Fibrous capsule containing ligaments, tendons etc..
b. Osteoarthritic Joint
Some laxity of capsule
Secondary synovitis of synovium
Focal erosion of articular cartilage
Slight increase in synovial fluid volume
New bone formation (osteophyte)
c. Rheumatoid Joint
Considerable laxity of joint capsule, supporting ligaments and their attachment to the bone
Inflamed and hypertrophied synovium with villi
Pannus overlying the periphery of the articular cartilage
Peripheral erosion of articular cartilage
Large increase in synovial fluid volume
Some softening of articular cartilage in central loadbearing areas
Fibrinoid deposit

both polar and non-polar regions in collagen, the polar regions have many repeating sequences of glycine-proline-hydroxyproline.

The fundamental unit of collagen is tropocollagen which is rod shaped (1.5 x 300 nm) and consists of three alpha-chains in triple helical configuration. Five different alpha chains with distinctive amino acid sequences are recognized: alpha 1 (I), alpha 2, alpha 1 (II), alpha 1 (III), and alpha 1 (IV). Each chain has a molecular weight of 95,000 - 100,000 daltons; four types of tropocollagen can be identified by their alpha chain content: type I contains (alpha 1 (I))$_2$ alpha 2; type II contains (alpha 1 (II))$_3$; type III contains (alpha 1 (III))$_3$; and type IV contains (alpha 1 (IV))$_3$.

These collagens demonstrate differences in carbohydrate content, the number of crosslinks and the degree of hydroxylation of prolyl - and lysyl residues. Tissue distribution of the four major types of collagen can be related to their structural differences and functional requirements within each tissue. In cartilage the collagen is type II and contains a high concentration of carbohydrates and is complexed with large amounts of proteoglycans. Chondroblasts are the only cells known to synthesize this form of collagen (Bole, 1979). The cellular and fibrillar components of connective tissue are embedded in an amorphous sol-gel continuum, known as ground substance consisting of proteoglycans.

Proteoglycans account for 5 - 20% of the dry weight of articular cartilage, but the actual amounts vary approximately inversely with the local concentration of collagen (Muir, Bullough and Maroudas, 1970). Proteoglycans consist of a long protein core to which glycosaminoglycans (GAG)are attached perpendicularly. 30 - 100 GAG chains are attached to the protein molecule. The GAG have been reported to have a molecular weight ranging from 2.3 x 10^5 daltons (Muir and Jacobs, 1967) to 3.2 x 10^6

daltons (Luscomb and Phelps, 1967). In cartilage GAG 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-glucoronic acid and N-acetyl-D-galactosamine in an alpha (1 - 3) linkage, each disaccharide unit is attached to the next by (1 - 4) linkage. Chondroitin sulphate has a sulphate ester group attached to either the 4 or 6 carbon position of N-acetly-D-galactosamine. In keratin sulphate the D-glucoronic 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. Synovial membrane is composed of two types of cells: Type A is phagocytic and the cells are lysosome rich with the appearance of macrophages and Type B cells which are rich in endoplasmic reticulum (Ziff, 1968). Type A cells carry out their phagocytic function as scavengers of waste materials, whereas Type B cells appear to be responsible for the synthesis of synovial fluid constituents (Hyaluronic acid and hyaluronate protein).

In rheumatoid arthritis the membrane becomes hypertrophic and oedematous with hypervasculation and the formation of large tufted villi of granulation tissue.

All joints contain a fluid, the synovial fluid, whose volume varies from joint to joint. In normal human adult knee the volume is 0.2 - 0.5 ml, but in rheumatoid arthritis there is usually a large increase to 5 - 30 ml (Caygill, 1968). The appearance of normal fluid is clear, colourless

or yellowish and viscid. Synovial fluid has two functions, firstly to provide nutrition to the avascular cartilage and secondly to act as a lubricant between cartilage surfaces.

Synovial fluid has a very high viscosity which decreases with the increasing shear rate and which may be greatly reduced in rheumatoid arthritis (Caygill, 1968). The high viscosity of synovial fluid is attributable to hyaluronic acid which is usually present as a sodium salt. The concentration of hyaluronic acid in normal fluid is about 0.3 - 0.4%, but this may be reduced to 0.07 - 0.1% in rheumatoid arthritis.

Electron microscopy indicates that synovial fluid is trapped in irregularities in the cartilage surfaces, thus maintaining a surface film even under high pressure. There may be an interaction between synovial fluid protein and hyaluronate-protein and cartilage (Hamerman, 1968). Under high pressure there may be a local slight increase in the concentration of hyaluronic acid protein complexes in the trapped pools by penetration of water into the cartilage (Hamerman, 1968). This may lead to an increase in the lubrication properties of synovial fluid (Hamerman 1968).

Normal synovial fluid contains about 2.5% protein which is considerably lower than the normal serum protein concentration of 7% (Norton and Ziff, 1966). Most of the protein components appear to be derived from serum (Norton and Ziff, 1966), but are present in different proportions. In general the concentration of high molecular weight molecules is lower in synovial fluid than in serum (Norton and Ziff, 1966).

In rheumatoid arthritis the synovial fluid protein concentration may be increased to 4.7% and the relative concentrations of individual components are nearer those in serum (Davies, 1970). The concentration of gamma-globulins (Ig) is particularly high in rheumatoid synovial fluid and some may be synthesized locally (Caygill, 1968).

The pH of normal synovial fluids is 7.8 when measured in vitro and 7.5 when measured in vivo and is only slightly lower in rheumatoid patients (Caygill, 1968).

Normal synovial contains a small number of leucocytes usually not exceeding 60 cells/mm^{-3}. The cell count is greatly increased in rheumatoid arthritis (Norton and Ziff, 1966), being 5,000 - 35,000/mm^{-3}. In most fluids more than 75% of the cells are polymorphonuclear leucocytes.

1.3.2 The Rheumatoid Joint

Human rheumatoid joints are generally swollen and inflamed. This is due to increased permeability (proliferation) of synovial tissue, leading to a build up of oedema fluid, the influx of inflammatory plasma cells and the release of inflammatory mediators.

In rheumatoid arthritis the development of earliest lesion, the pannus, is characterized by excess connective tissue proliferation of the synovial lining cells, extensive vascularization, increased number of synthetic mesenchymal cells and infiltration by lymphocytes, plasma cells, and macrophages (Pearson, Paulus, Machleder, 1975; Harris, 1976).

The first sign of cartilage destruction is the loss of proteoglycans, the hydrated "packing material" between the collagen fibrils. The degradation of proteoglycan can occur in several ways. The long hyaluronic acid backbone could be cleaved by hyaluronidase and so release individual proteoglycan subunits. Mammalian hyaluronidase is capable of attacking the chondroitin sulphate and keratin sulphate side chains releasing small fragments of four or more sugar molecules. However, hyaluronidase activity has not yet been detected in cartilage tissue (Woessner, Sapolsky, Nagase and Howell, 1977). Hyaluronidase tends to be active at slightly acid pH, indicating a role in intracellular digestion (Bollet, Bonner and Nance,

1963). Proteolytic enzymes digest the protein backbone of the proteoglycan subunit releasing peptide fragments containing one or more polysaccharide side chains, Malemud and Jonoff (1975) reported degradation of proteoglycans by elastase and cathepsin G. Cathepsin D which also degrades proteoglycans was detected in the pannus extracellularly (Poole, Hembry, Dingle, Pinder, Ring and Cosh, 1976). Sapolsky, Keiser, Howell and Woessner (1976) described three metalloproteases from cartilage which were able to digest the proteoglycans.

The depletion of proteoglycan is reversible, but until this component is replaced the collagenous framework of the tissue lacks its normal protection against mechanical stresses and enzyme attack. These processes can then cause damage to the basic structure of the tissue (cartilage) which is irreversible.

Morphological evidence confirms that cartilage destruction occurs exactly under the point of attachment of the pannus. Harris, Di Bona and Krane (1970) have shown that there is loss of collagen fibrils in the cartilage ahead of the advancing pannus, suggesting that enzymatic degradation of fibres is the first step in joint structural damage (Krane, 1974). Proteinase reaching the articular cartilage from the synovial fluid would cause generalized degradation of the tissue.

A thinning of the cartilage is commonly observed radiologically (Martell, 1974) in joints affected by rheumatoid arthritis and may precede the formation of an invasive synovial pannus (Hamerman, 1969).

The most likely cause of cartilage erosion is its degradation by enzymes. Enzymes could arise from tissue lining the joint space or from inflammatory cells that have entered from outside the area or from extravasation. Enzymes might also arise from the synvoial membrane that covers the cartilage or from fibroblast-like cells in the pannus that

overgrows the cartilage in rheumatoid arthritis. In osteoarthritis destructive enzymes arise from cartilage itself (Woessner, Sapolsky, Nagase and Howell, 1977).

Cytochemical techniques have demonstrated that rheumatoid synovial lining cells and the 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).

Several mechanisms have been proposed for the release of lysosomal constituents from polymorphonuclear leucocytes. One mechanism is "cellular death", when polymorphonuclear leucocytes are exposed to a variety of toxins. Injury to the plasma membrane is an early consequence and all intracellular materials are released from injured cells (Weissmann, 1977). Under these conditions, cytoplasmic enzymes, potassium and other cellular constituents, in addition to lysosomal hydrolases, find their way into the surrounding tissues.

Another mechanism is perforation of the cell by which materials gain access to the internal vascular system of the cell where they cause lysosomal membranes to rupture. Damage to the organelles leads to the release of their enzymes and the death of the cell. Crystalline substances, such as monosodiumurate, act upon phagocytic cells in this fashion (Weissmann and Rita, 1972).

When polymorphonuclear leucocytes ingest insoluble immune complexes or other particulates, a phagosome is formed which merges at its internal border with primary lysosomes to form secondary lysosomes (Cohn and Fedorka, 1969). Either because of incomplete fusion of vascular membrane or the persistance of endocytic channels, regurgitation of lysosomal hydrolases occurs and inflammatory materials are released into the

surrounding tissue without associated phagocytic cell death or release of cytoplasmic enzymes. This mechanism has been termed as "regurgitation during feeding". Weissmann, Zurier and Hoffstein (1972); Weissmann, Goldstein and Hoffstein (1975); and Ohlsson (1975) have shown that elastase and collagenase are regurgitated by this mechanism.

Another proposed mechanism of selective lysosomal enzyme release from polymorphonuclear leucocytes has been termed "reverse endocytosis" (Weissmann, Zurier and Hoffstein, 1972; Weissmann, Goldstein and Hoffstein, 1975) of "frustrated phagocytosis" (Henson, 1974). In this process material previously stored within lysosomes is exported to external milieu, where cells encounter immune complexes (both soluble and insoluble) or aggregated immunoglobulins deposited on solid surfaces (for example, collagen membranes) (Hawkins, 1971). They adhere to the surface of the immunoglobulins and selectively release their lysosomal constituents.

1.3a Cellular Origin of Degradative Proteinases in Joint Diseases

Degradative enzymes originate from almost any of the articular cells (resident cells: synovium, chondrocytes and non-resident cells in the rheumatoid synovial effusion, polymorphonuclear leucocytes (PMNs), lymphocytes).

There is a current debate on the origin of these enzymes in the arthritic joints, some theories suggest that the destructive enzymes causing erosion of cartilage and bone at the margins of the articulating surface in rheumatoid arthritis arise from adjacent soft tissue or from cells infilterated in the synovial fluid, that is the enzymes are extrinsic, arising outside the target cells. In osteoarthritis the lack of cellular infilteration of the synovium or synovial fluid makes an intrinsic breakdown by enzymes from chondrocytes themselves (Barrett and Saklatvala, 1981).

In cross sections through active rheumatoid synovial villi the heterogeneity of the cells are obvious, there are increased numbers of endothelial cells because there are many blood cells, phagocytic cells in the synovium could be both synovial lining cells and bone marrow-derived macrophages. The synovial lining cells show a marked increase in type B cells (fibroblast-like) and those containing Golgi cells and moderate numbers of lysosomes as well as developed endoplasmic reticulum. Type A cells (macrophage-like) in these tissues they have a different appearance from normal cells, they have fewer cell processes and contain swollen distorted mitochondria and numerous membrane-bound granular dense bodies which have been interpreted to be phagolysosomes (Harris, 1981).

In recent years the cells in the rheumatoid synovium have been characterized by studying them in freshly dissociated form, those adherent cells have a morphological appearance characterized by large and numerous branching processes. The terms dendritic and stellate have been used to describe those cells. Culture of those cells produce a high level of latent collagenase and prostaglandin (PGE_2) which play an important role in the degradation of extracellular matrix macromoleules (Krane, 1981).

Interaction of adherent stellate cells with lymphocytes and monocytes in culture could alter the funtion of the stellate cells, these interactions are at least in part mediated by soluble cell factors such as a monocyte derived factor termed as mononuclear cell factor (MCF).

This factor will stimulate stellate cells to produce more latent collagenase and PGE_2 (Krane, 1981). Both collagenase and prostaglandin production by these cells are inhibited by low doses of dexamethasone, colchicine has a stimulatory action on secretion by organ cultures of rheumatoid synovium. The majority of the adherent cells do not produce lysozyme. Macrophages which are one of the characteristic cell types in the inflammatory response produce lysozyme, many cells in the invasive pannus appear to be macrophages (Harris, 1981).

Activation of macrophage could occur in rheumatoid synovial tissue through several systems. Products of activated T-lymphocytes activate macrophages to release a number of mediators, including enzymes such as collagenase and plasminogen activator. Once the macrophages are activated in the synovial tissue the products of activation can have multiple roles in the production and perpetuation

of rheumatoid arthritis (Harris, 1981).

Macrophages can play an important role in modulating the response of lymphocytes to antigenic stimulation, interferon and polyamine oxidase released by macrophages suppress the immune response of lymphocytes, while proteinases or prostaglandins released by synovial cells or macrophages have a stimulatory effect (Denman, 1979).

Non resident cells in rheumatoid synovial inflammation

Polymorphonuclear Leucocytes (PMNs).

PMN leucocytes migrate immediately into the synovial fluid to which they are drawn by chemotactic factors. Active arthritis is accompanied by an increase in the volume and cellularity of synovial fluid. The mildly inflamed rheumatoid joint has a total cell count below 5,000 leucocytes per mm^3, while the actively inflammed joint commonly has a cell count in excess of 50,000 leucocytes per mm^3 of which over 80% of the cells are PMN leucocytes (Vernon-Roberts, 1979). It has been calculated that the larger number of PMN leucocytes could bring in large amounts of proteinases which eventually saturate the capacity of proteinase inhibitors present in synovial fluid, and enzyme activity (e.g. collagenase) will be unopposed.

Although PMN leucocytes are involved in phagocytosis in rheumatoid joints, there is evidence that the PMN leucocytes in peripheral blood from patients with rheumatoid arthritis are deficient in their capabilities for both phagocytosis and chemotaxis, this impairment can be attributed both to the prior ingestion of immune complexes and to cellular metabolic changes (Harris, 1981).

When PMNs are present in large amounts and active form in the

inflammatory sites, the superoxide ion (O_2^-) will be generated during phagocytosis which could damage other PMN leucocytes in the area leading to cell death and lysis and perpetuation of synovitis by irritating effect of lysosomal enzymes (Harris, 1981).

During acute gout chemotactic activity appears rapidly in the synovial fluid. This activity is due mainly to neutrophils phagocytizing monosodium urate crystals. Human neutrophils when exposed to urate or calcium pyrophosphate crystals release a glycoprotein (M.W. 8,400 daltons) which is chemotactic for neutrophils and monocytes (Goetzl, 1981). This factor appears in the lysosomal granular fraction during phagocytosis and is released when the crystals are taken up by endocytosis. It is of interest that the production and release of the factor can be inhibited by protease-esterase inhibitors (Spilberg, Gallacher, Mandell and Rosenberg, 1977).

In experiments with rabbits injection of the purified factor into joints led to an influx of neutrophils into the synovial tissues. The magnitude of the response was similar to that induced by an injection of monosodium urate crystals but the peak response for factor was achieved by 90 minutes compared to 300 minutes with the crystals (Goetzl, 1981).

Clearly in inflammatory disease PMN leucocytes contribute massively to the inflammation characteristics in specific disorders such as rheumatoid arthritis and gouty arthritis.

The Lymphocytes

Studies of lymphocytes in rheumatoid synovium and in rheumatoid blood and/or synovial fluid have generated evidence that these cells

are targets for the primary etiologic agent in rheumatoid arthritis. This evidence includes: the presence of lymphocytotoxic antibodies in blood, spontaneous activation of lymphocytes from rheumatoid synovium in vitro at a rate greater than that found in cultures of non-rheumatoid lymphocytes and the presence of an antibody to rheumatoid arthritis, nuclear antigen (RANA) found in lymphocytes transformed by Epstein-Barr virus, in many patients with rheumatoid arthritis (Harris, 1981).

The T-lymphocytes appear to be the predominant lymphocyte population and only a few B-lymphocytes are found (Thorsteinsson, Abrahamsen, Froland and Kass, 1981). T-lymphocytes sensitized by products of macrophages, or by processed antigen from macrophages, would have the capability to mediate a number of functions including the secretion of biologically active soluble products such as lymphokines. In rheumatoid arthritis there is evidence for lymphokine activity in synovium which includes the following effects:

1. increase in vascular permeability.
2. a lymphokine mediated chemotactic effect for granulocytes and monocytes.
3. activation of monocyte-macrophages, osteoclasts and synovial cells (Harris, 1981).

Immunoblasts (they are larger pyroninophilic lymphoid cells) are found in the synovial fluid of patients with active inflammatory arthritis (both seropositive and seronegative). These cells are not found in patients with non-inflammatory osteoarthritic effusions and they are not found in patients with crystal-induced arthritis (including gout). The origin of these cells in peripheral blood is unknown but similar cells are seen after infection or immunisation (Eghtedari, Bacon and Collins, 1980).

1.4 Role of Enzymes in Tissue Damage

A variety of proteinase are capable of breaking down cartilage at neutral pH and have been isolated from human polymorphonuclear leucocytes. They include a cartilage mucopolysaccharide degrading protease (Oronsky, Ignarro and Perper, 1973), an elastase (Janoff, 1973; Ohlsson and Ohlsson, 1974b), a collagenase (Ohlsson and Ohlsson, 1974a), chymotrypsin cationic proteins (Rindler-Ludwig and Braunsteiner, 1975) and cathepsin G (Schmidt and Havemann, 1974).

1.4.1 Collagenase

Collagenase plays a crucial role in the pathophysiology of rheumatoid arthritis. It was first detected in tadpole tails (Gross and Lapiere, 1962; Lapiere and Gross, 1963). It was later isolated from human skin (Fullmer, Lazarus, Gibson , 1966; Riley and Peacock, 1967); synovial tissue (Evanson, Jeffery and Krane, 1967; Lazarus, etal, 1968a); polymorphonuclear leucocytes (Lazarus, Brown, Daniels ., 1968b,) ; synovial fluid (Ohlsson, 1975; Harris and McCroskey, 1974; Woolley, Glanville, Grossley and Evanson, 1975); rheumatoid nodules (Harris, 1972) and rabbit synovial fibroblast-like cells (Werb and Burleigh, 1974).

Mammalian collagenase cleave the triple helix of tropocollagen at a single locus to produce characteristic TC^{A} and TC^{B} fragments (Steven, Torre-Blanco and Hunter, 1975) which are referred to as $TC^{\frac{3}{4}}$ and $TC^{\frac{1}{4}}$ respectively, at "neutral" pH (7 - 8) at 37°C. With human cartilage collagen fibrils and rheumatoid synovial collagenases (Harris and McCroskey, 1974; Harris and Krane, 1973), a 10 fold increase in the rate of collagen degradation was found with an increase in incubation temperature from 30 - 36°C. Since inflammation in rhemuatoid joints is associated with increases in

temperature, this may lead to an increase in the rate of degradation of important supporting tissues.

Two distinct synovial collagenases were found. One enzyme "A" with a high molecular weight (750,000 daltons), was resistant to inhibition by serum (Harris and Krane, 1974). The other, enzyme "B", was inhibited by serum and appeared identical to a collagenase found in synovial cell culture, with a molecular weight (33,000 daltons) (Woolley, Glan ville, Grossley and Evanson, 1975). The origin of the larger "A" enzyme is not known. It is possible that it is a dimer of the smaller enzyme or it could originate from polymorphonuclear leucocytes (Daniel and Lazarus, 1969).

Although there is strong evidence that collagenase is involved in the first stage of collagen degradation the biochemical mechanisms controlling the activity of this enzyme are not clear. It seems likely that collagenolytic activity is regulated by multiple pathways. Collagenase exists in latent and active forms; collagenases are released in latent precursor forms from cells which synthesize them. Dayer, Krane, Russell and Robinson (1976) have isolated latant collagenase from rheumatoid synovial cells, latent collagenases were also isolated from bone (Vaes, 1972; Vaes and Eechout, 1975; Eechout and Vaes, 1974) and polymorphonuclear leucocytes (Oronsky, Perper and Schroder, 1973; Kruze and Wojtecka, 1972; Williams, Lin and Perper, 1976).

Explanations for this latency have been of two kinds, the latent collagenase is either:

a. a pro-enzyme (Zymogen) form that needs proteinase for activation (Harper and Gross, 1972; Kruze and Wojtecka, 1972; Oronsky, Perper and Schroder, 1973), or

b. an enzyme inhibitor complex (Bauer, Stricklin, Jeffrey and Eisen, 1975; McCroskey, Richards and Harris, 1975; Woolley, Roberts and

Evanson, 1976; Shinkai, Kawamoto, Hori and Nagai, 1977). Extracellular activity of the collagenase might be controlled by another proteinase. Vaes (1972) reported the activation of latent collagenase by trypsin. Eechout and Vaes (1977) proposed that cathepsin B (lysosomal proteinase), Kallikrein and plasmin (humoral proteinase) may play a role in the physiological activation of latent collagenase. This may occur irrespective of the nature of procollagenase present, for example zymogen or enzyme inhibitor complex. It was found by Vater, Mainardi and Harris (1978) that latent rheumatoid synovial collagenase was capable of binding to fibrillar substrate while remaining incapable of catalysis of cleavage of the triple helix. Synovial collagenase like any other metalloproteinase could be inhibited by Ca^{2+} chelating agents, for example ethylenediamine-tetra-acetic-acid (EDTA), thiol agents (cysteine) (Evanson, Jeffrey and Krane, 1968) and by the serum components alpha-2-macroglobulin, alpha-1-antitrypsin and beta-1-anti-collagenase (Nagai, 1973; Werb, Burleigh, Barrett and Starkey, 1974; Ohlsson, 1975; Woolley, Roberts and Evanson, 1976).

1.4.2 Elastase

The elastase of neutrophil granulocytes is a serine proteinase that may play a part in important physiological processes such as the digestion of bacteria by phagocytes (Janoff and Blondin, 1973), the degradation of elastin in the arterial basement wall and emphysematous lung, the degradation of kidney basement membrane in glomerulonephritis and the destruction of articular cartilage in rheumatoid arthritis (Janoff, 1972). This cationic enzyme, together with collagenase, constitutes 5% of the dry weight of the polymorphonuclear leucocytes and was first of the proteinases to be well characterized (Janoff, 1972). An elastase with a molecular weight of 34,000 can be isolated from polymorphonuclear leucocytes (Ohlsson, 1975; Janoff, Blondin and Sandhaus, 1975). It hydrolyses typical pancreatic elastase substrates such as Boc-Ala-ONp and AC-$(ALa)_3$-NAN, and a variety of elastin

preparations from tendon, lung and basement membrane (Janoff, Blondin and Sandhaus, 1975). The pH optimum for elastase activity is 8.5 - 8.8. Elastase has broad substrate specificity; it appears to attack insoluble collagen (Barrett, 1975), and acts in concert with a specific collagenase. Both bacterial cell walls and cartilage proteoglycans can be digested by purified elastase (Janoff, Blondin and Sandhaus, 1975). Elastase is inhibited effectively by alpha-1-antitrypsin and alpha-2-macroglobulin; 92% of added elastase is bound by serum alpha-1-antitrypsin, 8% by alpha-2-macroglobulin (Ohlsson, 1975a). Polymorphonuclear leucocyte elastase is also inhibited by some of the chloromethyl inhibitors, especially N-acetyl-L-analyl-L-prolyl-L-alanine chloromethyl ketone (NAc AA PACK), gold thiomalate, and the newly described elastinal (Janoff, Blondin and Sandhaus, 1975; Umezawa, Aoyagi and Okura, 1973). Both collagenase and elastase are extruded into the incubation medium of polymorphonuclear leucocytes exposed to immune complexes (Ohlsson, 1975).

1.4.3 Cathepsin G (Chymotrypsin-like enzyme)

Polymorphonuclear leucocytes contain neutral proteinasein addition to collagenase or elastase. Cathepsin G was isolated by Janoff (1975), and characterized by Rindler and Braunsteiner (1973) from polymorphonuclear leucocytes. Cathepsin G is a basic protein of molecular weight 28,000. It is not active against elastin, and the pH optimum is near pH 7.5 (Barrett, 1975). The enzyme hydrolyses cartilage proteoglycan and attacks insoluble collagen (Barrett, 1975). It is inhibited by alpha-1-antitrypsin and di-isopropyl-fluorophosphate (Janoff, Blondin and Sandhaus, 1975).

1.4.4 Cathepsin D (Carboxyl proteinase)

This enzyme has been shown to be present in lysosomes (Weissman, Zurier and Hoffstein, 1972) and polymorphonuclear leucocytes (Ishikawa and Cimasoni, 1977). This enzyme has a pH optimum of 3.0 - 3.5 against denatured

haemoglobulin, but pH 5 against cartilage proteoglycan (Barrett, 1975). It has a molecular weight of 42,000. Poole, Hembry and Dingle (1974) were able to localize this enzyme not only in lysosomes of resting cells, but also in extracellular spaces following its secretion. Johnston and Greenbaum (1973) have shown that cathepsin D can cleave leukokininogens present in inflammatory exudates and generate pharmacologically active leukokinins.

1.5. Other Inflammatory Mediators

Prostaglandins are other inflammatory mediators found in synovial fluid. Husby, Bankhurst, Williams (1977) used an immunohistochemical technique to demonstrate the widespread distribution of prostaglandin E in lymphocytes, synovial lining cells and the intimal part of small blood vessels of rheumatoid synovial membrane.

In the early stages of the inflammatory response prostaglandins potentiate the process, causing vasodilation, increasing capillary permeability and to a certain extent facilitating cellular mobilization (Bonta and Parnham, 1978).

Prostaglandins in low doses (subthreshold) have been shown to produce sensitization of the inflammatory properties of other mediators, such as histamine, 5-hydroxy-tryptamine and bradykinin (Vane, 1976; Flower, 1977). Both prostaglandin E_1 and E_2 are suspected of being involved in the process of bone resorption (Robinson, McGuire and Levine, 1975).

Other mediators of inflammation which have been reported in the synovial fluids from patients with rheumatoid arthritis or gouty arthritis are the Kinins (Keele and Eisen, 1970). These are small peptides, formed by the action of specific proteolytic enzymes known as Kallikreins or Kininogens. Minute doses of Kinins act on the microcirculation causing capillary vasodilation, increased vascular permeability and pain (Lewis,

1963). In untreated rheumatoid patients, plasma kininogen levels are greatly raised but are rapidly returned to normal by treatment with indomethacin or aspirin (Brookes, Dick, Sharma and Zeillin, 1974).

1.6 Role of Rheumatoid Factor in Tissue Damage

Rheumatoid factors are immunoglobulins with antibody specificty for antigenic sites on the Fc part of the heavy chain of IgG.

Waaler (1940) observed a factor present in the sera of some patients with rheumatoid arthritis, which agglutinated sheep erythrocytes coated with anti-erythrocyte antibody from rabbit sera. The agglutinating factor (rheumatoid factor) was identified as an immunoglobulin (Ig) whose antibody activity was directed towards antigenic sites on IgG.

Rheumatoid factors that occur in the sera of patients or in apparently healthy subjects can belong to the IgM, IgG or IgA class. Rheumatoid synovium has been shown to produce IgM rheumatoid factor and IgG and to contain immune complexes consisting of IgM, IgG and C_3 (Schur , Britton, Franco, Carson, Sorman and Ruddy, 1975; Taylor-Upsahl, Abrahansen and Natvig, 1977). The concentrations of these complexes are greater in the synovial fluids than in the sera of rheumatoid patients.

In patients with rheumatoid arthritis, high titres of IgM rheumatoid factor show a correlation with the severity of disease and the frequency of complications (Sievers, 1965; Mongan, Cass, Jacox and Vaughan, 1969). Patients with nodules almost always are positive for IgM rheumatoid factor. In some patients negative tests may be due to "hidden rheumatoid factor" due to binding to autologus IgG and are only revealed to be positive after suitable treatment of sera (Allen and Kunkel, 1966).

The pathogenic role of rheumatoid factor in rheumatoid arthritis has not been clearly defined, although most investigators believe that it is intimately involved in the inflammatory reaction leading to tissue damage in this disease (Weissman, 1972) as shown in Figure 1.2.

Christian (1961) considered that rheumatoid factors were probably byproducts of the disease as in the case for the Wassermann antibody found in syphilis. Some evidence has been found to support this view, for example the transfusion of rheumatoid factor into healthy volunteers produced no ill effects (Harris and Vaughan, 1961).

Hollander, McCarty, Astroga and Castro-Murillo (1965) suggested that if native IgG is denatured due to some unknown pathological influence, antibodies such as IgM will be formed against the denatured IgG leading to the formation of high molecular weight antigen-antibody complexes of IgG-IgM. These complexes would be phagocytosed by leucocytes causing a consequential loss of lysosomal enzymes from leucocytes. This may be due to enhanced phagocytosis or to the lysosomes being unable to cope with high molecular weight complexes resulting in the release of enzymes into extracellular space.

Zvaifler (1965) suggested that some unknown event initiates inflammation causing the disruption of lysosomes and leakage of lysosomal enzymes. These enzymes could alter leucocyte nucleoprotein and consequently antibodies would be raised against the altered nucleoprotein. Antigen-antibody complexes would be formed and these would be engulfed by leucocytes leading to a further loss of lysosomal enzymes which would cause inflammation and tissue injury and complete a cycle by altering more nuclear material.

The role of complement activation in rheumatoid synovial fluid is probably central to understand certain inflammatory pathways in rheumatoid joint inflammation (Zvaifler, 1973). Synovial fluid complement has been

SEQUENCE OF TISSUE INJURY IN RHEUMATOID ARTHRITIS

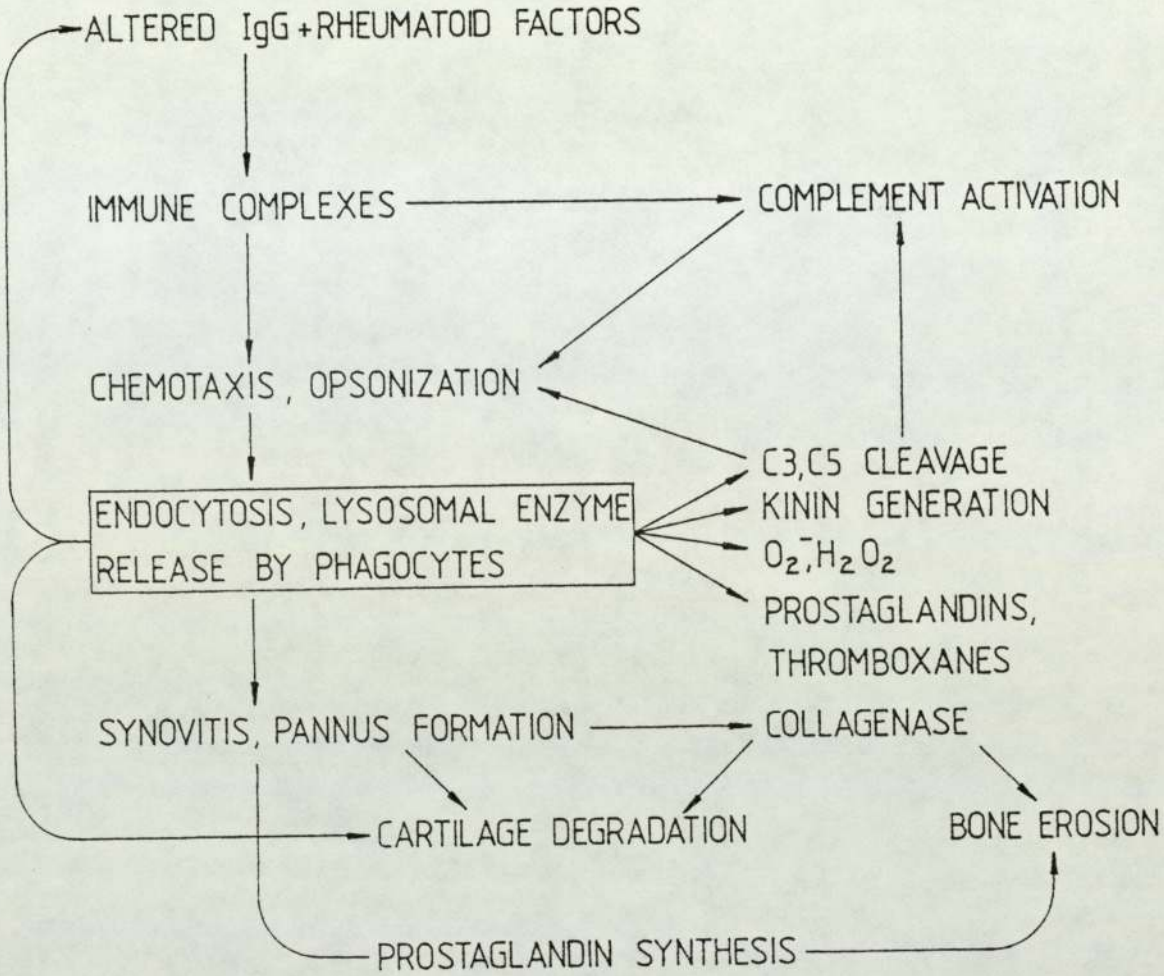


Fig 1.2 Role of lysosomal enzyme release in the pathogenesis of rheumatoid arthritis

shown to decrease in rheumatoid arthritis, which has been correlated with the severity of joint damage. Complexes of IgM, IgG and C_3 have been found in phagocytic cells in the joint fluid (Schur , Britton, Franco, Carson, Sorman and Ruddy, 1975; Zubler, Nydegger, Perrin, Fehr, McCormick, Lambert and Miescher, 1976; Carson, Lawrence, Catalno, Vaughan and Abraham, 1977).

The activation of complement produce chemotactic factors which attract polymorphonuclear leucocyte cells into the synovial effusion. The activation of complement also produces C_3a, which increases vascular permeability leading to an accumulation of fluid in the joint cavity (Ziff, 1977). IgM rheumatoid factor has been shown to fix complement, which enables it to participate in the inflammatory reaction (Tanimoto, Cooper, Johnson and Vaughan, 1975; Nydegger, Zubler, Gabay, Joliot, Karagerekis, Lambert and Miesher, 1977; Kaplan, DeHeer, Mullen and Vaughan, 1978).

Winchester etal (1970) have shown that IgG rheumatoid factors in rheumatoid synovial fluids are the main complement fixing constituents. Differences in complement fixing between IgM and IgG rheumatoid factors from different clinical sources have been found with the lowest complement fixing ability being associated with rheumatoid factors from apparently healthy sources (Schur , Bianco and Pansuh, 1975).

There is evidence that complexes containing IgG rheumatoid factor can be engulfed by synovial fluid leucocytes and synovial membrane macrophages irrespective of whether or not they contain IgM. This could be the reason why rheumatoid patients with no plasma IgM rheumatoid factor (seronegative) suffer from the disease, since they still have IgG rheumatoid factor in their plasma (Mellbye and Natvig, 1971).

1.7 Antiproteinases

Antiproteinases are considered as endogenous anti-inflammatory substances since they occur naturally and may play a role in inflammation. Human serum has long been known to inhibit the activity of several proteolytic enzymes. The overall activity of proteinases is the result of activity of the proteinases with any proteinase inhibitors present. During the past decade it has been possible to subdivide the antiproteinase activity of human serum into several individual inhibitors with different specificities (Heimburger, Haupt and Schwick, 1971).

1.7.1 Alpha-1-Antitrypsin (alpha-1-antiprotease)

Alpha-1-antitrypsin was first isolated, characterized and named by Schultz, Heide and Haupt (1962). Alpha-1-antitrypsin is normally present in human serum in a concentration of (54.0 mc mol/litre) which considerably exceeds the molecular concentrations of all other serum protease inhibitors (Heimburger,1975; Brackertz, 1978), and it is responsible for up to 90% of the trypsin inhibiting capacity of the plasma (Ganrot, 1972).

Alpha-1-antitrypsin is a low molecular weight (47,500 - 55,000 daltons) (Crawford, 1973; Musiani and Tomasi, 1976) glycoprotein, consisting of a single polypeptide chain with four carbohydrate side chains of two structural types (Chan and Rees, 1975). The carbohydrate portions comprise 12% of the total molecular mass (Heimburger, 1975) and contain D-galactose, D-mannose, N-acetyl-diglucosamine and sialic acid.

Alpha-1-antitrypsin is an"acute phase reactant" protein which appears to have a protective function in that it neutralizes proteolytic enzymes released into body fluids during inflammation. A rapid rise in alpha-1-antitrypsin has been observed during acute inflammation and severe

infection (Sharp, 1971).

A large molecular protein with the immunological specificity of alpha-1-antitrypsin, presumably a complex of the inhibitor and proteinase, was detected in the synovial fluid of subjects with confirmed or suspected rheumatoid arthritis (Brackertz, Hagmann and Kueppers, 1975).

Alpha-1-antitrypsin was found to inhibit and/or neutralize a large number of proteolytic enzymes extracellularly, such as granulocyte elastase and collagenase (Lieberman and Kaneshiro, 1972; Kueppers and Black, 1974; Ohlsson, 1971 and 1975), Kallikrein and cathepsin G and D (Brackertz, 1978) chymotrypsin, plasmin and thrombin (Rimon, Shamash and Shapiro, 1966) and synovial collagenases (Harris, DiBona and Krane, 1969),(all are enzymes associated with inflammation), in a molar ratio of 1:1. Not all collagenases are bound by alpha-1-antitrypsin, for example a neutral collagenase isolated from human gastric mucosa is not inhibited by alpha-1-antitrypsin (Woolley, Tucker, Green and Evanson, 1976), and neither are the acid proteases (Kopitar and Lebez, 1975).

Alpha-1-antitrypsin can exist in a large number of genetic varients of which the so called MMtype is the form found in normal individuals. A slowly migrating component called ZZ is seen electrophoretically in cases of genetic deficiency. Some deficient phenotypes of alpha-1-antitrypsin are important markers of susceptibility to diseases, for example severe alpha-1-antitrypsin deficiency has been traditionally associated with emphysema (Laurell and Eriksson, 1963) and chronic liver disease (Berg and Eriksson, 1972).

Recent information suggests that even a mild degree of alpha-1-antitrypsin deficiency is associated with many inflammatory and immunologically mediated conditions such as rheumatoid arthritis (Cox and Huber, 1976 Buisseret, Pembrey and Lessof, 1977).

Lewis and Capstick (1977) reported that there is an inverse relationship between alpha-1-antitrypsin and neutral proteinase levels in human rheumatoid synovial fluid. A similar relationship was demonstrated in the sera from adjuvant arthritic rats, the alpha-1-antitrypsin levels falling as the disease progressed (Parrot and Lewis, 1977).

Increased alpha-1-antitrypsin levels are also formed during pregnancy (Ganrot and Bj.erre, 1967; Fagerhol and Laurell, 1970), during use of contraceptive medication (Laurell, Kullander and Thorell, 1968; Lieberman and Mittan, 1973) and viral hepatitis (Kindmark and Laurell, 1972).

1.7.2 Alpha-2-Macroglobulin

Alpha-2-macroglobulin is a wide spectrum endopeptidase inhibitor (Barrett and Starkey, 1973). Its plasma concentration varies from (2.5 - 5 mc mol/litre), and it accounts for nearly 10% of the trypsin inhibitory capacity of plasma (Ganrot and Scherstén, 1967). Its molecular weight is in the range 725,000 - 820,000 daltons but the information about its quaternary structure is incomplete. Several studies suggest an eight chain subunit structure with dimers of these chains forming stable quarter molecular intermediators, each with a molecular weight of 196,000 daltons (Jones, Greeth and Ketwick, 1972). Because of its high molecular weight it is partially excluded form joint cavities (Kushner and Somerville, 1975). The smaller inhibitors might play important roles as antiproteases due to increased diffusion rate associated with their lower molecular weight.

Alpha-2-macroglobulin and alpha-2-macroglobulin-protease complexes have been identified in a variety of body fluids, including plasma, synovial fluid (Abe and Nagai, 1973; Hamberg, Vahtera and Moilanen, 1978), and peritoneal fluid (Aubry, Travis and Bieth, 1977). Some complexes are

thought to play a fundamental role in the activation of proenzymes (Aubry, Travis and Bieth, 1977). In a recent investigation by Cassiman, Leuven, Schueren and Berghe (1980) they demonstrated that alpha-2-macroglobulin can be detected in the connective tissues of most organs.

The complexes with proteinase are rapidly cleared from the blood by the reticuloendothelial cells of the liver (Ohlsson, 1971) and the degradation of the entrapped proteinase has been demonstrated to occur in the lysosomes of the Kupffer cells (Katayana and Fujita, 1974).

Alpha-2-macroglobulin levels are elevated in the plasma during pregnancy (Schumacher and Schulmberger, 1963). Alpha-2-macroglobulin was detected in synovial fluid, but in about 25% of the patients the inhibitor was present in an inactive form (Shtacher, Maayan and Feinstein, 1973).

Lewis and Parrott (1976) reported an increase in trypsin protein esterase (alpha-1- and alpha-2-macroglobulin) in plasma of rats with adjuvant induced arthritis, the antiprotease levels appeared to rise in response to inflammation.

1.7.3 Beta-1-Anticollagenase

Beta-1-anticollagenase has been discovered in human plasma by Woolley, Roberts and Evanson (1976). It migrates electrophoretically as a beta-1-globulin, with a molecular weight of about 40,000 daltons. It was found to be an effective inhibitor against neutral collagenases present in gastric mucosa, rheumatoid synovium, skin and granulocytes. Because of its smaller molecular size it is likely to be more diffusable than alpha-2-macroglobulin and are therefore more likely to reach inflammatory sites much more easily.

1.7.4 Other Antiproteinases

These are minor antiproteinases and their concentrations are extremely low and so they are of less importance. However, inter-alpha-trypsin inhibitor have been found in high concentrations in rheumatoid synovial fluid (Sandson and Hammerman, 1964; Brackertz, Hagmann and Kueppers, 1975).

Anti-chymotrypsin (alpha-1-glycoprotein) binds to chymotrypsin but not trypsin, and it behaves as an acute phase protein (Aronsen, Kelund, Kindmark and Laurell, 1972). Its concentration is also increased in serum and synovial fluid of patients with rheumatoid arthritis (Brackertz, Hagmann and Kueppers, 1975).

1.8 Endogenous Anti-Inflammatory Proteins

These substances have been found to inhibit granulation tissue deposition (Di-Pasquale, Girerd, Beach and Steinetz. 1963), carrageenan-induced edema (Billingham, Robinson and Robson, 1969), and delayed hypersensitivity reactions (Gaugs, Billingham and Rees, 1970).

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

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

Ford-Hutchinson, Insley, Elliot, Sturges 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 oedma and was resistant to acid and proteolytic digestion. This plasma fraction has a broad spectrum of anti-inflammatory activity ranging from inhibition of oedma formation to a reduction of the infilteration of leucocytes into inflammatory exudates (Smith and Ford-Hutchinson, 1975).

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).

The endogenous anti-inflammatory proteins offer a possible therapeutic advantage in that they are unlikely to have the side effects associated with anti-inflammatory drugs. Their most promising mode of use would appear to be a lysosomotrophic agents (De Duve, Barsy, Poole, Trouet, Tulkens and Van Hoof, 1974). However, they need considerably more investigation since the active anti-inflammatory molecule may be a degradation fragment of the proteins (Lewis, Best, Bird and Parrott, 1978).

1.9. Drugs Used in The Treatment of Rheumatoid Diseases

1.9.1 Non-Steroidal Anti-Inflammatory Drugs

The non-steroidal anti-inflammatory drugs (NSAID) have a well established role in the treatment of rheumatic diseases, especially rheumatoid arthritis. NSAID will often reduce the cardinal signs of inflammation. The early reduction of inflammation will reduce the tendency to deformity and possible cartilage and bone destruction by the inflammatory process.

Some of the common NSAID used in the treatment of rheumatoid arthritis are indomethacin, indoprofen, naproxen, ibuprofen, flufenamic acid, mefenamic acid, salicylates and acetaminophen.

One of the more important modes of action of the NSAID is the ability to inhibit prostaglandin synthesis in various tissues including the synovium (Mathur, Riley, Richardson, and Reavey-Cantwell, 1977; Newcombe and Ishikawa, 1977; Robinson, McGuire, Bastain, Kantrowitz and Levine, 1978) and in monocytes (Bray and Gordon, 1978).

Brown and Collins (1978) have shown that NSAID inhibits non specific

migration of human polymorphonuclear leucocytes. Other possible important actions are lysosomal membrane stabilization, inhibition of various mediators such as histamine, serotonin and kinins, and the reduction of free radicals such as O_2^- (the superoxide anion) (Famaey, 1978).

An important haematological effect is that most NSAIDs, again through prostaglandin inhibition, cause inhibition of platelet function. This effect is reversible with all but aspirin which is thought to permanently acetylate membrane-bound platelet cyclo-oxygenases (Samter, 1969).

McArthur, Dawkins and Smith (1971) found that L-tryptophan was displaced form binding sites of plasma proteins by NSAIDs and they proposed that such drugs might also displace certain peptides that could be anti-inflammatory in nature.

Most of NSAIDs are of comparable efficacy (Lee, Anderson, Miller, Webb and Bunchanan, 1976), and studies noting differences between drugs have been careful to point out the marked variation in individual responses to these agents (Huskisson, Woolf, Balme, Scott and Franklyn, 1976).

The incidence of side effects varies with the individual drugs but most of these drugs share similar problems. Dyspepsia, gastrointestinal errosions and ulceration are the major side effects.

1.9.2 Steroidal Anti-Inflammatory Drugs

Corticosteroids are effective agents in reducing inflammation in rheumatoid arthritis (Thompson, 1970; Cope, 1972), but the potential side effects restrict their routine use.

Corticosteroids have been found to reduce the number of circulating T-lymphocytes after a single or repeated dose which returns to normal after 12 - 24 hours (Cooper, Petts, Luckhurst and Penny, 1977). Corticosteroids

also supress antigen-processing by macrophages due to a substantial fall in blood monocytes from a reduced delivery from the bone marrow and to an altered distribution of cells between tissue and blood. Monocytes emigration, chemotaxis, phagocytosis and bactericidal functions are all impaired by steroids (Fauci, 1976).

Corticosteroids cause a less pronounced fall in B-cell numbers (Cooper, Pelts, Luckhurst and Penny, 1977) but in the long term immunoglobulin synthesis is depressed with a marked fall in serum immunoglobulin levels. Corticosteroid administration results in a profound impairment of neutrophil phagocytosis, degranulation and bactericidal capacities (Fauci, 1976). This explains the high prevalence of bacterial infection in corticosteroid treated patients.

Lewis and Piper (1975) reported that cortisol and prednisolone can inhibit prostaglandin release from the tissues.

No uniform agreement exists with respect to the stabilization of lysosomal membranes. Lewis and Day (1972) demonstrated that therapeutic concentrations of corticosteroids inhibited the release of lysosomal enzymes from rheumatoid synovial membrane. While Persellin and Ku (1974) claimed that corticosteroids did not stabilize lysosomes isolated from human polymorphonuclear leucocytes against lysis by heat or detergent either in vitro or in vivo.

1.9.3 Second Line Agents (Slow Acting Anti-Rheumatic Drugs).

This group includes gold, D-pencillamine, chloroquine, and recently levamisole was introduced to this group. These drugs have generally been used in the past following failure of NSAIDs because of their toxicity. All have a considerable "lag time" between the commencement of their use

and the onset of their action.

The mechanism of action of these drugs is not clearly understood. Gold has been found concentrated in synovial tissue, especially type A synovial lining cells and other phagocytes where it may stabilize lysosomal membranes and inhibit sulphydryl dependent enzymes (Gerber and Paulus, 1977).

The exact mode of action of D-pencillamine in rheumatoid arthritis is not known although it chelates heavy metals, prevents reduction of mature insoluble collagen fibres and reduces titres of rheumatoid factor and immunoglobulin (Brookes, 1978).

The mechanism of action of antimalarial drugs in rheumatoid arthritis is also not understood. Levamisole was first used as an antihelmintic agent and was first noted to have a beneficial effect on patients with rheumatoid arthritis in 1875 (Schuermans, 1975). Levamisole has been shown to have a profound action on immunological functions, possibly through its effect on T-cells (Symons and Rosenthal, 1977).

1.9.4 Immunosuppressive Drugs

Immunosuppression has in recent years assumed a major role in the management of patients with life-threatening or vital organ-threatening diseases (Pearson and Levy, 1975).

Azathioprines, cyclophosphamide and methotrexate are the most widely used agents in this class. Azathioprines were noted to be purine inhibitors. Cyclophosphamide like any alkylating agent alters nucleic acid (for example, DNA) structure, thereby potentially altering the function of the cells.

These agents are reserved for severe cases of rheumatoid arthritis because of the toxicity and the undetermined effects of long term immunosuppression therapy, for example the development of neoplasms.

1.10 Animal Models for the Study of Rheumatoid Arthritis

Animal models can be useful in studying the pathogensis and aetiology of some diseases and for testing new drugs.

One unusual factor in rheumatoid arthritis is its rarity in other animal species. Many different techniques have been used, reflecting the different approaches given to possible mechanisms of inflammation in different laboratories.

Animal models can be divided into five main groups according to Gardner (1960): infective, immunological, chemical, endocrinological and and physical.

One of the most used models is an immunological one where polyarthritis is induced in rats by the injection of Freund's complete adjuvant into the animal tail or hind foot pad (Pearson, 1956). After an initial inflammation in the paw the arthritis develops after a latent period of about ten days from the injection of the adjuvant. Articular lesions develops in the paws and tail and occasionally in the spine. The disease reaches a peak about twenty five days and may then regress slightly in some animals, although the condition is chronic (Pearson and Wood, 1959; Pearson and Wood, 1963).

It has been shown recently that the severity of the arthritis increases with decreased size of the mycobacterial particles (Liyanage, Currey and Vernon-Roberts, 1975).

The synovial tissue of the affected joints proliferates and pannus tissue invades the joint space causing severe bone erosion. Inflammatory cells such as mononuclear cells are also present in the synovium (Pearson and Wood, 1963; Pearson, 1963). In this rat model no rheumatoid factor is present (Glenn, Gray and Kooyers, 1965) and also there are few polymorpho-

nuclear leucocytes present in the affected joints (Pearson, 1963).

Dumonde and Glynn (1962) developed an immunological model of a chronic antigen-induced arthritis in rabbits. The rabbits are initially immunized with fibrin in Freund's adjuvant. Several weeks after fibrin is injected into a joint which then developed a chronic monoarticular arthritis. The joint lesion closely resembles that of human arthritis with synovial hypertrophy and hyperplasia, pannus formation and erosion of articular cartilage and bone. There is prolonged local synthesis of immunoglobulins, which takes place in the inflammed synovium (Glynn, 1968; Glynn, 1969). There is also a considerable influx of polymorphonuclear leucocytes, lymphocytes and plasma into the affected joints (Dumonde and Glynn, 1962).

Another popular animal model for inducing acute inflammation is carrageenan induced rat paw oedema as described by (Winter, Risley and Nuss, 1962). Carrageenan is a mucopolysaccharide derived from Irish sea moss which produces oedema upon injection into the hind foot pad of the rat. Carrageenan has no antigenic properties, therefore the inflammatory response is due to local stimulus and is highly reproducable (Gardner, 1960).

Another model used to study the sequence of events during the production of granulation tissue is the cotton wool pellet granuloma test (Meir, Schuler and Desaulles, 1950). In this model cotton wool dental pellets of known weight are implanted sub-dermally into rats and left for seven days, after which the animals are killed and the pellets recovered, dried and weighed. The increase in weight being due to granulation tissue being deposited in the pellets.

Three phases of inflammatory response in this model have been described by Swingle and Shideman (1967). In the early phase, the pellet

absorbs fluid of low protein content, followed by an invasive phase where the pellet is filled with inflammatory cells. Finally, the pellet has increased numbers of fibroblasts which synthesize granulation tissue.

In summary since there is no animal model completely satisfactory for rheumatoid arthritis screens for anti-inflammatory and anti-arthritic drugs employ a variety of animal models relevant to the type of inflammation being studied.

1.11 Aims of The Research

It is known that both collagenase and elastase play a central role in the destruction of articular cartilage in joints affected by rheumatic diseases.

Therefore, this investigation was concerned with a study of the levels of these enzymes and total proteinases in human rheumatic synovial fluids, to determine whether there is a correlation between these proteinases, alpha-1-antitrypsin (which is a major anti-proteinase) and alpha-2-macroglobulin.

There is evidence that both collagenase and elastase exist in a pro-enzyme form, so it was decided to study if these enzymes exist in a pro-enzyme form in human rheumatic synovial fluid. Another study was concerned with the inhibiting capacity of human rheumatic synovial fluid for both collagenase and elastase.

It is not normally possible to study the complex biochemical changes that take place in humans during the onset of rheumatoid arthritis because the disease is difficult to diagnose in its early stages. It is also difficult to study the natural development of rheumatic disease without the

added effects of drugs used in the treatment. Therefore it was proposed to measure the changes of collagenase, elastase, alpha-1-antitrypsin and alpha-2-macroglobulin levels in rat serum during the onset and the development of adjuvant induced arthritis.

2. METHODS AND MATERIALS

2.1. Collection of Samples

2.1.1 Collection of Synovial Fluid

Synovial fluids were aspirated from the knees of patients with a rheumatic disease. The fluids were centrifuged at 6,000 r.p.m. for 25 minutes to remove cells and debris. The cell free fluids were stored at -30°C, in airtight containers, until required for use. The following tests were carried out on the fluid samples:-

a) Total protein levels (See Section 2.2.4)
b) Collagenase activity (See Section 2.2.1)
c) Elastase activity (See Section 2.2.2)
d) Proteinase levels (See Section 2.2.5)
e) Acid phosphatase activity (See Section 2.2.3)
f) TIC levels (See Section 2.2.6)
g) TPE levels (See Section 2.2.7)

2.2. Methods Used in Assay of Human Rheumatic Synovial Fluid, Pregnant Plasma, and Post-Parturient Plasma

2.2.1 Collagenase Activity

Collagenase activity was measured according to the method of Steven, Torre-Blanco, and Hunter (1975). A series of reaction tubes were set up.

Series A contained: 7 mg of substrate (fluorescein-isothiocyanate FITC), labelled collagen fibrils prepared by the method of Steven etal (1975), 5 ml of 50 mM Tris buffer/5 mM $CaCl_2$, pH 7.5, and 100 mcl of the synovial fluid sample.

Series B contained: 7 mg of substrate (FITC, labelled collagen fibrils), 4 ml of 50 mM Tris buffer/5 mM $CaCl_2$, pH 7.5, and 1 ml of ethylene diamine tetra acetic acid (EDTA), pH 7.5, to give a final concentration of 20 mM in the reaction mixture plus 100 mcl of the synovial fluid sample. The reaction mixture was then placed in a shaking water bath at 37° C for twenty four hours. After incubation 1 ml of the supernatant was diluted to 10 ml with distilled water. The absorbance was read at 495 nm in a Pye-Unicam Sp 500 spectrophotometer. The difference between the readings of series A and series B gave the collagenase activity. Duplicate determinations for both series were run. A standard curve was prepared by using a reference collagenase (type III: Fraction A from Clostridium histoliticum, Sigma Chemical Company, U.S.A.).

2.2.2 Elastase Activity

a) Elastase activity was measured according to the method of Sachar etal (1955) where 20 mg of elastin-orcein (Sigma Chemical Company, U.S.A.) was placed in small bottles and 1 ml of 0.2 M Tris buffer, pH 8.8, and 1 ml of distilled water plus 100 mcl of synovial fluid sample was added to each bottle. The mixtures were then incubated for three hours at 37°C, in a shaking water bath. At the end of the incubation period 2 ml of 0.7 M phosphate buffer, pH 6.0, was added to each bottle, the mixtures were filtered and the absorbance read in a Pye-Unicam spectrophotometer Sp 500 at 595 nm. A standard curve was prepared by incubating different amounts of elastase (Sigma, elastase no. E-1250 pancreatopeptidase E; E.C. no. 3.4.21.11 from Hog pancreas type I, 15 units per mg of protein). One unit of elastase will solublize one mg of elastin in twenty minutes at pH 8.8 and 37°C) with 20 mg of elastin-orcein.

b) Elastase activity was measured at the beginning of the work according to the method of Naughton and Sanger (1961). Using elastin-congored as a substrate, where 4 mg of dyed substrate was suspended in 3.5 ml of 0.05 M Na_2CO_3-HCL, pH 8.8, to which 0.5 ml of the sample was added. The mixture was incubated at room temperature for three hours and then filtered and the filt rate read in the Pye-Unicam sp 500 spectrophotometer at 495 nm. This method was not used in later experimaents because it was found that trypsin also degraded this substrate and the results obtained were not satisfactory.

2.2.3 Acid Phosphatase Activity

Acid phosphatase activity was determined by the method described by Symons, Lewis and Ancill (1969).

A 0.1 ml sample was incubated with 0.5 ml of 15 mM p-nitrophenyl phosphate and 0.5 ml of 90 mM sodium-citrate buffer, pH 4.8, for thirty minutes in a shaking water bath at 37°C. The reaction was stopped by the addition of 5 ml 0.1 M sodium hydroxide solution. The p-nitrophenol released was determined by measuring the absorbance of the solution of 410 nm against a blank, which was prepared by the addition of 0.1 ml of distilled water in the place of the sample. A standard curve of the absorbance of p-nitrophenol (at various concentrations) at 410 nm was also prepared.

2.2.4 Estimation of Protein Concentration

Protein concentration was determined by a modification of the method of Lowry, Rosebrough, Farr and Randall (1951).

The following reagents were prepared:-

5% w/v Copper Sulphate

10% w/v Sodium Potassium Tartrate

2% w/v Sodium Carbonate in 0.1 N Sodium Hydroxide

1 ml of the copper sulphate solution and 1 ml of the sodium-potassium tartrate solution were mixed and diluted accurately to 10 ml. 1 ml of this solution was then diluted to 50 ml with sodium carbonate solution and 0.2 ml of suitably diluted sample were pipetted into a tube. To this 2 ml of the alkaline reagent mixture was added, mixed, and allowed to stand for ten minutes at room

temperature. After this time 0.2 ml of Folin-Ciocalteu's phenolic reagent (BDH, Chemical Company) was added, mixed and allowed to stand for twenty minutes and then the absorbance was determined at 700 nm.

A standard curve was prepared by using bovine serum albumin (Sigma Chemical Company, U.S.A.) at different concentrations and the absorbance was read at 700 nm in a Pye-Unicam Sp 500 spectrophotometer.

2.2.5 Proteinase Activity

Proteinase activity was measured by a modification of the method of Rinderknecht, Geokas, Silverman, Lillard and Haverback (1968). 0.5 ml of a suitably diluted sample (synovial fluid) and 2 ml of 0.1 M Tris-HCl buffer, pH 7.4, were incubated together with 20 mg of Azure blue impregnated hide powder (Calbiochem, U.S.A.) for twenty four hours at 37°C in a shaking water bath. The solution was then cooled and filtered to remove residual hide powder. The absorbance of the dye released into the solution was read at 595 nm in a Pye-Unicam Sp 500 spectrophotometer, water was used on a blank.

A standard curve of the absorbance at 595 nm of completely dissolved Azure blue impregnated hide powder was also prepared.

2.2.6 Trypsin Inhibitory Capacity (TIC)

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

A 2 ml of trypsin solution (20 mcg/ml in 0.1 M Tris-HCl buffer, pH 8.2, and 0.02 M Calcium Chloride) was added to 10 mcl of the sample. A 2 ml of trypsin solution was also added to 10 mcl of 40 mg/ml bovine serum albumin solution (Fraction V) (Sigma Chemical Company, U.S.A.) as a control. The solutions were then allowed to stand at room temperature for 10 - 15 minutes.

To activate the trypsin inhibitory proteins, 0.5 ml of this mixture was then added to 2.5 ml of the BAPNA substrate (0.45 mg/ml in a 0.1 M Tris-HCl buffer, pH 8.2, and 0.02 M Calcium Chloride) and incubated for ten minutes in a shaking water bath at 37°C. 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 nm in a Pye-Unicam Sp 500 spectrophotometer. The blanks were prepared by adding the sample trypsin solution to the BAPNA substrate after the incubation and addition of the 30% (v/v) acetic acid.

2.2.7 Trypsin-Protein Esterase (TPE) Activity

The method used to determine the T.P.E. activity of various samples was developed by Ganrot (1966), which enables the concentration of certain large molecular weight proteinase inhibitors to be determined. These inhibitors can bind and inhibit trypsin so that it cannot break down large molecular weight substrates, such as BAPNA. The activity of the bound trypsin is unaffected by soybean trypsin inhibitor. A 0.5 ml portion of trypsin solution (100 mcg Trypsin per ml of 0.1 M Tris-HCl buffer, pH 8.2, and 0.2 M Calcium Chloride) was added to 25 mcl of the sample fluid, 0.5 ml of Tris-HCl buffer alone was added to 25 mcl of 40 mg/ml of bovine serum albumin

solution as a blank. After 10 seconds, 1.5 ml of the BAPNA solution containing the soybean trypsin inhibitor (Type II-S, Sigma Chemical Company, U.S.A.) (0.9 mg of BAPNA and 66.8 mcg of soybean trypsin inhibitor per ml of 0.1 M Tris-HCl buffer, pH 8.2, and 0.02 M Calcium Chloride) was also added. The mixture was incubated for 10 minutes at 37°C in a shaking water bath. The reaction was stopped by the addition of 0.5 ml of 30% (v/v) acetic acid. The samples were read against the blank at 400 nm in a Pye-Unicam Sp 500 spectrophotometer.

2.3 The Detection of Pro-enzymes in Synovial Fluids

2.3.1 The Detection of Pro-elastase in Synovial Fluids

The object of this experiment was to release elastase by pre-incubation with trypsin. A stock solution of trypsin (from bovine pancreas, twice crystallized) (Sigma Chemical Company, U.S.A.) 1 mg/ml was prepared and mixed with synovial fluids in test tubes as below:

Synovial fluid		Trypsin (stock)
0.5 ml	+	0.5 ml (0.5 mg trypsin)
0.5 ml	+	1 ml (1 mg trypsin)

The volume was completed to 2 ml with 0.2 M Tris-HCl buffer, pH 8.8, and the mixture was incubated for thirty minutes at 37°C. 100 ml of the incubation mixture was used to assay elastase as in section (2.1.2).

2.3.2 The Detection of Pro-collagenase in Synovial Fluids

The object of this experiment was to release collagenase by pre-incubation with trypsin. A stock solution of trypsin (from bovine pancreas, twice crystallized) (Sigma Chemical Compnay, U.S.A.) 5 mg/ml was prepared and mixed with synovial fluid as below:

Synovial fluid		Trypsin (stock)
100 mcl	+	100 mcl (0.5 mg trypsin) T_1
100 mcl	+	200 mcl (1 mg trypsin) T_2

The volume was completed to 1 ml with 50 mM Tris-HCl buffer, pH 7.5, and 5 mM Calcium Chloride, the mixture was incubated for

thirty minutes at 37°C. 100 mcl of the incubation mixture was used for collagenase assay as in section (2.1.1). 100 mcl of the synovial fluid was diluted to 1 ml and used as a control.

2.4 Detection of Elastase and Collagenase Inhibitory Action of Human Rheumatic Synovial Fluid on Elastase and Collagenase

2.4.1 Detection of Elastase Inhibitory Action of Human Rheumatic Synovial Fluid on Elastase

This experiment was carried out to determine the elastase inhibitory action of human rheumatic synovial fluid. The scheme of the experiment is summarized as follows:-

Test tube no.	1	2	3	4	5
Elastin-Orcein (mg) (Sigma Chemical Company, U.S.A.)	20	20	20	20	20
0.2 M Tris-HCl buffer pH 8.8 (ml)	1.95	1.9	1.85	1.8	2.0
Sample (mcl) (synovial fluid)	50	100	150	200	-
Elastase (ml) (pancreatopeptidase E, E.C. no. 3.4.21.11 from Hog pancreas type I) (7.3 unit/ml)	1	1	1	1	1

The tubes were then incubated in a shaking water bath for three hours at 37°C. Then the solutions were filtered and read in a Pye-Unicam Sp 500 spectrophotometer at 595 nm.

The same experiment was carried out to determine the elastase inhibitory capacity of normal human serum.

2.4.2 Detection of Collagenase Inhibitory Action of Human Rheumatic Synovial Fluid on Collagenase

This experiment was carried out to determine the collagenase inhibitory action of human rheumatic synovial fluid. The scheme of the experiment is summarized as follows:-

Series A

Test tube no.	1	2	3	4
FITC-labelled collagen fibrils (mg)	7	7	7	7
50 mM Tris buffer/5 mM $CaCl_2$, pH 7.5, (ml)	3.95	3.9	3.85	4
Synovial fluid (mcl)	50	100	150	-
Collagenase (ml) (form II exColstridium Hystolyticum. 286 Collagenase mcg/mg, from Koch-light laboratories Ltd). Each 1 ml contained 2.86 units.	1	1	1	1

In Series B the same amounts of substrate, sample and collagenase were used. 1 ml of ethylene diamine tetra-acetic acid (EDTA), pH 7.5, to give a final concentration of 20 mM in the reaction mixture, was added to each test tube, and 50 mM of the Tris buffer/5 mM $CaCl_2$, pH 7.5, was added to each test tube to give a total volume of 5 ml in each tube. The tubes were then incubated in a shaking water bath for 24 hours at 37°C. Then the solutions were filtered and read at 495 in a Pye-Unicam Sp 500 spectrophotometer.

2.5 The Induction and Measurement of Adjuvant Arthritis in Rats

Adjuvant Arthritis was induced into 150 - 200 gm male Wistar Rats using the method of 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 glass pestle and mortar, and suspended in liquid paraffin at a concentration of 5 mg/ml. The rats were anaesthetised using a mixture of 3% halothane in oxygen (flow rate 300/minute) and nitrous oxide (flow rate 1,500 cc/minute) produced by a Boyles veterinary anaesthetic trolley (British Oxygen Ltd.).

0.03 ml of the adjuvant suspension was injected into the left hind foot, and control animals were injected with 0.9% (w/v) sterile saline. The inflammatory response was estimated by measuring the increase in volume of the hind feet of the rats. The volume of each paw was measured with a plethysmograph using a modification of the method described by Harris and Spencer (1962). The hind feet were immersed separately up to the hair line in a mercury bath which was linked to a blood pressure transducer and an oscillograph recorder (Searle Bioscience, Kent). The measurement of the hind feet of the rats were taken just prior to the injection of the adjuvant and again at various intervals during the experiment; 3-4 ml of blood samples were taken by cardiac puncture before injection of the adjuvant and at different intervals throughout the experiment. The blood samples were centrifuged at 3,000 r.p.m. for twenty minutes and the serum was used for collagenase assay in section (2.1.1) and elastase assay as in section (2.1.2).

2.6 Separation of Proteinase by Electrophoresis

Electrophoretic separation of proteinases were performed by gel electrophoresis.

2.6.1 Agar Gel Electrophoresis

1% (w/v) agarose (Sigma Chemical Company, U.S.A.) in 0.075 M sodium barbitone buffer, pH 8.6, was liquified in a water bath at 100°C. When fully liquified, and free from lumps, the agarose solution was allowed to cool to 55°C and poured on to an electrophoresis tray (Shandon, Cheshire) fitted with microscope slides. A perspex scraper (Shandon) was then passed over the tray to remove the surplus liquid agarose. The agarose was then allowed to set leaving a 1 mm thick layer of gel covering the slides. Slots with a capacity to hold up to 7 mcl were cut with a pattern cutter. 3-4 mcl of synovial fluid was applied to each slot using a microsyringe (Hamilton Micromeasure, Switzerland). The slide tray was then placed in a Shandon electrophoresis tank which contained 0.075 M sodium barbitone buffer, pH 8.6. A current of 8 volt/cm was applied across the agarose gel such that the proteins migrated towards the anode. The migration of protein was visualised by a drop of bromophenol blue and the electrophoresis was stopped after two hours. The slide trays were then incubated with 0.5% (w/v) casein solution for 45 minutes at room temperature. After incubation the gel trays were fixed overnight in 50% (v/v) ethanol containing 3% (v/v) acetic acid. The gels were then stained in a solution of 1% (w/v) amido-black in 7% (v/v) acetic acid for thirty minutes. The background staining in agarose gel was then removed by

washing the gel in 7% (v/v) acetic acid until the background was clear.

2.6.2 Polyacrylamide Gel Electrophoresis

8% (w/v) acrylogel (BDH, Chemical Company) (which consists of a mixture of acrylamide monomer with N,N`-methylene-bis-acrylamide) was dissolved in distilled water, the solution was then filtered to remove any impurities. 0.2 ml of N,N,N`,N`-tetramethylenediamine (TEMED) was added to the acrylogel solution followed by 7 ml of 1% (w/v) ammonium persulphate. Then the mixture was immediately poured into perspex gel moulds, 10 cm x 14 cm x 2 mm dimensions with a capacity of 16 slots each holding 8 mcl. When the gel was set it was placed in plates filled with 0.02 M Tris buffer for two hours, then the gels were transfered to Shandon electrophoresis tanks fitted with a Grant F H 15 cooling system. 4-6 mcl of samples were loaded into the slots using a microsyringe (Hamilton Micromeasure, Switzerland). The electrophoresis tanks were filled with 0.025 M of Tris buffer and a current of 8 volt/cm was applied and the temperature was maintained at 4°C throughout the run. The migration of proteins were visualised by mixing a drop of bromophenol blue with one of the synovial fluid samples as a marker. After three hours run the gels were either stained for proteinase as mentioned in section (2.6.1) or stained for protein as below.

The gels were fixed first with 50% (v/v) ethanol in 10% (v/v) acetic acid for thirty minutes followed by immersion in a 0.1% (w/v) Coomassie Brilliant Blue R (Sigma Chemical Company. U.S.A.) solution in 25% (v/v) ethanol in 10% (v/v) acetic acid. The gels in the

staining solutions were shaken for one hour at 37°C. The gels were destained using 25% (v/v) ethanol in 10% (v/v) acetic acid.

The gels were stained for alpha-1-antitrypsin using the method of Hercz and Barton (1978). After the run the gel was immersed in a solution of (125g/litre) trichloroacetic acid for 5 - 10 minutes and then transfered to a solution of (5 gm/litre) periodic acid for 30 minutes followed by a solution of sodium arsenite in (95/5 v/v) water/ acetic acid solution. Within a few minutes the gels became dark brown but after an additional 20 - 30 minutes they became colourless again. The gels were then washed with two consecutive treatments of (1 gm/ litre) sodium arsenite in (95/5 v/v) water/acetic acid, each wash lasting for 10 minutes. Then the gels were finally immersed in a solution of basic fuchsin prepared according to the method of Kodousek (1969) and within half an hour red bands started to appear, reaching full intensity within 1 - 2 hours. These bands were alpha-1-antitrypsin.

2.6.3 Electrophoretic Separation of Esterase

The gel was prepared as previously described in section (2.6.2). After a stiff gel had been formed the gel was immersed in 76 mM Tris/ 5 mM citric acid, pH 8.9, and shaken for two hours. 0.5 M sodium borate, pH 9.0, was used as the electrode buffer. A current of 8 volts/cm was applied for a two hour run, after which the gel was stained for esterase by incubating the gel with a mixture consisting of 2% (w/v) napthylacetate in acetone and fast red T.R. salt in 0.15 M phosphate buffer, pH 6.5 at 37°C until the red crimson bands reached full intensity. The gel was then preserved in 10% (v/v) acetic acid.

2.6.4 Quantitative Estimation of Specific Proteins by Electroimmuno Assay

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

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

1% (w/v) agarose gel in 0.07 M barbital buffer, pH 8.6, containing 2 mM calcium lactate was liquified in a water bath at 100°C. The agarose solution was allowed to cool to 40°C and the requisite amount of antiserum added. The antiserum concentration used was the minimum one that had previously been found to form a distinct preciptin line with the antigen. The antisera to human alpha-1-antitrypsin and human alpha-2-macroglobulin (Dako Immunoglobulin, Denmark) was mixed with agarose in the ratios of 1:50 and 1:75 respectively. A 15 ml portion of agarose containing the antiserum was then poured into a mould consisting of two identical rectangular glass plates, 20 cm x 10 cm, spaced 1 mm apart by a U-shaped former manufactured from 1 cm wide polythene strips. The mould was clamped and held upright until the gel had set. The clamps were removed and one glass plate slid away to leave a slab of gel 18 cm x 8 cm x 1 mm. Holes 4 mm in diameter were cut in the gel along the longest edge, 2 cm from the edge, with the centres of the holes a minimum of 8 mm

apart. The gel was placed in a Shandon electrophoresis tank with the line of holes perpendicular to the electric field and furthest from the anode. Eight microlitres of the solution to be tested were pipetted into separate holes. For this procedure human synovial fluid and serum was diluted, 1:15 and 1:10, to assay alpha-1-antitrypsin and alpha-2-macroglobulin respectively, the dilution being made with the electrode buffer which consisted of 0.07 M barbital buffer, pH 8.6. A power pack was then used to apply a voltage of 70 - 80 across the gel. The electrophoresis was allowed to proceed for 18 hours by which time the height of the "rockets" remained constant. The voltage was then switched off and the height of the "rockets" measured from the centre of the holes to the peak tips using a dark field illuminator to easily visualise the "rockets". Standard antigen solutions were examined during each electrophoretic run so that the results for different samples on different runs could be directly compared.

2.7 Chromatographic Fractionation of Synovial Fluid

The synovial fluid was partially fractionated by eluting 3 ml of the synovial fluid through a Pharmacia (Uppsala) column (K 2640), 2.5 cm in diameter and 35 cm long, packed with Sephadex G-150 (Pharmacia). Using 0.1 M Tris buffer, pH 8.6, as eluant. The technique of downward elution was utilized. The column eluate was monitored at 280 nm using a Uvicord II flow through cell (L.K.B. Instruments, England) connected to a strip chart recorder and collected in 10 ml fractions by a Ultrorac 7000 automatic fraction collector (L.K.B.). The fractions were dialysed against distilled water for 24 hours at 4°C and then assayed for collagenase as in section (2.2.1). The void volume of the column was determined using Dextran blue, and the column was colibrated using albumin (M.Wt = 66,000), ovalbumin (M.Wt = 44,000), pepsin (M.Wt = 34,700), trypsinogen (M.Wt = 24,000) and lysozyme (M.Wt = 14,300). (Sigma Chemical Company Ltd., U.S.A.)

2.8 The Preparation of Fluorescein-Isothiocynate Labelled Collagen Fibrils

2.8.1 The Preparation of Collagen Fibrils

Collagen fibrils were prepared according to the method of Steven (1967).

Rat tail tendons were cleaned from adhering tissue and cut into small fragments. The fragments were disintegrated by using a glass pestel and mortar, and the disintegrated tissue was defatted in acetone and then re-equiliberated with water. Samples of disintegrated tendons were shaken for eighteen hours at room temperature with approximately 20 volumes of ethylenediamine tetra-acetic acid (EDTA), pH 7.5. The reaction mixture was then washed and dialysed against distilled water free of the chelating agent (EDTA), the fibrous mass of treated tendons were then suspended in one litre of 0.2 M acetic acid and stirred for 3 hours followed by centrifugation at 8,000 Xg at $4^{\circ}C$.

The supernatant containing dispersed polymerised collagen fibrils was collected and the insoluble residue was re-extracted with 0.2 M acetic acid for a further three hours. After centrifuging of the second polymerised collagen fibrils, the two supernatants fibril fractions were combined and the polymerised fibrils were collected after raising the reaction pH to 6.7 with 1 N sodium hydroxide.

2.8.2 Preparation of FluoresceinLabelled Polymeric Collagen Fibrils

The fluoresceinlabelled polymeric collagen fibrils were prepared according to the method of Steven, Torre-Blanco and Hunter (1975).

The purified polymeric collagen fibrils were suspended in 0.2 M sodium acetate and the pH adjusted to 9.5 with 0.1 M sodium hydroxide. The polymeric collagen was then washed with distilled water, and the water replaced by acetone. The acetone damp fibrils were cut into small pieces and placed in 500 ml acetone containing 0.1 gm FITC (Isomer I, B.D.H. Ltd.) (0.1 gm FITC was used for every 10 gm of acetone damp fibrils). The reaction mixture was shaken for 24 hours at 4°C, when the acetone was decanted the collagen fibrils were retreated with a further 500 ml acetone containing 0.1 gm FITC. The yellow insoluble product was washed with acetone (three times), 1% (w/v) sodium bicarbonate (four times), followed by 50 mM Tris/5 mM $CaCl_2$ buffer, pH 7.5, at 37°C, until all traces of excess reagent had been washed from the FITC labelled polymeric collagen. Finally the product was washed with water followed by acetone, then the FITC labelled collagen fibrils were dried in a vacuum desiccator and stored in dark bottles at -30°C.

3. RESULTS

3.1 The Levels of Proteinasesand Anti-Proteinases in Human Rheumatic Synovial Fluid Samples

Table 3.1 shows the clinical notes of the synovial fluid samples used. Table 3.2 shows the levels of collagenase, elastase, proteinase, trypsin inhibitory capacity (TIC), acid phosphatase, and total protein in samples of synovial fluid withdrawn from patients of both sexes, suffering from a variety of rheumatic conditions.

The results from the study carried out above were entered into a computer which has been programmed to analyse groups of data by linear regression and calculate the significance of these results.

Table 3.3 shows the significance levels calculated by the computer.

Table 3.1 Showing sex, age, type of rheumatic disease, and drug used for treatment for each synovial fluid sample

Sample Number	Clinical Notes
1	Female, aged 48, suffering from ankylosis spondylitis with peripheral arthropathy; drugsused for treatment was cimitidine and indocid.
2	Female, aged 76, suffering from sero +ve rheumatoid arthritis; drugsused for treatment was aspirin and steroids.
3	Male, aged 66, suffering from sero -ve rheumatid hips and varicose veins, fluid was aspirated from left knee clear of pus cells; drug used for treatment was Depo-medrone.
4	Female, aged 65, suffering from sero -ve rheumatoid arthritis; drugs used for treatment were Gold and Indocid.
5	Male, aged 53, suffering from sero -ve polyarthritis; drugsused for treatment were Gold, Indocid and Prednisolone.
6	Male, suffering from gout, sample was aspirated from left knee; drug used for treatment was allopurinol.
7	Male, suffering from gout, fluid was aspirated from right knee clear of pus cells and organisms; drug used for treatment was Butazolidin.
8	Male, no information.
9	Male, aged 37, suffering from gout; drug used for treatment was Indocid.
10	Male, no information.
11	Male, suffering from unknown type of Still's

Table 3.1 continued

Sample Number	Clinical Notes
	disease, duration of the disease is 9 years, no drugs were used for treatment, fluid was aspirated from both knees. White blood cell count (WBC): Right knee - $6.2 \times 10^6/cm^3$ Left knee - $14.8 \times 10^6/cm^3$
12	No information
13	Female, aged 14, suffering from Still's disease, fluid was aspirated from both knees.
14	Male, aged 48, suffering from rheumatoid arthritis, duration of the disease is 20 years; drugs used for treatment are dapsone and indocid.
15	No information
16	Male, aged 61, suffeing from osteoarthritis, fluid was aspirated from left knee. White blood cell count: $140/cm^3$
17	Male, suffering from rheumatoid arthritis, fluid was aspirated from both knees; drugs used for treatment were prednisolone and penicllamine.
18	Male, aged 32, suffering from sero +ve rheumatoid arthritis; drug used for treatment was Indocid. White blood cell count (WBC): $3.6 \times 10^6/ cm^3$
19	Female, suffering from rheumatoid arthritis, duration of the disease is 17 years, fluid was aspirated from right knee. White blood cell count (WBC): $24,700\ cm^3$
20	Female, aged 35, suffering from rheumatoid arthritis. fluid was aspirated from both knees; drug used for treatment was Gold.

Table 3.1 continued

Sample Number	Clinical Notes
21	No information.

Table 3.2 The Levels of Proteinase, Collagenase, Elastase, TIC, Total Protein and Acid Phosphatase in Human Rheumatic Synovial Fluid

Sample Number	Proteinase Activity mg substrate broken down in 24 hours	Collagenase Activity mcg/100 mcl	Elastase Activity u/100 mcl	TIC mc mol/min/ml	Total Protein mg/ml	Acid Phosphatase Activity mcg-P-nitrophenol/ml
1	8.0	2.85	1.8	1.6	6.5	0.9
2	7.5	3.0	1.64	1.8	5	0.56
3	5.1	1.5	0.97	2.9	3.75	0.4
4	6.8	2.4	2.9	0.9	6.5	0.8
5	6.0	1.6	1.2	4.3	6.75	0.3
6	7.9	3.0	2.64	0.63	6.2	0.3
7	8.1	3.4	2.34	2.2	5.9	0.55
8	7.5	2.5	2.8	2.4	5.4	0.6
9	7.8	3.6	2.8	2.5	6.5	0.72
10	8.0	3.2	2.6	1.9	6.1	0.9
11 R	5.35	2.85	2.7	1.1	4.0	17
11 L	5.85	2.15	2.3	1.4	4.5	16

Table 3.2 continued

Sample Number	Proteinase Activity mg substrate broken down in 24 hours	Collagenase Activity mcg/100 mcl	Elastase Activity u/100 mcl	TIC mc mol/min/ml	Total Protein mg/ml	Acid Phosphatase Activity mcg-P-nitrophenol/ml
12 R	4.4	2.4	2.43	2.43	3.1	34.5
12 L	4.0	2.6	2.5	2.5	3.5	32
12 S	1.05	0.7	1.2	2.8	4.5	nd
13 R	3.59	2.7	3.5	1.3	4.5	0.4
13 L	3.05	2.9	3.0	1.9	4.57	0.45
14	6.72	2.5	2.7	2.3	5.5	0.3
15	7.52	3.2	2.8	3.8	3.2	0.2
16	2.05	0.9	1.2	1.8	3.1	0.45
17 R	8.05	3.0	2.6	1.4	4.5	0.49
17 L	8.5	3.4	2.1	1.3	4.8	0.45
18	7.5	3.5	3.2	2.2	4.8	0.55
19	7.56	3.8	2.6	0.65	6.3	3.1
20 R	7.2	5.1	2.5	2.6	4.4	0.3

Table 3.2 continued

Sample Number	Proteinase Activity mg substrate	Collagenase Activity mcg/100 mcl	Elastase Activity u/100 mcl	TIC mc mol/min/ml	Total Protein mg/ml	Acid Phosphatase Activity mcg-P-nitrophenol/ml
20 L	7.5	7.2	3.0	2.2	4.5	0.36
20 S	4.0	1.2	1.1	3.15	6.5	0.5
21	7.00	4.0	2.0	0.8	3.15	0.55
Mean ± S.D.	6.199 ± 2.04	2.89 ± 1.28	2.33 ± 0.68	1.92 ± 1.0	4.92± 1.2	3.14 ± 7.58
Mean RA ± S.D.	6.16 ± 1.64	3.1 ± 1.3	2.5 ± 0.67	1.95 ± 0.85	4.8 ± 1.5	0.78 ± 0.94
Mean Gout ± S.D. O.A. (16)	7.9 ± 0.92 2.05	3.54 ± 1.6 0.9	2.6 ± 1.0 1.2	1.83 ± 0.74 1.8	6.2 ± 0.3 3.1	0.52 ± 21 0.45

R, Right knee,

L, Left knee

S, Serum,

nd, not detected

RA, Rheumatoid arthritis

Table 3.2a Levels of Proteinase, Collagenase, Elastase, CRP, ESR and WBC counts in Human Rheumatic Synovial Fluid

Patient No./ Disease State	Proteinase Activity mg substrate broken down in 24 hrs	Collagenase Activity mcg/100 mcl	Elastase Activity U/100 mcl	CRP* mg/L	ESR+ mm/hr	WBC• Count cell/cm^3
11R (RA)	5.35	2.85	2.7	25	30	6.2×10^6
11L	5.85	2.15	2.3	-	-	14.8×10^6
13R (RA)	3.95	2.7	3.5	10	33	10.3×10^6
13L	3.05	2.9	3.0	-	-	5.4×10^6
14 (RA)	6.7	2.5	2.7	33	55	9.2×10^6
17R (RA)	8.05	3.0	2.6	62	52	6.34×10^3
17L	8.5	3.4	2.1	-	-	-
18 (RA)	7.5	3.5	3.2	84	100	3.8×10^6
19 (RA)	7.56	3.8	2.6	120	80	24.7×10^6
20R (RA)	7.2	5.1	2.5	16	59	5.04×10^4
20L	7.5	7.2	3.0	-	-	-
16 (OA)	2.05	0.9	1.2	Neg.	4	160

*CRP: C-reactive protein

+ESR: Erythrocytes sedimentation rate

•WBC: White blood cell

CRP and ESR were measured in patients serum.

Values for CRP, ESR and WBC counts were kindly provided by the rheumatism research group in Birmingham University.

No CRP, ESR or WBC counts were provided for gout samples because those samples were from the Royal National Hospital for Rheumatic Diseases in Bath.

Table 3.2b A Comparison Between Disease Activity and Mean Values of Proteinases

Disease Activity	Mean of Proteinase Activity	Mean of Collagenase Activity	Mean of Elastase Activity
Non-active (OA)	2.05	0.9	1.2
Low activity (RA)	4.55	2.65	2.9
High activity (RA)	7.57	4.07	2.6

Non-active: With negative or very low values for CRP and ESR

Low activity: CRP and ESR values below 30

High activity: CRP and ESR values above 30

Table 3.3 The Significance Levels Between Various Parameters as Determined by Computer Analysis

	Proteinase	Collagenase	Elastase	TIC	Total Protein	Acid Phosphatase
Proteinase	-	99.6%	73.3%	22.8%	96.1%	77.0%
Collagenase	99.6%	-	99.4%	40.8%	19.2%	48.4%
Elastase	73.3%	99.4%	-	89.8%	2.3%	8.5%
TIC	22.8%	40.8%	89.8%	-	69.3%	92.5%
Total Protein	96.1%	19.2%	2.3%	69.3%	-	48.4%
Acid Phosphatase	77.0%	48.4%	8.5%	92.5%	48.4%	-

Values over 95.0% are taken as being statistically significant.

Table 3.4

This table shows another group of synovial fluid analysed for total alpha-1-antitrypsin, free alpha-1-antitrypsin, total alpha-2-macroglobulin, free alpha-2-macroglobulin, protease level and total protein. The result was entered into a computer which has been programmed to analyse groups of data by linear regression and to calculate the significance of the result. Table 3.5 shows the significance levels calculated by the computer.

Table 3.4 The Levels of Total Alpha-1-Antitrypsin, Total Alpha-2-Macroglobulin, TIC, TPE, Proteinase Level, and Total Protein in another set of Human Rheumatic Synovial Fluid

Sex/Disease State	Total alpha-1-antitrypsin Peak heights mm	Total alpha-2-macroglobulin Peak heights mm	Free alpha-1-antitrypsin TIC (mc mol/min/ml)	Free alpha-2-macroglobulin TPE absorbance units/ml	Proteinase Level mg/ml/hr	Total Protein g/100 ml
M, RA	20	6	1.92	0.095	0.032	5.28
F, RA	13	4	0.86	0.051	0.68	5.68
F, RA	18	4	1.05	0.063	0.064	4.44
M, RA	14	5	1.11	0.082	0.778	5.26
F, RA	27	9	2.08	0.017	0.571	7.49
M, RA	22	4.5	1.18	0.094	1.59	7.18
F, RA	11.5	7	0.85	0.03	0.096	6.12
F, -	6.5	2	0.33	0.039	0.059	3.36
M, OA	9	2	0.84	0.034	0.044	5.19
F, RA	11.5	3	1.29	nd	0.088	5.35
F, RA	13	4	1.23	nd	0.974	8.53
F, CH	8	2	0.63	0.043	0.077	5.52
F, -	8.5	3	0.55	nd	0.103	5.08

Table 3.4 continued

Sex/Disease State	Total alpha-1-antitrypsin Peak heights mm	Total alpha-2-macroglobulin Peak heights mm	Free alpha-1-antitrypsin TIC (mc mol/min/ml)	Free alpha-2-macroglobulin TPE absorbance units/ml	Proteinase Level mg/ml/hr	Total Protein g/100 ml
F, RA	7	6	0.02	nd	0.22	4.73
M, RA	4	4	nd	nd	0.22	5.19
F, RA	9	3	0.12	nd	0.158	5.97
F, RA	12	6	nd	nd	0.237	5.78
F, RA	9	3	0.7	0.029	0.229	4.18
F, -	7	2	0.65	nd	0.601	5.33
M, RS	10	13	0.93	0.08	0.246	7.18
Mean ± SD	12 ± 5.8	4.6 ± 2.7	0.81 ± 0.57	0.035 ± 0.034	0.313 ± 0.38	5.64 ± 1.216
Mean RA ± SD	13.1 ± 6.2	4.8 ± 1.8	1 ± 0.67	0.056 ± 0.03	0.29 ± 0.29	5.66 ± 1.23
Plasma Pooled	14.6*	16.6+	3.25	0.152	nd	7.3

M, Male;

F, Female;

RA, Rheumatoid arthritis;

OA, Osteoarthritis;

CH, Chondrocalcinosis;

RS, Reiter's Syndrome;

nd, not detected;

*, Equivalent to 0.21g/100 ml alpha-1-antitrypsin;

+, Equivalent to 0.20g/100 ml alpha-2-macroglobulin.

The ratio of the mean values for $\frac{\text{total}}{\text{free}}$ antiproteinase were for alpha-1-antitrypsin 4.5 (blood), 14.8 (Synovial fluid), and for alpha-2-macroglobulin 109 (blood), 131 (Synovial fluid).

Table 3.5 The Significant Levels of Various Parameters shown in Table3.4 as Determined by Computer Analysis.

	Alpha-1-antitrypsin	Alpha-2-macroglobulin	TIC	TPE	Proteinase	Total protein
Alpha-1-anti-trypsin	-	90.5%	100%	95%	96.9%	95.8%
Alpha-2-macro-globulin	90.5%	-	81.4%	73.7%	29.8%	97.9%
TIC	100%	81.4%	-	-	78.5%	-
TPE	95%	73.7%	-	-	24.6%	-
Proteinase	96.6%	29.8%	78.5%	24.6%	-	99.6%
Total Protein	95.8%	97.9%	-	-	99.6%	-

Values over 95.0% are taken as being statistically significant.

3.2 Pro-Enzymes Activities in Human Rheumatic Synovial Fluid

3.2.1 Pro-Elastase Activity in Human Rheumatic Synovial Fluid

From Table 3.6 it was found that there was an increase in elastase activity in synovial fluids treated with trypsin, but this increase was not uniform with different samples of synovial fluids; the increase in samples treated with 0.5 mg trypsin varied from 12.3 - 54.9%, and the increase in samples treated with 1 mg of trypsin varied from 42.8 - 95.4%.

3.2.2 Pro-Collagenase Activity in Human Rheumatic Synovial Fluid

Table 3.7 shows the increase in collagenase activity in synovial fluid treated with trypsin, like that of elastase the increase varied with different samples; the increase in samples treated with 0.5 mg trypsin varied from 27.8 - 76.9%, and the increase in samples treated with 1 mg trypsin varied from 66.7 - 161.5%.

Table 3.6 The Pro-Elastase Activity in Some Human Rheumatic Synovial Fluid

Sample No (S)	absorbance (a_1)	absorbance (a_2)	mean (a_1, a_2) (m)	(m)-blank	S'-T_1	S"-T_2	%increase in activity
12 R	0.076	0.079	0.0775	0.065	-	-	-
12' R	0.11	0.106	0.108	0.0955	0.0905	-	39.2
12" R	0.148	0.15	0.149	0.137	-	0.127	95.4
13 R	0.037	0.038	0.038	0.0255	-	-	-
13' R	0.055	0.058	0.057	0.0445	0.0395	-	54.9
13" R	0.071	0.07	0.071	0.0585	-	0.0485	90.1
14	0.082	0.079	0.0815	0.069	-	-	-
14'	0.094	0.096	0.095	0.0825	0.0775	-	12.3
14"	0.12	0.122	0.121	0.1085	-	0.0985	42.8
15	0.044	0.046	0.045	0.0325	-	-	-
15'	0.065	0.066	0.066	0.0535	0.0485	-	49.2
15"	0.081	0.084	0.083	0.0705	-	0.0605	86.2
T_1	0.018	0.017	0.0175	0.005	-	-	-
T_2	0.022	0.023	0.0225	0.01	-	-	-
Blank	0.013	0.012	0.0125	-	-	-	-

S', synovial fluid treated with 0.5 mg trypsin,

S", synovial fluid treated with 1 mg trypsin,

T_1, 0.5 mg trypsin

T_2, 1 mg trypsin

Water was used as a blank.

Table 3.7 The Pro-Collagenase Activity in Some Human Rheumatic Synovial Fluid

	Series A			Series B			(C)				
Sample (S)	a_1	a_2	Mean (m)	a_1	a_2	Mean (m)	MeanA-MeanB	C-blank	S'-T_1	S"-T_2	%increase in activity
11 R	0.044	0.046	0.045	0.01	0.012	0.011	0.034	0.026	-	-	-
11' R	0.073	0.075	0.074	0.018	0.016	0.017	0.057	0.049	0.046	-	76.9
11" R	0.099	0.096	0.097	0.02	0.02	0.021	0.076	0.068	-	0.062	161.5
12 R	0.054	0.056	0.055	0.011	0.01	0.011	0.044	0.036	-	-	-
12' R	0.075	0.078	0.077	0.02	0.019	0.02	0.057	0.049	0.046	-	27.8
12" R	0.096	0.095	0.096	0.021	0.023	0.022	0.074	0.066	-	0.06	66.7
17	0.035	0.037	0.036	0.017	0.015	0.016	0.02	0.012	-	-	-
17'	0.049	0.047	0.048	0.02	0.023	0.021	0.027	0.019	0.016	-	33.3
17"	0.066	0.067	0.067	0.026	0.027	0.027	0.04	0.032	-	0.026	116.7
18	0.063	0.065	0.064	0.012	0.014	0.013	0.051	0.043	-	-	-
18'	0.091	0.095	0.093	0.019	0.018	0.019	0.0745	0.067	0.064	-	48.8
18"	0.11	0.108	0.109	0.023	0.021	0.022	0.087	0.079	-	0.073	69.8
T_1	0.024	0.025	0.025	0.015	0.013	0.014	0.011	0.003	-	-	-
T_2	0.03	0.031	0.031	0.017	0.017	0.017	0.014	0.006	-	-	-
Blank	0.015	0.011	0.013	0.004	0.006	0.005	0.008	-	-	-	-

S', synovial fluid treated with 0.5 mg trypsin,

S", synovial fluid treated with 1 mg trypsin,

T_1, 0.5 mg trypsin,

T_2, 1 mg trypsin,

Water was used as a blank.

3.3 Detection of Elastase and Collagenase Inhibitory Action of Human Rheumatic Synovial Fluid on Elastase and Collagenase

3.3.1 Detection of Elastase Inhibitory Action of Human Rheumatic Synovial Fluid on Elastase

Table 3.8 shows to what extent different amounts of human rheumatic synovial fluid inhibited the activity of pure elastase. The inhibitory capacity varied with different samples, some showed a degree of saturation where by increasing the amount of synovial fluid showed no definite increase in the inhibitory capacity.

Table 3.9 shows the elastase inhibitory capacity of normal human serum. From the data it was noticed that there was complete inhibition of elastase activity.

Table 3.8 Elastase Inhibitory Action of Human Rheumatic Synovial Fluid on Elastase

Sample (Synovial fluid)	% Inhibition of Elastase Activity			
	50 mcl	100 mcl	150 mcl	200 mcl
11 R	26.2	57.1	90.1	98.1
12 R	6.1	13.77	22.13	28.52
13 R	7.89	33.34	36.72	39.54
14	92.9	97.2	99.3	99.5
17	31.9	79.5	95.5	97.7
21	48.1	91.22	97.7	99.7

R = Right knee

Table 3.9 Elastase Inhibitory Action of Normal Human Serum on Elastase

Sample (Normal serum)	% Inhibition of Elastase Activity			
	50 mcl	100 mcl	150 mcl	200 mcl
1 (F)	97.6	98.4	99.2	99.5
2 (M)	81.8	95.6	97.2	98.9
3 (M)	72.86	92.7	98.9	99.9
4 (F)	93.6	96.3	98.5	98.8
5 (F)	96.5	97.2	98.5	99.1
6 (M)	88.9	97.6	99.3	99.8
7 (M)	96.6	98.6	99.2	99.6

F = female,

M = male.

3.3.2 Detection of Collagenase Inhibitory Action of Human Rheumatic Synovial Fluid on Collagenase

Table 3.10 shows to what extent different amounts of human rheumatic synovial fluid inhibited the activity of pure collagenase. There was no complete inhibition for the collagenase activity as the amount of synovial fluid increased, and the percentage of inhibition is low compared to that of elastase.

Table 3.10 Collagenase Inhibitory Action of Human Rheumatic Synovial Fluid on Collagenase

Sample (Synovial fluid)	% Inhibition of Collagenase Activity		
	50 mcl	100 mcl	150 mcl
11 R	2.9	11.43	22.86
12 R	2.33	39.54	46.51
15	4.35	41.31	69.6

3.4 Adjuvant Induced Arthritis in the Rats

3.4.1 The Assessment and Development of Adjuvant Arthritis

The development of adjuvant induced arthritis in the injected left hind feet of male Wistar rats is shown in Figure 3.5. The intensity of the arthritis was assessed by the increase in volume of the hind feet. There was a sharp increase in volume in the injected feet and a noticeable increase in the non-injected in the hind feet of rats injected with the adjuvant.

3.4.2 Serum Collagenase Patterns of Adjuvant Arthritic Rats

The effect of adjuvant arthritis on collagenase activity of rat serum is shown in Figure 3.6.

The collagenase activity sharply decreased in test rats after ten days when compared with the controls.

3.4.3 Serum Elastase Pattern of Adjuvant Arthritic Rats

The effect of adjuvant arthritis on elastase activity is shown in Figure 3.7. There was a decrease in elastase activity in test rats when compared with the controls.

3.4.4 The Trypsin Inhibitory Capacity (TIC) and The Trypsin Protein Esterase (TPE) Activities of Adjuvant Arthritic Rats Serum

The effect of adjuvant arthritis on TIC and TPE activities is shown in Figure 3.8 and Figure 3.9 respectively. The TIC activity

was depressed in test rats while the activity increased in control rats. The TPE activity was greatly increased in the test rats, while the activity in the control rats slightly decreased.

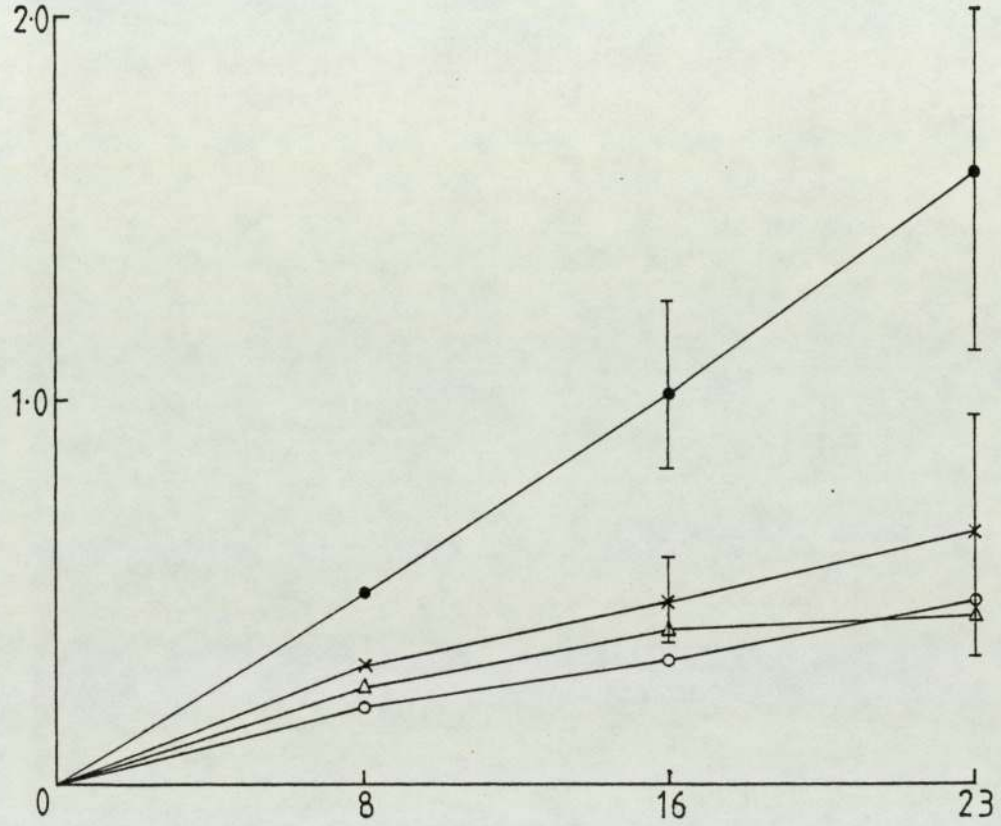


Figure 3.5 Increase in foot volume of left ●——● and right ×——× hind feet of rats after injection of mycobacterial adjuvant into left hind feet. Increase in foot volume of left △——△ and right ○——○ hind feet of rats after injection of 0.9% saline into left hind feet. Each result is a mean of seven animals and vertical lines indicate S.D.

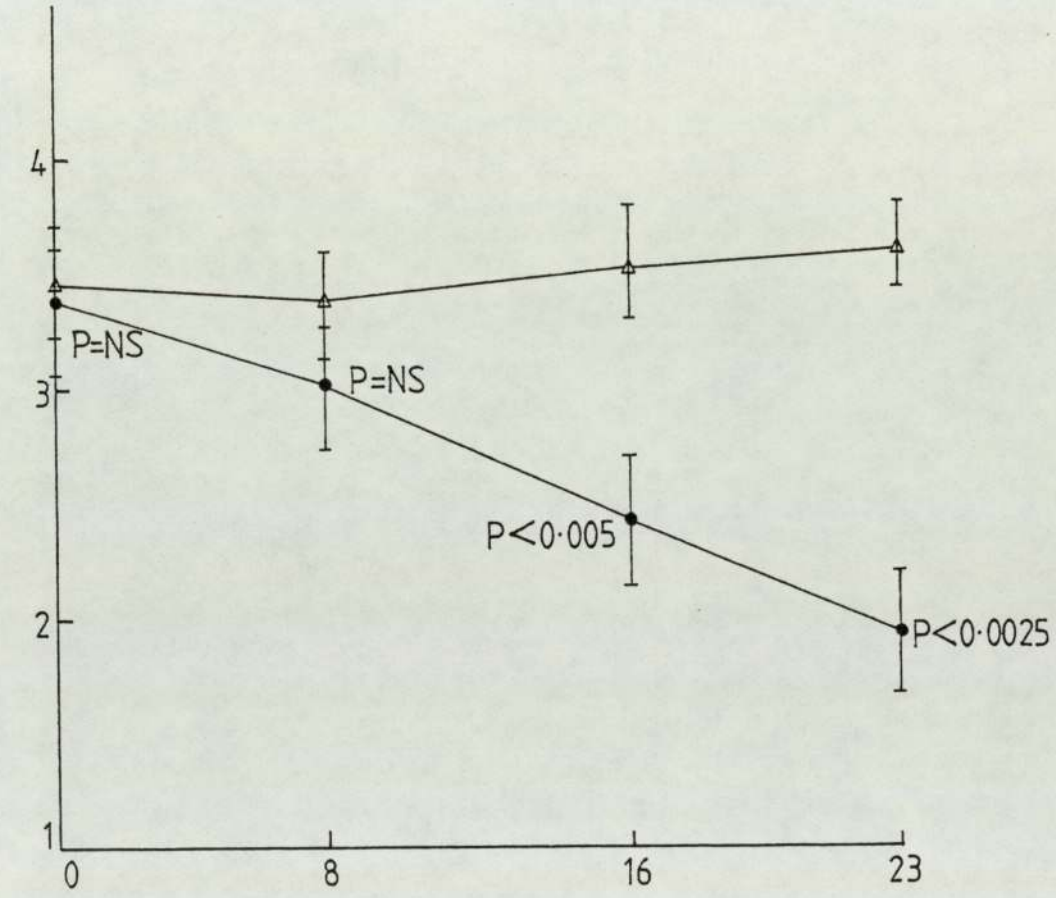


Figure 3.6 Collagenase activity of serum of rats injected with mycobacterial adjuvant ●——● , and rats injected with 0.9% saline △——△ , each result represents a mean of seven animals and verticle lines represent ± S.E.M.

NS: Non Significant

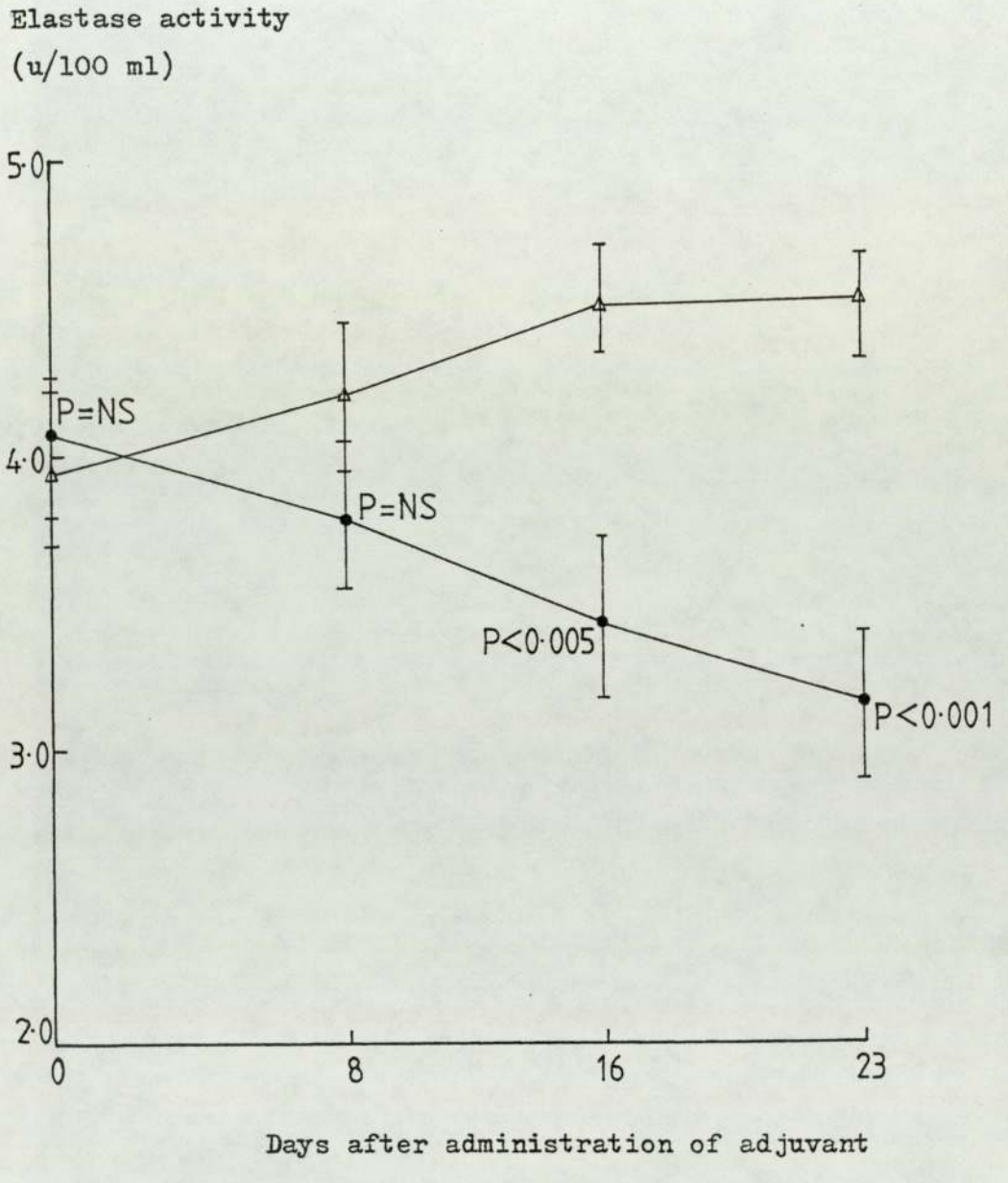


Figure 3.7 Elastase activity of serum of rats injected with mycobacterial adjuvant ●——● , and rats injected with 0.9% saline △——△ , each result represents a mean of seven animals and vertical lines represent S.E.M.

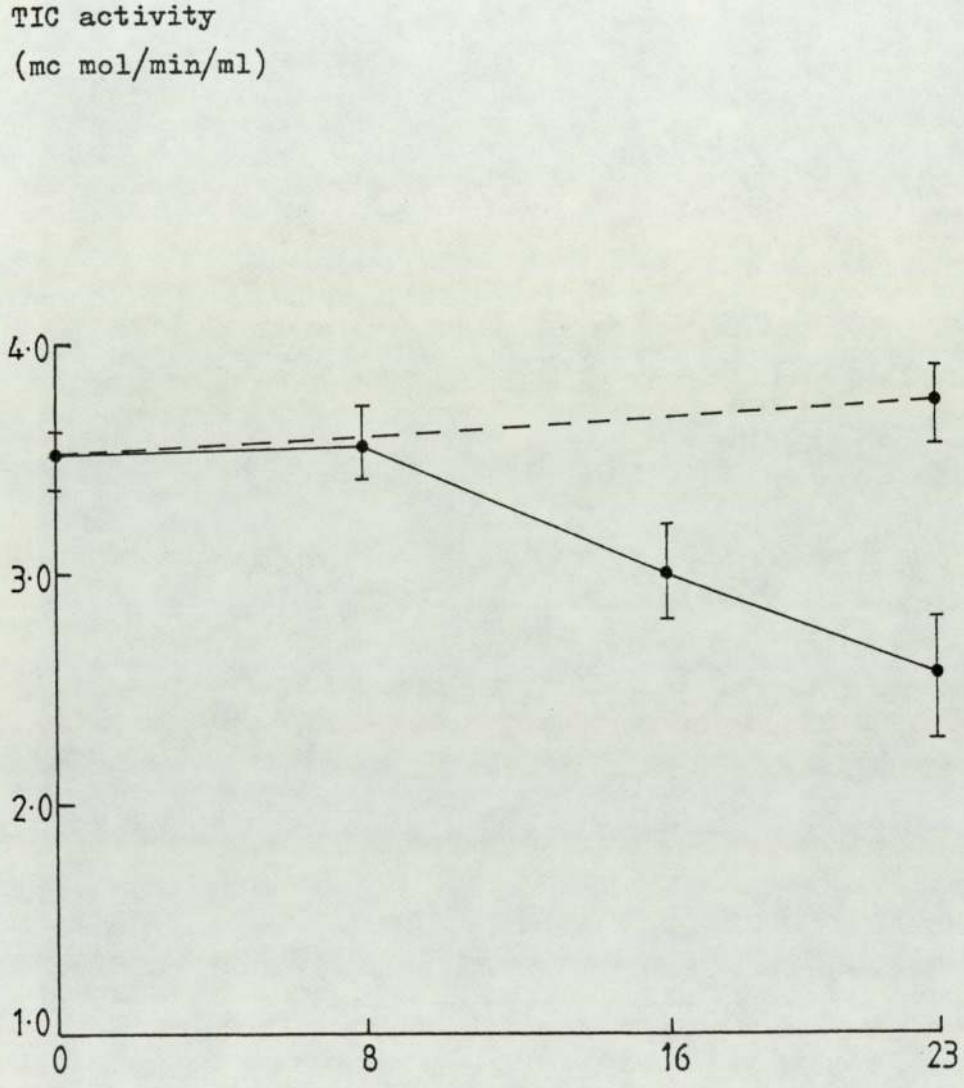


Figure 3.8 Trypsin inhibitory capacity of serum of rats injected with mycobacterial adjuvant ●——● , dotted lines represent non-arthritic control rats. Each result represents the mean of five animals and vertical lines represent S.E.M.

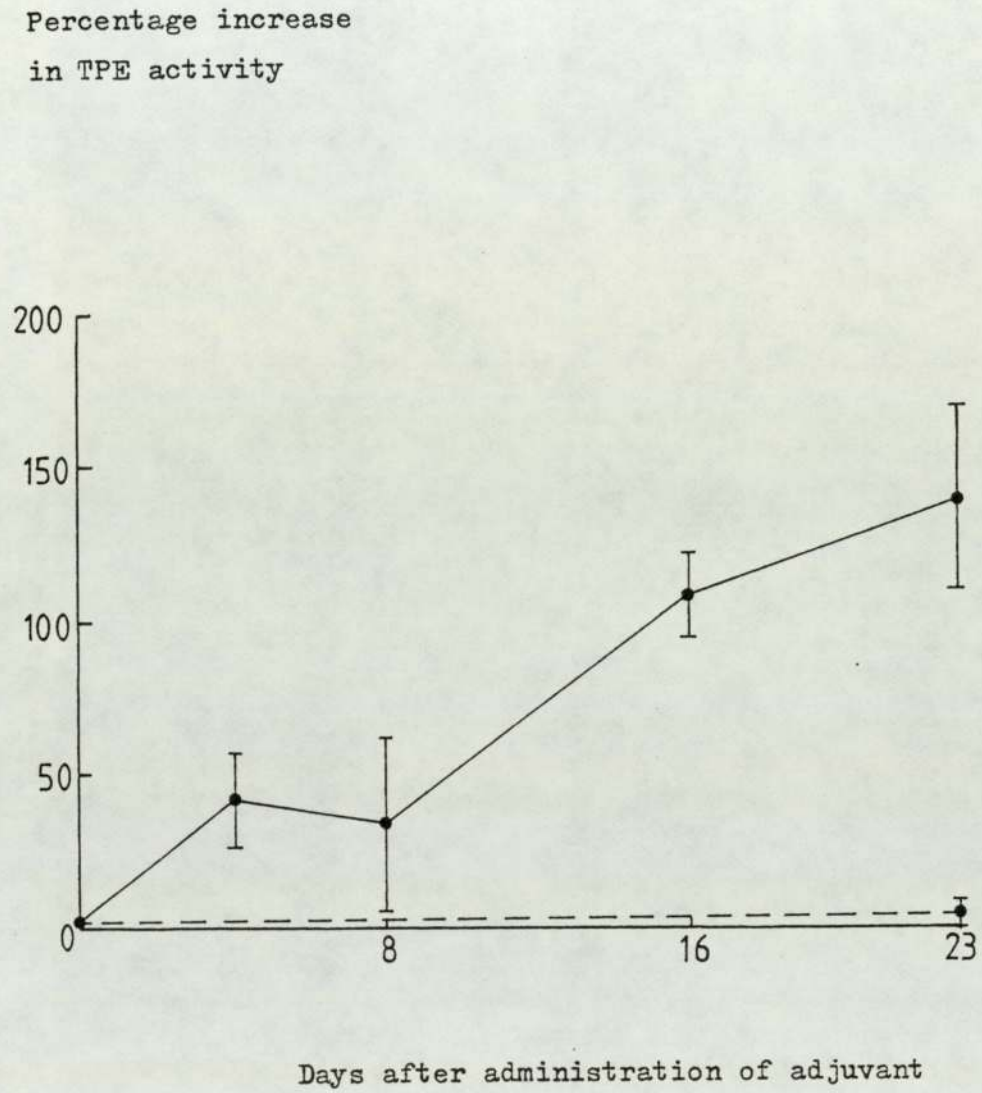


Figure 3.9 Trypsin-protein esterase activity of serum of rats injected with mycobacterial adjuvant •——• . Dotted lines represent non-arthritic control rats. Each result represents the mean of five animals and vertical lines represent S.E.M.

3.5 Separation of Human Rheumatic Synovial Fluid by Electrophoresis

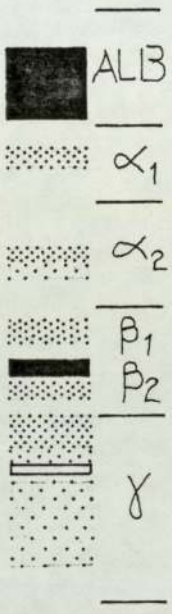


Figure 3.10 Electrophoresis pattern of normal human serum

From the electrophoresis patterns shown in figure 3.11 and 3.12 it was shown that synovial fluid has a similar protein pattern to serum, and treatment of samples with trypsin does not unmask new proteins which can be detected by methods used.

Trypsin appears to digest many of the proteins in the synovial fluid, especially beta one and beta two proteins, see figure 3.11, 3.12, 3.14A and 3.16. Trypsin itself migrates towards the cathode. Also treatment of synovial fluid with trypsin results in the loss of esterase activity as in figure 3.13, 3.14 and 3.15. Apart from

the general loss of proteins after tryptic digestion, there was no evidence found to suggest that trypsin unmasked new proteolytic enzymes. From the sketch shown in figure 3.17 it was noticed that treatment of synovial with trypsin results in the appearance of protein bands which can be detected by Schiffs reagent which detects glycoproteins such as alpha-1-antitrypsin.

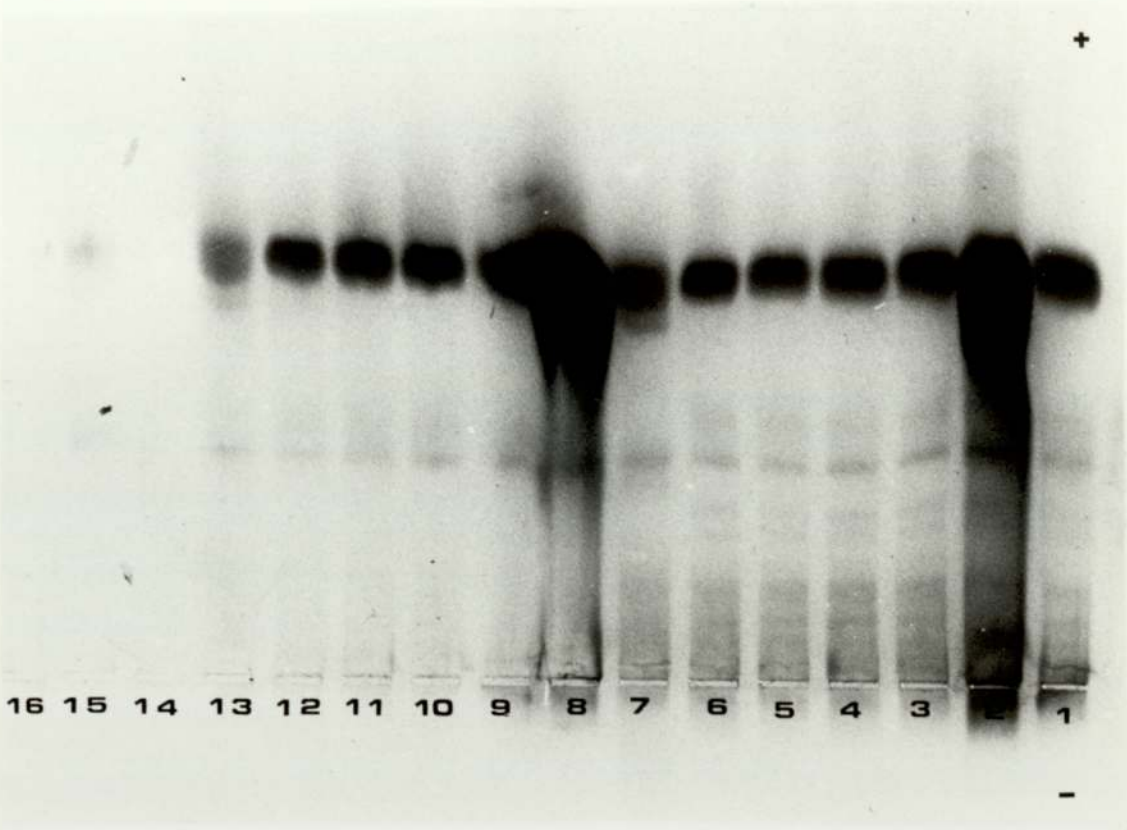


Figure 3.11 Electrophoresis of 1, 2 Synovial Fluid (SF); 3, 4, 5, 6, 7 matching serum of the same SF; 8, 9, SF + 0.1 mg trypsin; 10, 11, 12, 13 serum + 0.1 mg trypsin; 14, 15, 16 trypsin.
Gel was stained for protein with Coomassie Brilliant Blue R after the run.

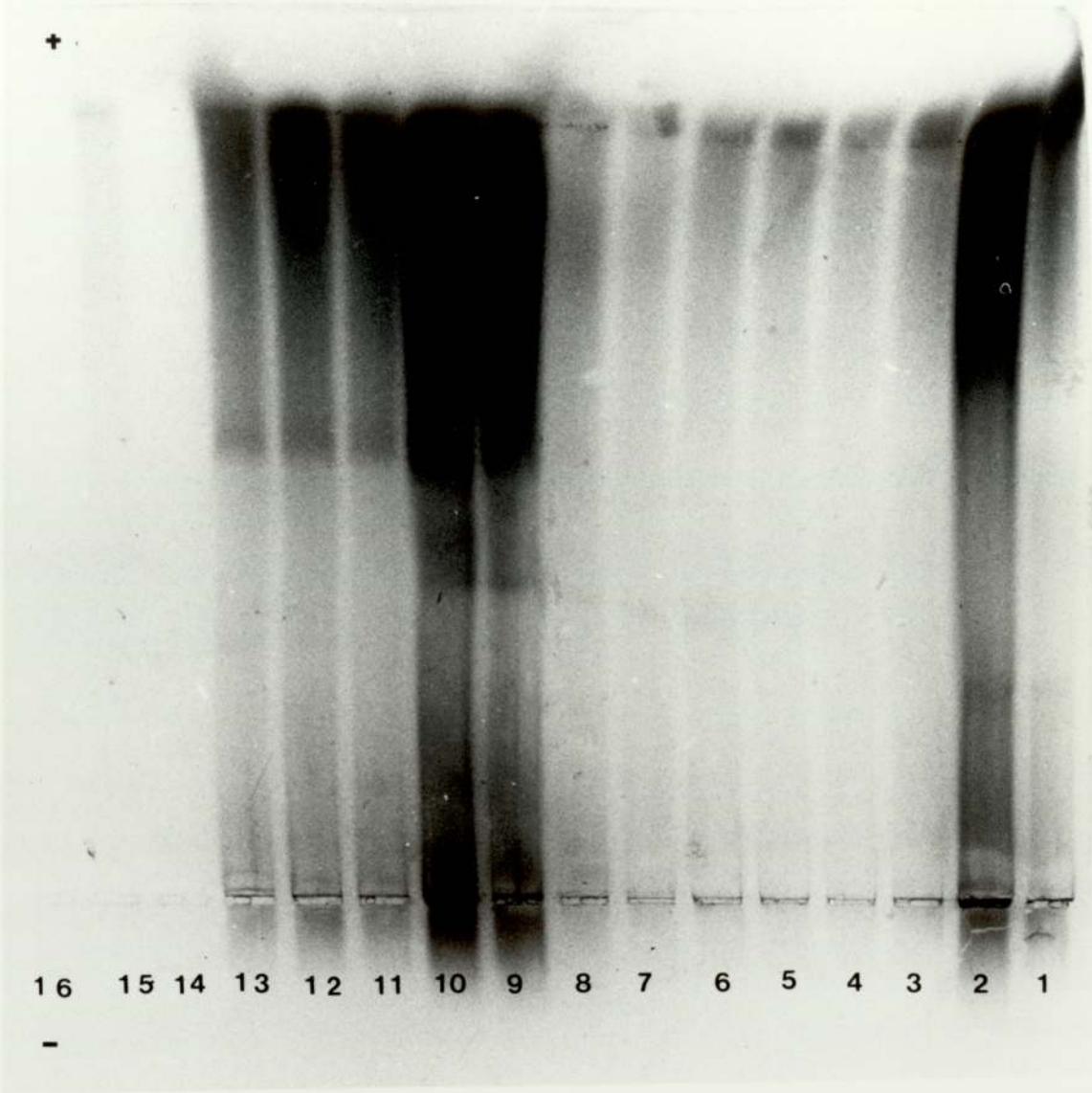


Figure 3.12 Electrophoresis of 1, 2 SF; 3, 4, 5, SF + 0.5 mg trypsin; 6, 7, 8 SF'+ 1 mg trypsin; 9, 18 SF; 11, 12, 13 SF' + 0.5 mg trypsin; 14, 15, 16 trypsin.
Gel was stained for protein with Coomassie Brilliant Blue after the run.

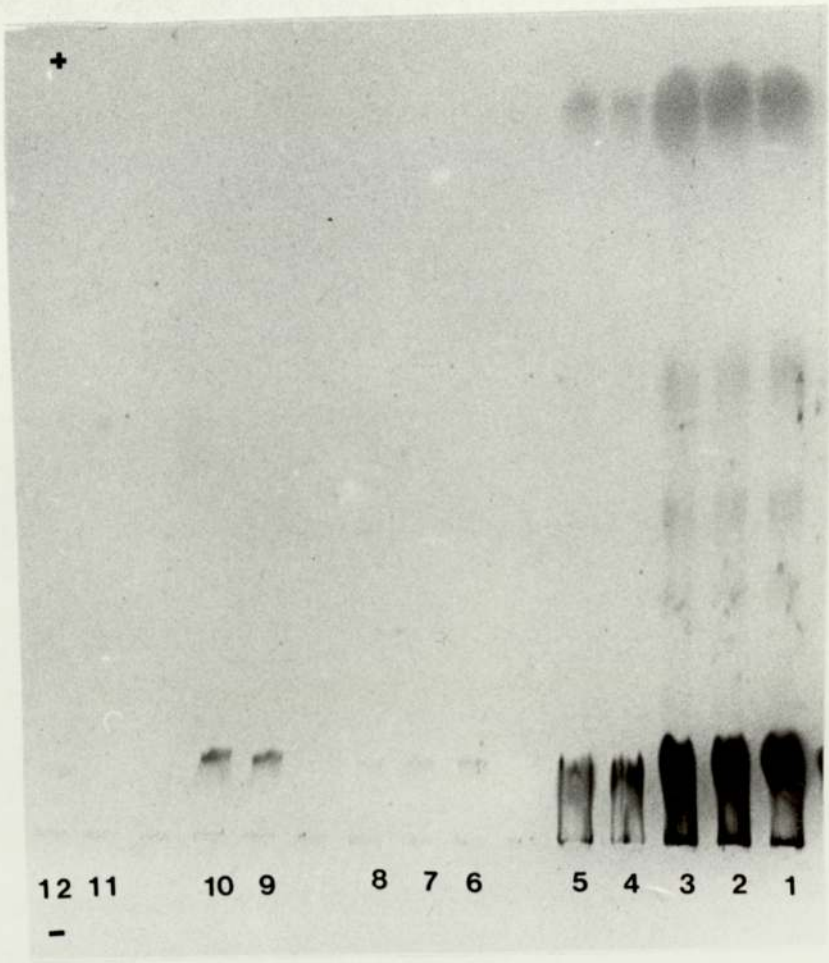


Figure 3.13 Electrophoresis of 1, 2, 3 SF; 4, 5, SF + 0.1 mg trypsin; 6, 7, 8 SF' + 1 mg trypsin; 9, 10 SF' + 0.5 mg trypsin; 11, 12 trypsin.
Gel was stained for esterase after the run.

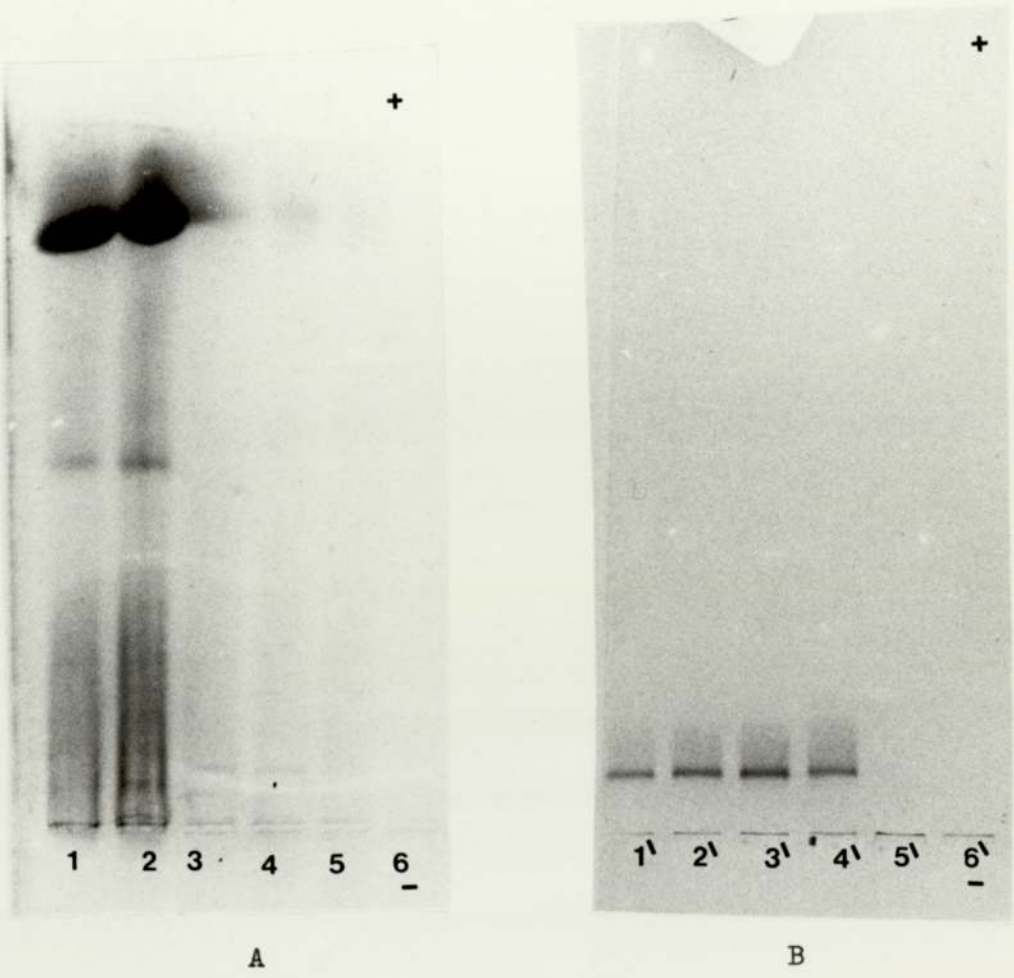


Figure 3.14 Electrophoresis of 1, 1', 2, 2', SF; 3, 3', 4, 4' SF + 0.5 mg trypsin; 5, 5', 6, 6' SF + 1 mg trypsin.
A was stained for protein.
B was stained for esterase.

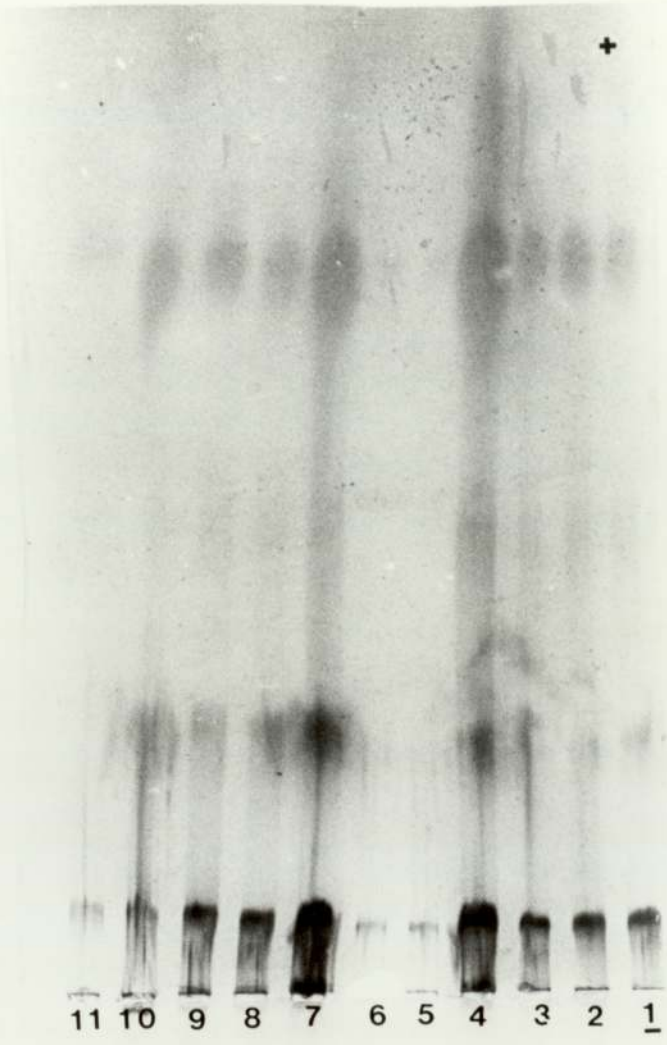


Figure 3.15 Electrophoresis of 1, 2, 3, 4 SF; 5, 6 SF + 0.5 mg trypsin; 7, 8, 9, 10 SF; 10, 11 SF + 0.5 mg trypsin. Gel was stained for esterase after the run.

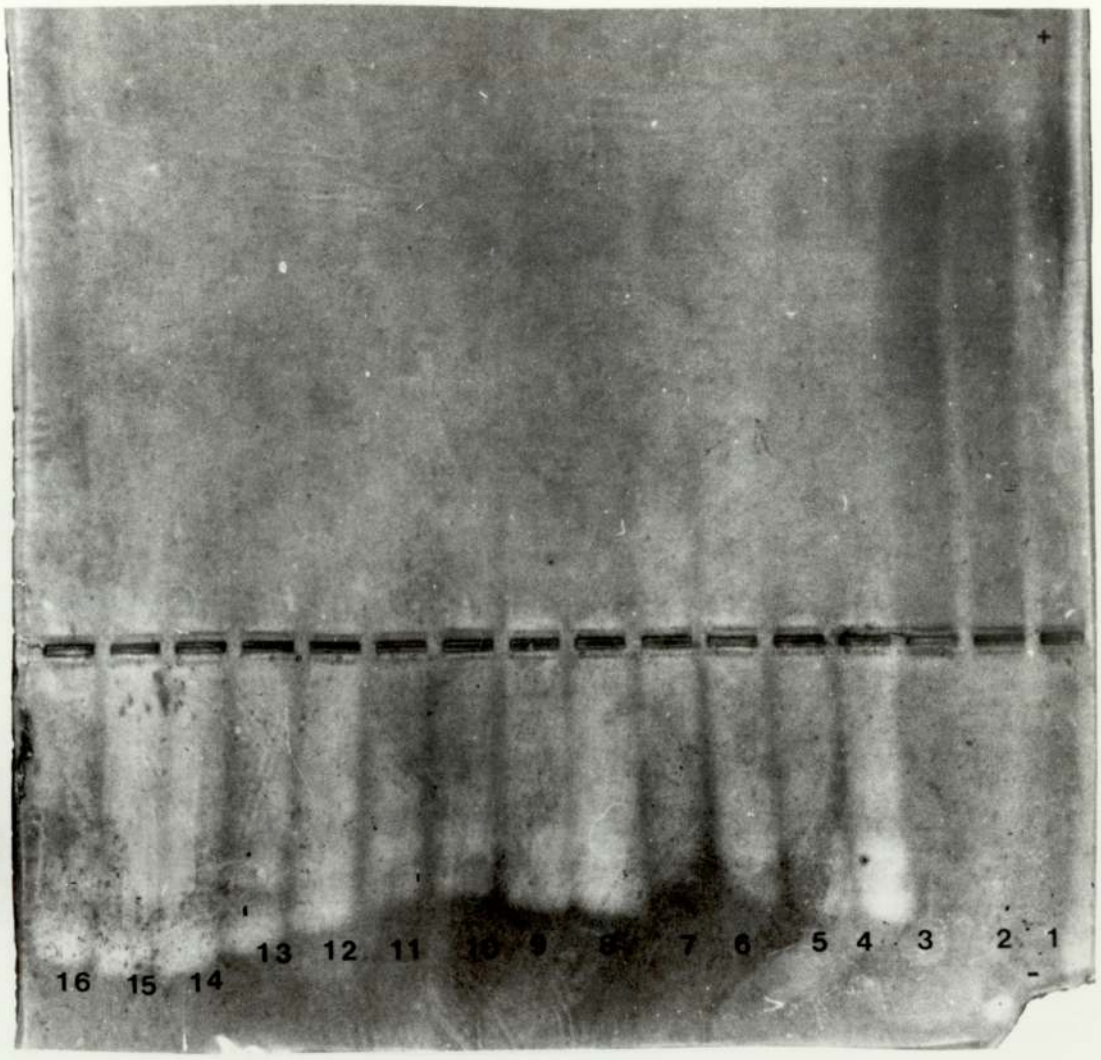


Figure 3.16 Electrophoresis of 1, 2, 3, SF; 4, 5 100 mcl SF + 0.1 mg trypsin; 6, 7 100 mcl SF + 1 mg trypsin; 8, 9 SF + 2 mg trypsin; 10, 11 50 mcl SF + 0.1 mg trypsin; 12, 13 50 mcl SF + 1 mg trypsin; 14, 15, 16 trypsin. Gel was stained for proteinase after the run.

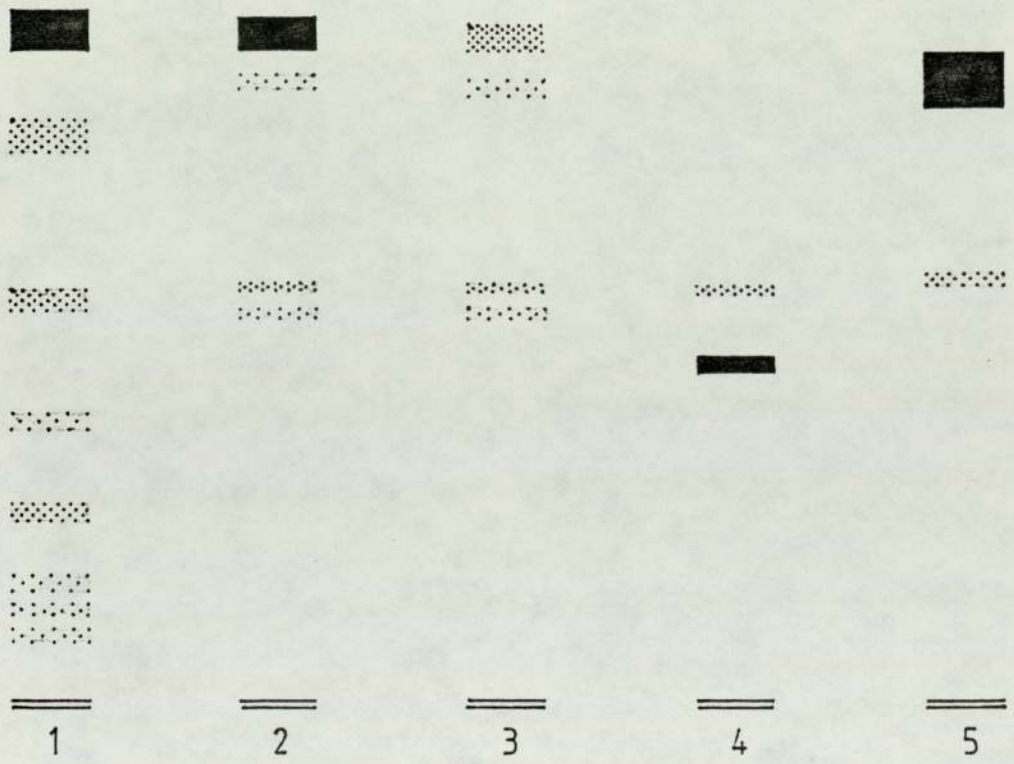


Figure 3.17 Sketch of electrophoresis diagram in which 1, SF; 2, SF + 2 mg trypsin; 3, SF + 4 mg trypsin; 4, 1 mg trypsin + 1 mg alpha-1-antitrypsin; 5, alpha-1-antitrypsin.

3.6 Chromatographic Fractionation of Human Rheumatic Synovial Fluid

The elution pattern obtained when 3 ml of human rheumatic synovial fluid was fractionated by gel filteration on a sephadex G-150 column is shown in Figure 3.18. The elution pattern consisted of an initial broad peak followed by a small secondary peak. The column fractions were assayed for collagenase and the pattern obtained indicates the prescence of a broad peak of collagenase activity. The molecular weight (M.Wt.) of each fraction was determined from the calibration curve, Figure 3.19. M.Wt. of fraction 1 (F_1): 50,000 Daltons, M.Wt. of fraction 2 (F_2): 20,000 Daltons and M.Wt. of collagenase: 34,700 Daltons.

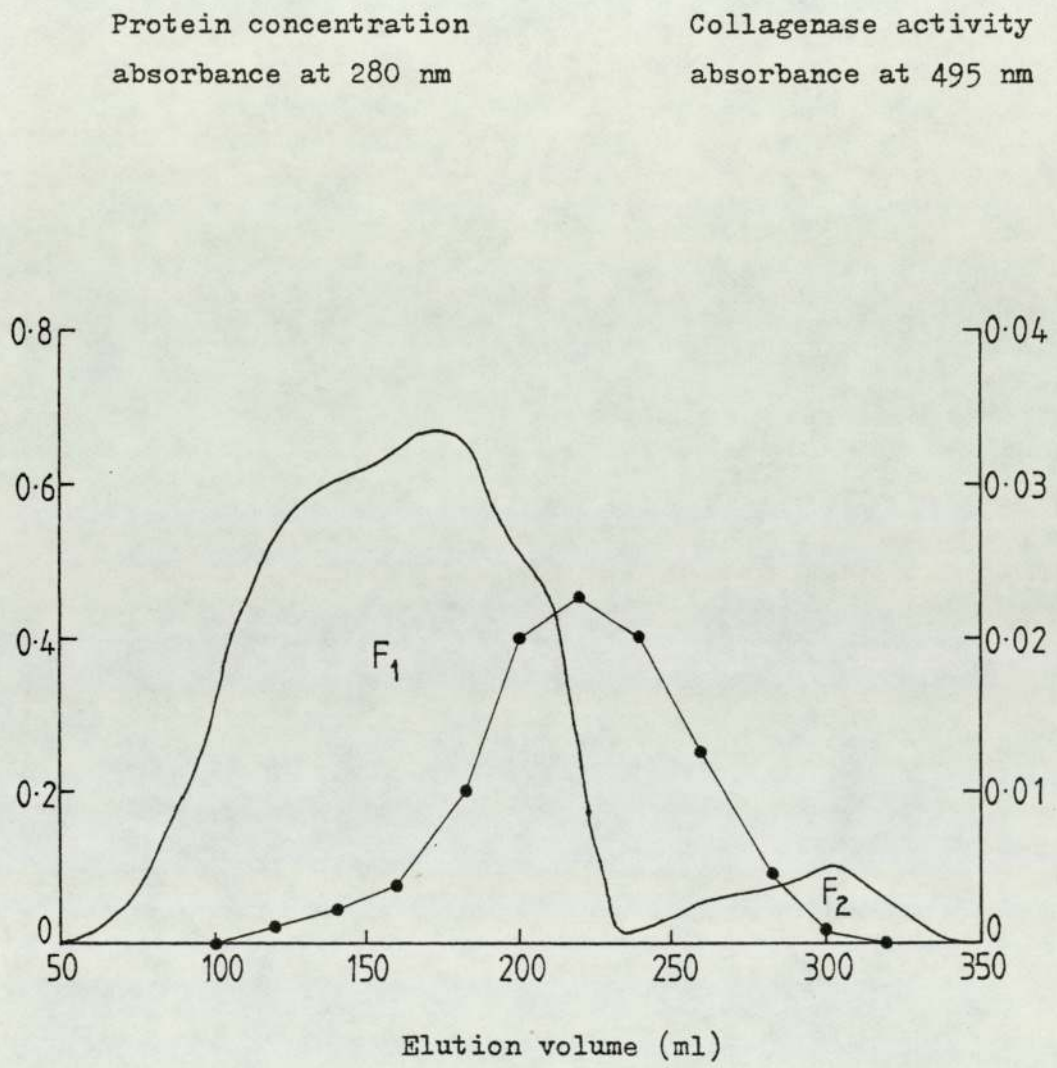


Figure 3.18 The protein elution profile of synovial fluid through a **Sephadex** G-150 column in 0.1 M Tris buffer, pH 8.6. The absorbance at 280 nm ———— , and absorbance at 495 nm ●————● .

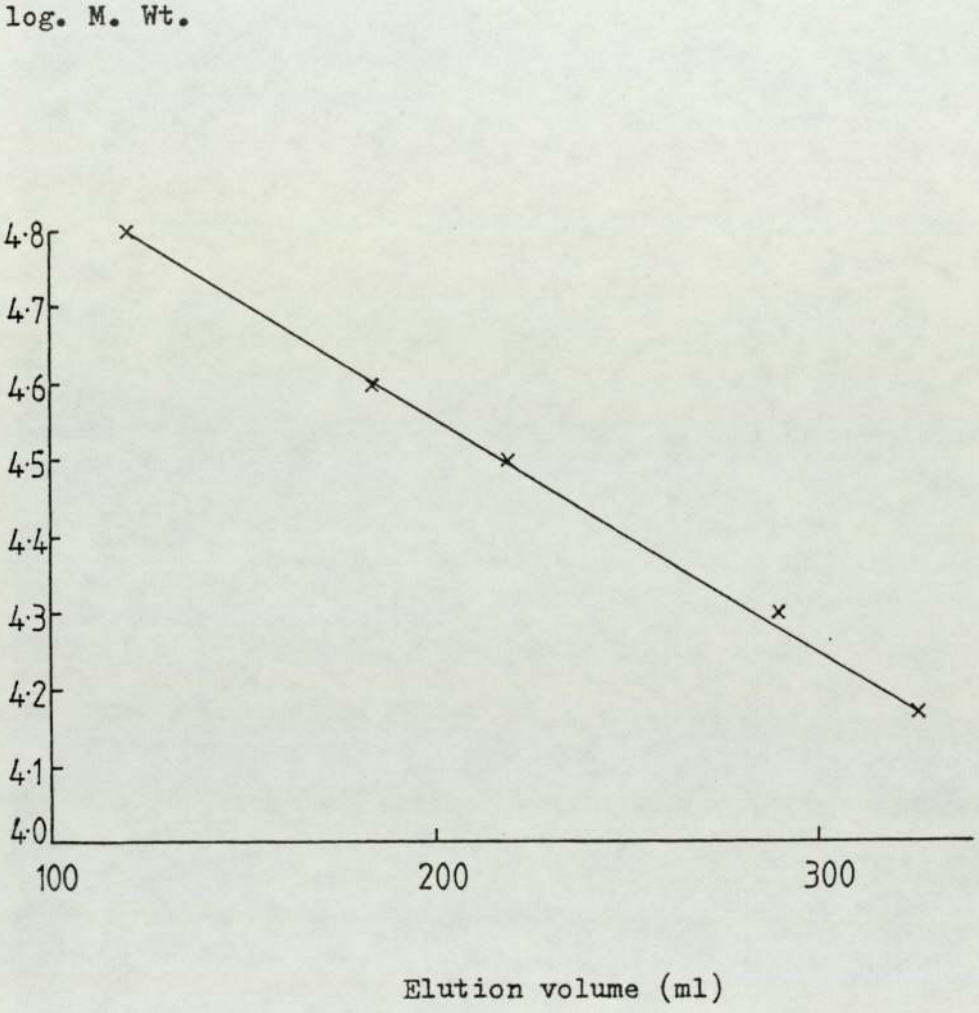


Figure 3.19 Calibration curve of column of Sephadex G-150 in 0.1 M Tris buffer, pH 8.6, proteins used for the calibration Albumin, ovalbumin, pepsin, trypsinogen and lysozyme.

4. DISCUSSION

4.1 Levels of Proteinases and Anti-proteinases in Human Rheumatic Synovial Fluids

This study has been mainly concerned with the role of collagenases and elastases in inflammation and the role of alpha-1-antitrypsin inhibitor and therefore a possible endogenous anti-arthritic agent.

In this study significant positive relationships were found between proteinase and collagenase, collagenase and elastase, and proteinase and total protein. Obviously collagenase and elastase activity is parallel and this would be destructive in the arthritic joint since these structures are rich in collagen fibres and proteoglycans. Therefore proteolytic action is likely to be general rather than selective. Obviously other proteinases were present since these parallel total protein levels. This may reflect a plasma origin or increased inflammation due to a progressive migration of polymorphonuclear leucocytes into the joint. Total protein levels in synovial fluid reflect the increase in permeability of membranes associated with inflammation (Vernon-Roberts, 1978), and therefore an index of severity of the inflammation.

Neither collagenase, elastase or total protein activity paralleled lysosomal breakdown since no correlation was found with these parameters and acid phosphataselevels. This may indicate at least in part a non-lysosomal origin for collagenase and elastase.

These results were compared with those of an earlier study (Lewis and Bird, unpublished). Where again total anti-proteinase

levels paralleled total protein and also total alpha-1-antitrypsin and total alpha-2-macroglobulin levels. Significant positive correlations were found between proteinase and total alpha-1-antitrypsin, proteinase and alpha-2-macroglobulin and total protein and proteinase.

In this case the two anti-proteinases were obviously of plasma origin and formed part of the total protein levels in the fluids. The levels of alpha-1-antitrypsin in sera and synovial fluid from patients with rheumatoid arthritis and other joint diseases have been found significantly elevated (Brackertz, Hagmann and Kueppers, 1975). It was suggested by these workers that higher than normal concentrations of alpha-1-antitrypsin could help to control tissue damage by inhibiting proteolytic enzymes released from lysosomes of polymorphonuclear leucocytes.

According to Ohlsson (1975a) the inactivation of proteinases occurs via the following mechanism:

1. Proteinase + Alpha-1-antitrypsin (active) $\longrightarrow$ Alpha-1-antitrypsin-proteinase (inactive)

2. Alpha-1-antitrypsin-proteinase + Alpha-2-macroglobulin $\longrightarrow$ Alpha-2-macroglobulin-proteinase + Alpha-1-antitrypsin (active)

3. Alpha-2-macroglobulin-proteinase + phagocytes $\longrightarrow$ degradation

Therefore, whilst alpha-1-antitrypsin levels would remain high, alpha-2-macroglobulin would deplete and could only be replenished by migration of fresh alpha-2-macroglobulin from the plasma.

Since alpha-2-macroglobulin is a high molecular weight protein its rate of diffusion into joint tissues would be low in contrast to

alpha-1-antitrypsin with a much lower molecular weight and consequently a high diffusion rate. In the Table (3.4) alpha-1-antitrypsin levels are higher than alpha-2-macroglobulin. It would be expected that total alpha-1-antitrypsin would parallel total protein, since its rate of diffusion would be similar to that of albumin, whereas that of alpha-2-macroglobulin would be much slower due to its increased molecular size.

The amount of active alpha-1-antitrypsin would depend on the kinetics of its reaction with proteinases which will vary with different proteinases and this may explain why no significant correlation was found between alpha-1-antitrypsin and collagenase and elastase, but a positive correlation was found between proteinases and total alpha-1-antitrypsin.

The activity of both total alpha-2-macroglobulin and free alpha-2-macroglobulin will depend on rate of diffusion, rate of reaction and rate of phagocytosis. Since these may vary in patients this may be the reason for the lack of significance between alpha-2-macroglobulin and proteinases.

It has been shown in Table (3.2) that mean values for proteinase activity and total protein levels differ between different rheumatic diseases. Samples from gout patients showed the highest mean values for proteinase and total protein, where the proteinase activity range was (7.8 - 8.1 mg substrate broken down in 24 hrs) and total protein was (5.9 - 6.2 mg/ml). Gouty arthritis is an acute inflammation with infiltration by a large number of PMNs which will breakdown, due to ingestion of the monosodium urate crystals, releasing high levels of lysosomal enzymes. Consequently high levels of proteinases and proteins would be expected and the results confirm

that observation.

In rheumatoid arthritis the range of proteinase activity was (4 - 8.5 mg substrate broken down in 24 hrs) and total protein was (3.1 - 6.75 mg/ml). These wide ranges can be explained on the basis of disease activity, patients with active rheumatoid arthritis have highest levels of proteinases and protein.

Only one sample with osteoarthritis was provided and the proteinase activity and total protein value in that sample was much lower than that of gout and rheumatoid arthritis. This result was consistant with the disease pattern since osteoarthritis is recognized dominantly as a non-inflammatory disease.

The results are consistant with the proteinase and protein values in synovial fluid being a useful biochemical indicator of the severity of inflammation, and emphasise on the important role of proteinases in tissue destruction in inflammatory rheumatic diseases.

The erythrocyte sedimentation rate (ESR) and the C-reactive protein (CRP) are well established indicies for rheumatic diseases (Kushner, 1981). Measurement of CRP and ESR are good indicators of the degree of activity and severity of rheumatoid arthritis.

From Table (3.2 a) it was found that the increase in CRP and ESR was accompanied by an increase in proteinase activity. High values for CRP and ESR are indicies for severe inflammation and the results show that proteinase activity increases as well. Proteinases are responsible for tissue damage and their activity is accelerated during severe inflammation.

It was found that high levels of proteinases were accompanied by high white blood cell counts (WBC) which may reflect a cellular origin of enzymes found in synovial fluid. This was clearly shown

in sample 19 where higher levels of proteinases were accompanied by high WBC counts. In contrast in sample 16 (osteo-arthritis, which is non-inflammatory) low levels of proteinases were accompanied by low WBC counts.

Table (3.2 b) shows clearly the relationship between disease activity and mean values for proteinases. Samples with high activity showed high mean values for proteinase and collagenase. In contrast samples with low activity showed low values for proteinase and collagenase and the non-active sample showed the lowest value for proteinases.

4.2 Electrophoretic Separation of Proteins in Human Rheumatic Synovial Fluid

The electrophoretic protein pattern obtained for the synovial fluids is similar to that of serum, this could indicate that the bulk of total protein present is of plasma origin.

Digestion of total protein was observed after treatment with trypsin and no pro-enzymes were observed by this method, which could be due to the insensitivity of the technique used. Only added trypsin in high concentrations could be observed by this method. Digestion of esterase was also observed after treatment with trypsin.

4.3 Separation of Human Rheumatic Synovial Fluid by Gel Chromatography

By using this technique for separation synovial fluid was separated into two protein fractions, one with a high molecular weight 50,000 daltons, and the other with a molecular weight approximately 20,000 daltons. Both collagenase and elastase were assayed in each fraction. Collagenase was detected in a fraction with a molecular weight of 37,000 daltons. Elastase activity could not be detected in the fractions.

4.4 Pro-Collagenase and Pro-Elastase Activities in Human Rheumatic Synovial Fluid

In joint diseases such as rheumatoid arthritis and osteo-arthritis the destructive process starts with the gradual disappearance of proteoglycans of articular cartilage, the collagen fibrils are left naked and susceptible to attack by lytic enzymes and to mechanical stress.

These enzymes can exist in latent forms which is either pro-enzymes (Werb, Mainardi and Vater, 1977), or enzyme inhibitor complex (Woolley and Evanson, 1977). These latent enzymes can be activated by using proteinases such as trypsin (Vaes, 1972a).

Peltonen (1978) reported no significant increase in collagenase activity after treatment with trypsin. It was found from the results shown in Table (3.7) and (3.6) that there was an increase in both collagenase and

elastase after treatment with trypsin and this increase in activity increased with increasing amounts of trypsin.

It was found that there was individual variation in the increase of activity between samples. Although no correlation was found between inflammatory arthritis (rheumatoid arthritis) and non-inflammatory arthritis (osteoarthritis), it must be remembered that single samples were examined.

Single samples reflect the state of the disease at that time and may well be influenced by other factors which vary during the disease process, for example drugs, anti-proteinase levels etc. An examination of samples taken serially throughout the course of the disease would provide more information on the proteolytic variations between specific rheumatic disorders.

4.5 Collagenase and Elastase Inhibitory Capacity of Human Rheumatic Synovial Fluid

Human rheumatic synovial fluid was shown to inhibit both pure collagenase and elastase. This inhibitory capacity was diminished after treatment of synovial fluid with trypsin.

In the case of elastase the activity of the enzyme was largely inhibited by 50 mcl of normal human serum. With synovial fluid, apart from sample 14, considerably more fluid was needed to inhibit the enzyme as effectively as serum. Therefore synovial fluid inhibitory capacity towards elastase was considerably less than that of serum.

In the case of collagenase the inhibitory capacity is very low compared to that of elastase and only higher amounts of the synovial can inhibit the activity of pure collagenase.

However, since both enzymes were inhibited by synovial fluid, therefore the presence of inhibitors in joint fluid must represent an endogenous anti-inflammatory action. It is likely then that the inhibitory action was due to alpha-1-antitrypsin or alpha-2-macroglobulin or a combination of both.

4.6 Levels of Proteinases and Anti-Proteinases in Rat Serum with Adjuvant Induced Arthritis

The fall in collagenase and elastase activity in the serum of adjuvant arthritic rats coincided with a fall in alpha-1-antitrypsin values. Since alpha-1-antitrypsin can react with these enzymes, then mutual interactions between proteinase and alpha-1-antitrypsin may have been responsible for the fall in serum levels of both. Polymorphonuclear leucocytes contain both collagenase and elastase and these cells accumulate in the affected joints in response to inflammation. As inflammation increases in severity these cells release their enzymes into the joint which probably leak into the general circulation.

Lewis and Collins (1971) observed a general rise in acid phosphatase activity and proteinase activity (Parrott and Lewis, 1977) in the serum of adjuvant rats. Consequently the fall in collagenase and elastase serum levels may reflect some specificity of alpha-1-antityrpsin for these enzymes. Alpha-1-antitrypsin also will migrate into affected joints and since the fluid in these joints are rich in proteolytic enzymes further alpha-1-antitrypsin-proteinase interactions will also deplete alpha-1-antitrypsin levels.

Although alpha-1-antitrypsin is an acute phase protein (APR) in humans there is no evidence that it is APR in the rat (Parrott, 1976).

Ganrot (1973) has demonstrated that there is a direct correlation between 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 and the levels of alpha-2-acute-phase-globulin. Both alpha-1-macroglobulin and alpha-2-acute-phase-globulin are APR (Parrott, 1976) in the rat and it is possible that the elevated TPE levels in serum reflect an APR response to inflammation. Therefore, the difference between serum alpha-1-antitrypsin (TIC values) and alpha-1-macroglobulin and alpha-2-acute-phase-globulin (TPE values) may be due to different rates of synthesis in the liver.

The adjuvant arthritis is a well established model for chronic inflammation. It is mainly used in pharmaceutical industries for evaluation of new anti-inflammatory drugs.

In the experiments carried out there was a fall in the serum levels of collagenase, elastase and alpha-1-antitrypsin. In humans with rheumatoid arthritis the serum and synovial fluid levels of alpha-1-antitrypsin rise. These findings may reflect species differences, but it is also possible that the formation of proteinase-antiproteinase complexes was responsible for the depression of the levels of proteinases and antiproteinase in rats serum. Parrott and Lewis (1977) have shown an increase in proteinase levels in fluids and tissue minces of arthritic joints of adjuvant rats; this could explain the fall in collagenase and elastase levels in these animals, due to difficulties in the aspiration of synovial fluid from the inflamed joints of arthritic rats, no collagenase or elastase activities estimation were done

on synovial fluid.

The Freund's adjuvant infected rat shares a number of similarities with patients with rheumatoid arthritis. These include clinical evidence of joint swelling, subcutaneous nodules, histopathological evidence of proliferative synovitis and ultimately progression to cartilage and bone erosion; however, major differences do exist such as the absence of rheumatoid factor (Ackerman, Rooks, Shott, Genant, Maloney and West, 1979). The histopathology of the synovium in adjuvant arthritis by light and electron microscopy is indistinguishable from inflammatory synovitis in man (Hadler, 1976).

The adjuvant induced disease is thought to be caused by a cell mediated immune response, circulating antibody and immune complexes are not found. Recently T-lymphocytes has also been demonstrated in the adjuvant arthritic rat which further substantiates the importance of cell mediated response (Ackerman, Rooks, Shott, Genant, Maloney and West, 1979).

The adjuvant arthritis is characterized by a rapid steady, evolution to advanced destruction in 3 - 4 weeks, leaving residual joint destruction, deformity and bony ankylosis, where as the human rheumatoid arthritis characterized by a progressive or remitting course extending over periods of months to years and the outcome is variable. For these reasons and considering potential species differences, one should be cautious in attempting to extrapolate rat experiment results to human rheumatoid arthritis. Adjuvant arthritis is a laboratory phenomenon peculiar to the rat and has no direct clinical couterpart in man, however, in the narrower concept of providing information on proteinase-

antiproteinase interaction *in vivo* the arthritic rat model is useful as a comparison to the human results.

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