IN VIVO AND IN VITRO STUDIES OF ENCAPSULATED ALPHA-1-ANTITRYPSIN AND CORTICOSTEROIDS AS ANTI-RHEUMATIC AGENTS

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SUMMARY

The anti-inflammatory effect of α_1-antitrypsin was investigated against rat carrageenan oedema, rat adjuvant arthritis, and rabbit monoarticular arthritis. Anti-inflammatory activity was proved only in rat adjuvant arthritis, and some anti-rheumatic action was evident from the biochemical tests on rabbit synovial fluid.

Corticosteroids treatments of rats elevated plasma α_1-antitrypsin levels. This effect was dose responsive as were the performed liver function tests of plasma and histological evidence of liver damage. A conclusion whether α_1-antitrypsin synthesis was due to a direct action of the steroids on the liver or indirectly due to liver damage could not be made with certainty.

The effect of intra-articular administration of α_1-antitrypsin and cortisol phosphate in a free and an erythrocyte-encapsulated form was tested on rabbit monoarticular arthritis. α_1-Antitrypsin did not prove anti-inflammatory on its own, but a combination of α_1-antitrypsin with cortisol phosphate gave superior results to either substance on its own.

A method of encapsulation of substances into intact erythrocytes was developed, which leaves the cells with their full circulating lifetime, in contrast to previous preparations. The survival of such preparations was monitored in the circulation of rats, guinea-pigs and rabbits by cell counts after labelling with fluorescein isothiocyanate.

Two steroids were encapsulated into erythrocytes at different doses and used as intravenous treatments against adjuvant arthritis in the rat. The encapsulated steroids were superior to the free steroids in controlling the disease.

In vitro tests were carried out to investigate the slow release of encapsulated preparations, the biochemistry of the encapsulating agent and the destruction of the preparation by phagocytosis. The concentration of encapsulated materials was assayed.

KEYWORDS

alpha-1-antitrypsin corticosteroids erythrocyte encapsulation anti-rheumatic

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1 INTRODUCTION

1.1 Rheumatoid diseases and rheumatoid arthritis

'Rheumatoid diseases' is the overall name given to diseases characterized primarily by pain and stiffness in joints, bones, muscles, tendons or ligaments. These diseases include more than 90 disorders, such as rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, rheumatic fever, gout etc.

In rheumatoid arthritis itself, chronic inflammatory lesions occur. These are distributed through a wide variety of tissues but particularly affect the joints. The lesions cause pain, swelling and deformity in the affected parts, and may lead to considerable destruction of tissues and cause lifelong crippling. Arthritis and rheumatism have been aptly described as the "diseases which kill the fewest but cripple the most".

The severity usually fluctuates, giving some remission followed by exacerbation. The disease is common, with world wide distribution. Its incidence is maximal in early adult life, but the effects are more severe in old age. Some forms occur in children. Women are generally more susceptible than men.

1.1.1 Cause of the disease

After many years of research, the exact cause of rheumatoid arthritis remains unknown. Numbers of factors have been proposed as the cause of this disease, among them: infectious organisms, heredity, hormonal abnormalities, metabolic defects, nervous ailments, emotional stress, diet, climate, physical injury and hypersensitivity disorders including autoimmune responses.

Over the years many scientists have attempted to isolate micro-organisms from the tissue or fluid in inflamed arthritic joints. Organisms such as bedsonia, diptheroids, mycoplasmas and viruses have been isolated or demonstrated. However, these results have not been successfully reproduced by other workers, and the possibility of contamination must not be dismissed. Marmion (1976) concluded that there was no direct evidence that infectious organisms were the missing exogenous antigens in rheumatoid arthritis. However, there is some indirect evidence in that many cases of rheumatoid disease are clearly associated with prior infection, and it seems that such factors contribute to the natural history of the disease, particularly in its chronic character.

At the present time it is widely believed that the inflammation in rheumatoid diseases may well be triggered by immune processes. However, it is not established whether such processes involve autoimmunity (i.e. directed against the antigenic constitution of the patient's own tissue) or if foreign antigenic material, due to the presence of a physical or an infectious agent, may be required.

A model of an initiation process in rheumatoid arthritis has been proposed by Hollander and also independently by Zvaifler. Hollander et al. (1965) suggests that the initiating factor may be a virus, which alters the immunoglobulin IgG, which causes it to become antigenic. Zvaifler (1965) does not specify the trigger in his theory, but does agree that IgG is the resultant antigen.

1.1.2 Rheumatoid factor

A substance or group of substances known as the rheumatoid factor (or factors) is often associated with rheumatoid arthritis, though its identity and role (if any) in the disease is controversial.

Diagnosis of rheumatoid arthritis frequently involves detection of the rheumatoid factor in the serum or the joint fluid, although the factor does not always occur even in patients with obvious rheumatoid arthritis (Mellbye & Natvig 1971). It is also unfortunately the case that the factor is not exclusively associated with arthritis, and has even been detected in the sera of some 'normal' individuals (Wolfe 1968).

Many studies of rheumatoid factor have suggested that it is a macroglobulin antibody (γM) which reacts with a gamma globulin (γG) of small molecular weight (7 S). There is much evidence that it reacts only with altered γG, i.e. changed by contact with antigens, heat or other agents. This aggregated γG then might assume the role of an antigen in susceptible individuals, to which a 19 S macroglobulin rheumatoid factor is formed as a secondary antibody.

Reaction of rheumatoid factor with the aggregated γG forms a loosely bound molecular aggregate (of size 22 S) which is readily reversible. The complex is deposited as particulate matter in the joint, and its phagocytosis by leucocytes would lead to a consequential loss of some lysosomal enzymes from the leucocytes. These enzymes would give rise to tissue inflammation and may also produce altered 7 S γG, completing the cycle of a self-perpetuating inflammatory process. This essentially is the theoretical process suggested by Hollander et al. (1965) for inflammation in rheumatoid arthritis.

Zvaifler (1965) proposed a very similar hypothesis, as follows. An unknown event initiates inflammation, causing disruption of lysosomes and leakage of lysosomal enzymes. These enzymes could alter leucocyte nucleoprotein and thus antibodies would be raised against the altered protein. Antigen-antibody complexes would be formed, and in the presence of rheumatoid factor inclusion bodies could develop. These would be engulfed by leucocytes, leading to a further loss of lysosomal enzymes, which would cause inflammation and tissue destruction, and complete the cycle by altering more nuclear material. The initiating factor may even disappear, leaving the disease in a self-perpetuating state.

1.1.3 The inflammatory process

The most common manifestation of rheumatoid diseases is inflammation. Inflammation involves changes in connective tissues and the vascular supply of damaged tissue. This process may be considered as a protective function designed to minimize or eliminate the cause of damage and subsequently to remove the damaged tissue and lead to its repair.

The acute inflammation following a stimulus involves two mechanisms, the most immediate being a fluid process, shortly followed by a cellular one. Both arise from vascular alterations. The vascular events in inflammation generally start with a short-lived (a few seconds) arteriolar constriction due to vasomotor reflex. The injured tissue itself liberates vasoactive compounds which cause a more persistent localized vasodilation and increased blood flow in arteries, capillaries and venules (Spector & Willoughby 1968).

The process of vasodilation is accompanied by an increased permeability of the vessels to larger plasma protein molecules (Cotran 1967) due to the effect of endogenous mediators. The ultrastructural features that account for the permeability changes are the openings of the endothelial junctions, and the quantitative or

qualitative changes in the cytoplasmic vesicles (Böhm 1977). All these events lead to a loss of plasma-like fluid in the surrounding tissues, producing a swelling (oedema) in the injured area. The accumulation of fluid itself has a function in diluting toxic materials such as irritants or bacterial toxins, thereby reducing the intensity of tissue damage.

Capillary and arteriolar responses and permeability changes are almost entirely mediated by histamine released from mast cells (present in almost all tissue). Evidence for the involvement of histamine is available: Spector & Willoughby (1957) found histamine in the exudate after intrapleural injection of turpentine for up to 30 minutes after initiation of the pleurisy. Vascular permeability in the initial phase of inflammation can be inhibited by anti-histamine treatment (Hurley & Spector 1965). Sheldon & Bauer (1960) have shown that tissue depletion of histamine delayed the onset of inflammation produced by injection of bacteria. Thus histamine appears to be the major mediator in the early phase of increased vascular permeability, although its contribution in later phases is probably insignificant (Spector & Willoughby 1968). The initial phase of this response can also be inhibited by known antagonists of endogenous permeability factors (Logan & Wilhelm 1966).

Other mediators are involved in the later phases. A number of systems producing active substances have been indicated, of which kinin, prostaglandin and complement systems seem most important.

Kinins are vasodilation and exudation promoting polypeptides, the most effective in this respect being bradykinin and kallidin. Kinins are formed by enzymatic degradation of a precursor (a kininogen) such as plasma α-2-globulin by a proteolytic lysosomal enzyme called kallikrein.

The involvement of kinin-like substances in inflammation was shown by Di Rosa & Sorrentino (1968) in a study of antiprotease inhibition of rat paw carrageenan oedema. Kinins have been shown to participate in maintaining oedema in the rat paw carrageenan model (Di Rosa, Giroud & Willoughby 1971; Vinegar, Macklin, Truax & Selph 1971). However, no overall correlation has been found between the amount of kinins in synovial fluid and the clinically assessed amount of inflammation (Melmon, Webster, Goldfinger & Seegmiller 1967).

Prostaglandins are long chain fatty acids which have vasodilating and exudate promoting properties (Lewis 1971) which may play a major part in the later stages of acute inflammation. In inflammation, phospholipase-A released from damaged cells converts phospholipids to arachidonic acid, a prostaglandin precursor. Subsequent action on this precursor by prostaglandin synthetase (an enzyme with very wide distribution) gives rise to various prostaglandins.

Prostaglandins may potentiate the effects of other mediators such as kinins (Ferreira & Vane 1974; Robinson & Levine 1974). Prostaglandin-like substances have been implicated in the second phase of oedematous response (Di Rosa & Willoughby 1971) and prostaglandin-E_2 has been shown to be synthesized by rheumatoid synovial tissue in culture (Robinson, McGuire & Levine 1975).

Many of the non-steroidal anti-inflammatory drugs are known to inhibit prostaglandin synthesis, which is thought to be their mode of action (Vane 1971); and the anti-inflammatory potency of these drugs seems to parallel their relative potency as inhibitors of prostaglandin synthetase (Paulus & Whitehouse 1973).

The cellular complement system is a special group of normal serum proteins which interact sequentially to effect a variety of inflammatory events. Products elaborated during these reactions mediate changes in vascular permeability (Lepow 1971). The products also attract both polymorphonuclear and mononuclear leucocytes, influence the release of lysosomal enzymes, enhance phagocytosis (by promoting the adherence of complement-coated complexes to formed elements in the blood) and damage cell membranes, ultimately inducing osmotic lysis and cell death.

The vascular changes eventually lead to increased viscosity of the blood and cell packing - hence PMN leucocytes are pushed against the blood vessel walls where they adhere to the endothelium and eventually penetrate the wall, migrating into the surrounding tissues by an amoeboid action. Later increased numbers of mononuclear cells (monocytes, lymphocytes and plasma cells) infiltrate into the lesion (Ryan 1967). One factor probably assisting in this action is chemotaxis, the chemical attraction of cells by mediators. An important group of substances thought to induce the directed migration of leucocytes from the blood through the extravascular spaces are the chemotactic factors derived from the complement system (Nelson 1974).

After infiltration, polymorphonuclear cells have a secretory role, providing enzymes for the destruction of cellular and other material. The monocytes (macrophages) ingest particulate matter or cell debris by phagocytosis.

Once foreign material has been removed, the repair process begins by the development of organized granulation tissue. Fibroblast cells migrate into the area from surrounding connective tissue and deposit a layer of collagen fibres around the lesion. Capillary loops grow into the fibrous layer (giving a granular appearance) to establish a vascular network.

After the granulation phase, the tissue to some extent reverts to a normal condition, with the addition of considerable deposited fibres, forming scar tissue. In very persistent inflammation, the site may become calcified.

1.1.4 Lysosomes in inflammation

The large quantities of destructive enzymes present in PMN leucocyte lysosomes include at least 20 different substances, such as acid phosphatase, lipase, β-glucuronidase, sulfatases, esterases and proteases (Weissmann 1974). These enable the white cell to destroy a wide variety of foreign matter. Evidence that lysosomal enzymes are probably important mediators of both tissue destruction and inflammation was presented by Weissmann, Spilberg & Krakauer (1969). They found they were able to induce chronic inflammation and cartilage degradation by injecting lysates of purified lysosomes into the joints of experimental animals.

Large quantities of lysosomal enzymes are found in rheumatoid synovial fluid, compared with normal synovial fluid and also compared with other inflamed tissues (Luscombe 1963; Coppi & Borardi 1968; Anderson 1970). It has been shown that these enzymes are selectively released when PMN leucocytes or macrophages phagocytose particles such as immune complexes, micro-organisms or zymosan fragments (Weissmann et al. 1971; Wright & Malawista 1973; Ringrose, Parr & McLaren 1975). Several mechanisms have been proposed to explain this selective release of enzymes.

The physical process of phagocytosis involves the particle firstly being surrounded by a cellular membrane, the enclosure being called a phagosome. These phagosomes merge with the primary lysosomes to form secondary lysosomes or phagolysosomes. In this combined enclosure the lysosomal enzymes are able to digest the foreign particle (Cohn & Fedorka 1969).

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

Another mechanism which has been proposed considers that the membranes of the secondary lysosomes might also merge with the outer cell membrane during the process of digestion, thus releasing the lysosomal constituents. This is termed 'reversed endocytosis' or 'frustrated phagocytosis' (Henson 1971; Weissmann, Zurier & Hoffstein 1972).

1.2 Pathology of joints

1.2.1 The normal joint

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

The joint itself is enclosed in a capsule made of tough fibrous tissue. The inner surface of this capsule is lined with a soft material called the synovial membrane. Within the joint space is found the synovial fluid, which consists of a dialysate of plasma, with the addition of hyaluronic acid and hyaluronate protein (Sanderson & Hamerman 1962). The normal synovial membrane controls the influx of plasma proteins, and the synovial lining cells themselves synthesize the hyaluronate protein and hyaluronic acid (Ibid)

Synovial lining cells were found to be of two types (Barland, Novikoff & Hamerman 1962). Type A are phagocytic with a prominent Golgi apparatus and with finger-like extensions (filopodia). It was suggested that they have a dual function, both secretory and phagocytic (Ibid).

The type B cell is structurally adapted for a synthetic role, producing hyaluronic acid and hyaluronate protein. Coulter (1962) noted a well-developed endoplasmic reticulum, charateristic of protein producing cells.

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

1.2.2 The rheumatoid joint

Lesions occur mainly in the synovial membranes of the smaller joints. The condition starts as acute inflammation of the joint. The earliest changes are capillary and arteriolar vasodilation, followed by vascular congestion and oedema with subsequent development of a cell-rich infiltrate. The inflamed membrane shows hyperplasia and hypertrophy (Hamerman, Barland & Janis 1969), with multilayered cell proliferation to the extent that displaced tissue forms folds or fronds (villi) on the surface.

Initially the membrane becomes infiltrated by PMN leucocytes, but later heavy infiltration by lymphocytes and plasma cells occurs. Thus the early stages are typical of inflammation generally.

The rheumatoid synovial tissue shows an increased metabolic activity and an increased rate of glycolysis (Falchuck, Goetze & Kulka 1970). The synthesis of hyaluronic acid is also increased, but it is not polymerized to the normal extent and has a lower molecular weight (Castor, Prince & Hazelton 1966). This causes a fall in the viscosity of the synovial fluid, and can lead to increased stress and friction on the cartilage surfaces (Walker et al. 1970).

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

Fibroblast infiltration of the damaged membranes leads to a vascular granular tissue formation, which is termed pannus. The pannus can extend from the inflamed synovium between the joint space over the cartilage, eventually replacing the normal articular surface membrane. This further impairs the relative movement and establishes fibrous links between opposing surfaces. Pannus formation can lead to distortion of the joint resulting in permanent deformity and eventual solidification of the joint (ankylosis). Histologically, pannus consists of proliferating fibroblasts, numerous small blood vessels, inflammatory plasma cells and some collagen fibres (Hamerman, Barland & Janis 1969).

It has been suggested that the pannus is the cause of cartilage erosion in the rheumatoid joint (Barland, Novikoff & Hamerman 1964) although pathological changes in cartilage have also been found well away from the pannus (Hamerman, Janis & Smith 1968). Other factors such as a deficiency of oxygen in the rheumatoid synovial fluid (Falchuck, Goetze & Kulka 1970) or a lack of glucose (Ropes & Bauer 1953) may lead to changes in cartilage metabolism and turnover.

While the foregoing may contribute, it is, however, more likely that the major cause of cartilage erosion is degradation by lysosomal enzymes. Rheumatoid synovial lining cells and pannus have both been found to have greatly increased numbers of lysosomes (Hamerman, Stephens & Barland 1961). An even greater source of lysosomes is infiltrating PMN leucocytes (Weissmann 1966; Zvaifler 1971). The mechanism of release of lysosomal enzymes has been mentioned in the section of this thesis on the inflammatory process.

Hamerman, Janis & Smith (1968) demonstrated that the protein-polysaccharide portion of cartilage could be disrupted by cleavage of the polysaccharide chain by enzymes such as hyaluronidase and by proteolytic enzymes that attack the protein core.

A variety of lysosomal enzymes which play a very active role when released into joints have been isolated or demonstrated. Evanson, Jeffrey & Krane (1968) described the release of collagenases in cultures of rheumatoid synovial tissue. Cartilage mucopolysaccharide degrading protease (Oronsky, Ignarro & Perper 1973), a collagenase (Ohlsson & Ohlsson 1974) and an elastase (Janoff 1973) have been isolated from human PMN leucocytes as well as from macrophages.

Evidence has been presented (Mohr, Westerhellweg & Wessinghage 1981) that polymorphs accummulate at the cartilage-pannus junction and within the cartilaginous matrix. This localization of the inflammatory cells may be regarded as a possible way by which enzymes from PMNs (capable of degrading proteoglycans and collagen fibres) may attack the cartilage in a microenvironment in which serum inhibitors (antiproteases) are substantially absent. Although it was previously believed that the destruction by pannus is confined to previously damaged or necrotic cartilage (Chaplin 1971), it has recently been shown (Mohr, Wild & Wolf 1981) that the pannus containing polymorphs can invade living as well as necrotic cartilage.

1.3 Animal models in rheumatoid disease

Knowledge about the pathogenesis of certain human diseases and the mechanisms that induce them has in general been advanced by the presence of reasonably similar disease counterparts in some animal species. However, it is generally stated that in no other species apart from man and perhaps the primates is there a naturally occurring disease which resembles rheumatoid arthritis (Pearson 1964; Dumonde & Glynn 1962).

In fact, there are animal arthritides of veterinary importance, such as spontaneous Erysipelothrix insidiosa arthritis in swine and dogs (Hadler 1976). Although these arthritides may offer an insight into the mechanisms of articular destruction and in some aspects (e.g. histopathology, titres of rheumatoid factor) parallel the human condition, for various reasons they have not been considered suitable as models of rheumatoid arthritis in man (Gardner 1960; Hadler 1976).

Inflammation is important in, but is not restricted to rheumatoid diseases, and is therefore well enough defined to be of use in the search for drugs that modify the inflammatory process.

The complexity of the inflammatory process and the diversity of the drugs that have been found effective in modifying it have resulted in the development of numerous methods of assay capable of detecting anti-inflammatory substances (Gardner 1960; Pearson 1964; Turner 1965; Swingle 1974). Some of these methods have become popular because of their simplicity, economic feasibility and ability to select drugs known to give some benefit in the clinical management of rheumatoid diseases.

Screening procedures that have been used in an attempt to assess the anti-inflammatory/anti-rheumatic potential of drugs can be divided broadly into acute and chronic categories. The acute models are concerned mainly with the manifestation of one of the cardinal signs of inflammation (such as swelling or reddening) without the involvement of joint pathology. In the chronic models the major emphasis is on the articular disease where destructive changes occur in the articular cartilage and bone. Anti-inflammatory agents may then be assessed by their ability to diminish or prevent such chronic or acute changes.

Gardner (1960) divided the methods of experimental production of arthritis into five groups: induction by infective agents, chemical agents, altered endocrine mechanisms, immunological methods , or physical means.

In a review of methods of evaluating anti-inflammatory activity, Swingle (1974) classifies the methods of assay as follows: the modification of one of the cardinal signs of inflammation; the modification of one of the events occurring during the inflammatory process; the possession of a property that has been associated with a class of drugs known to be anti-inflammatory; and the modification of syndromes purported to be animal models for human rheumatoid diseases.

The work described in this thesis required the use of three animal models of inflammation. These were:

(1) carrageenan induced oedema in the paw of the rat
(2) adjuvant arthritis in the rat
(3) antigen induced monoarticular arthritis in the rabbit.

1.3.1 Carrageenan induced oedema in the rat paw

The method is based on the inhibition of an induced swelling of the rat's paw. The swelling is produced by an injection of a small amount of a suspension or solution of an oedemogen into the plantar tissue of a hind paw. Assessment of the response is usually made at the time of maximal swelling.

Methods for measuring the amount of swelling of the paw can be made by determining the paw thickness (Brownlee 1950), its weight (Mielens et al. 1969) or its volume, this being found by displacement of water (Vinegar, Schreiber & Hugo 1969) or mercury (Winter, Risley & Nuss 1962; Van Arman et al. 1965). The mercury displacement method is most commonly used in a device incorporating a pressure transducer to give a readout on a suitable galvanometer (see Section 2.5).

Many oedemogens have been employed, such as ovalbumen, dextran, mustard, formalin (Turner 1965; Gardner 1960) but in recent years the most widely used has been carrageenan (replacing formalin) possibly because it is the least affected by non-specific influences (Garattini et al. 1965). Carrageenan is not known to be antigenic and therefore the inflammatory response is entirely due to the local stimulus, and is highly reproducible (Gardner 1960).

Carrageenan is a mixture of polysaccharides composed of sulphated galactose units, and is derived from Irish sea moss (Chondrus crispus) (Smith, O'Neill & Perlin 1955). Its use as an oedemogen was introduced by Winter, Risley & Nuss (1962). Not all samples are equally effective in production of the inflammatory response: the effective preparations are those containing the λ- type galactan (McCandless 1962). The structural integrity of the polysaccharide is essential for its oedemogenic activity, shown by the loss of some activity by heat denaturation (Vinegar, Schreiber & Hugo 1969).

The development of oedema in the rat paw following introduction of carrageenan has been described as a biphasic event (Vinegar, Schreiber & Hugo 1969). The initial phase of the oedema, lasting about an hour, has been attributed to the release of histamine and serotonin. The oedema is maintained during a plateau region of the swelling/time curve by kinin-like substances until the second phase (of much greater oedema) is under way, this being due to the release of prostaglandin-like substances (Di Rosa & Willoughby 1971; Di Rosa, Giroud & Willoughby 1971). The involvement of kinin-like substances in theresponse to carrageenan was suggested by the data of Van Arman et al. (1965) and Di Rosa & Sorrentino (1968) who were able to substantially inhibit the oedema with anti-proteases.

The recognition of different mediators for different phases of the oedema has important implications for interpreting the effects of drugs. The existence of two phases of swelling probably explains the failure of standard anti-inflammatory drugs to effect a complete inhibition of the oedema. Vinegar, Schreiber & Hugo (1969) have found that it is the second phase of the oedema that is sensitive to such drugs as hydrocortisone, phenylbutazone and indomethacin. The evaluation of drugs against the second (responsive) phase of the oedema seems to be a more sensitive assay for anti-inflammatory drugs.

The apparent importance of leucocytic exudation for the full development of the oedema by carrageenan was recognisedby Vinegar et al. 1971; Van Arman, Risley & Kling 1971). These investigators were able to correlate either the number of PMN leucocytes that emigrated into the carrageenan injected paw or the peripheral leucocyte count with the amount of the oedema . Di Rosa & Willoughby (1971) suggested that there is an inter-relationship between leucocyte emigration and prostaglandin activation in the later phase of carrageenan induced oedema, and suggested that the predictive value of the assay resides in the involvement of leucocytes in the response. They also suggest that because the

standard non-steroidal drugs inhibit leucocyte migration and also the 'prostaglandin phase' of the carrageenan oedema, this assay can be successfully used to search for effective anti-inflammatory compounds.

The carrageenan induced oedema model has the drawback of a certain non-selectivity in that it can be inhibited by a number of drugs not usually thought of as anti-inflammatory (Silvestrini 1965; Niemegeers, Verbruggen & Janssen 1964). It has been pointed out (Winter 1966) that many of these drugs were given at doses that would be expected to produce behavioral or autonomic effects. Another possible limitation is the toxicity (via gastro-intestinal damage) of some orally active drugs (aspirin, phenylbutazone, indomethacin) at the doses required for inhibition of the oedema (Walz et al. 1970; Green & Green 1971).

Formanek, Forster & Stoklaska (1968) have found marked differences in the effect of anti-inflammatory compounds dependent on their time of administration. This was true for oedemas caused by three different irritants: though carrageenan was not one of them, there is an obvious inference that this effect is general for irritant-induced oedemas.

Swingle et al (1971) found that pretreatment with any of six anti-inflammatory drugs inhibited the oedema caused by carrageenan. However, treatment 15 minutes before the carrageenan injection was more effective than administration 60 minutes before the injection. Levy (1971) obtained inhibition with lower doses of aspirin and phenylbutazone when the drugs were administered some 2 hours after the carrageenan, rather than immediately after. (Measurements were 4 hours after the carrageenan injection.) This confirms the suggestion that the second phase of the oedema is more sensitive to most anti-inflammatory drugs (Vinegar, Schreiber & Hugo 1969).

Despite the limitations which have been mentioned, the carrageenan oedema response is suited for comparative bioassay of anti-inflammatory drugs, and the estimates of relative potency thereby obtained do seem to reflect clinical experience of the drugs. Whether substances shown to be anti-inflammatory by this test prove in fact to be anti-rheumatic must, of course, be determined by clinical trials.

1.3.2 Adjuvant - induced arthritis in the rat

The rat adjuvant-induced arthritis model has been extensively used for the screening of drugs of potential use in the treatment of rheumatoid arthritis.

The adjuvant production of arthritis in the rat was first described by Stoerck, Bielinski & Budzilovich (1954) and later elaborated by Pearson (1956).

In this procedure, arthritis is produced in the rat by the injection of Freund's complete adjuvant (a dispersion of killed mycobacterium tuberculosis in mineral oil) intra-dermally into one hind foot. Following injection, the foot rapidly increases in volume during the first three days. Thereafter the swelling diminishes slightly until after about 7 or 8 days after the injection, when further swelling occurs. At approximately the tenth day after injection, inflamed lesions appear in areas of the body remote from the injection site: the ears, the tail and in joints in the forepaws and hind feet. Fourteen days after the injection these secondary lesions have usually proliferated extensively; both hind feet including the ankle joints are usually red and swollen. Animals with severe involvement of the extremities also lose weight, develop coarse ruffled fur, and generally appear to be unwell.

Beyond this stage, the inflammation subsides, leaving pale granulomatous swelling around the joints, although in some animals the condition may persist to become chronic (Pearson & Wood 1959, 1963) and progress to the destruction of the joints involved with ankylosis.

The development of the arthritis syndrome in rats may be conveniently followed by measuring the changes in thickness or volume of both hind feet (Newbould 1963).

The arthritic response to the adjuvant has been suggested to be a delayed hypersensitivity response to mycobacteria antigen(s) (Waksman, Pearson & Sharp 1960; Pearson & Wood 1964) although some workers have suggested an autoimmune disease, in which the responsible antigen is altered collagen (Mackay & Burnet 1963; Steffen & Wick 1971).

The arthritic activity of Freund's adjuvant seems to reside in the wax D fraction of the mycobacteria (Waksman, Pearson & Sharp 1960) with the presence of a peptidoglycan moiety, which bears a close resemblence to a fundamental component of mycobacterium cell walls (Migliore & Jolles 1968).

The rat is the only species in which this polyarthritis syndrome induced by Freund's adjuvant has been observed; attempts to induce the disease in other species such as the rabbit, guinea-pig and mouse have been unsuccessful (Glenn & Gray 1965).

Various strains of rat differ in their response to the induction of adjuvant arthritis. For example, Buffalo rats are particularly resistant (Swingle, Jaques & Kvam 1969), while in outbred Sprague-Dawley rats the incidence of arthritis approaches 90% following adjuvant injection. (Ibid).

The resistance of certain strains of rat may have an anatomical rather than a physiological basis. Whitehouse, Pearson & Paulus (1972) suggest that the apparent resistance of the Buffalo strain is caused by the relatively poor lymphatic drainage of the site of injection, since full scale polyarthritis could be produced by injecting the adjuvant directly into a lymph node of these rats.

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

The most popular sites for injection of adjuvant are the tail and the hind paw. The most successful route for producing the disease has been intradermal (Glenn & Gray 1965). Since the intradermal route gives ready access to the lymph system, and considering the previously mentioned observations on the Buffalo strain (Whitehouse, Pearson & Paulus 1972), it is likely that the regional lymph nodes play a leading part in the development of a hypersensitivity to the adjuvant. In support of this, Newbould (1964) has shown that the passage of some of the injected material into the lymphatic system is a prerequisite for the development of secondary lesions. Furthermore, it has been shown (Ward & Jones 1962; Newbould 1963) that removal of the depot within two hours of injecting adjuvant into the tail prevented the development of secondary lesions. However, removal of the popliteal lymph node before or after injecting adjuvant did not inhibit development of the disease (Waksman, Pearson & Sharp 1960).

Injection directly into the lymph node has been used, and may be the most reliable method of induction (Whitehouse, Pearson & Paulus 1972). It is in fact possible to transfer the disease to normal rats in a highly inbred strain by the transfer of large numbers of viable lymph node or spleen cells collected from several animals from the 8th to 12th days, though the transferred polyarthritis is usually less severe than the original (Pearson & Wood 1964).

The amount of adjuvant injected (ranging from 0.1 to 10 mg per rat) was not found to cause any difference in the incidence or severity of the resulting arthritis (Glenn & Gray 1965). However, the size of the tubercle particles in the injection was found to influence the induction of the disease (Fujihara, Mori & Nakazawa 1970). It has been found (Liyanage, Currey & Vernon-Roberts 1975) that to produce the disease it is essential that the Freund's adjuvant incorporates tuberculous aggregates smaller than 90 μm. In fact, large aggregates appeared to have an inhibitory effect on the incidence and severity of the induced arthritis (Ibid).

The histopathology of the synovium in rat adjuvant arthritis is indistinguishable from inflammatory synovitis in man by light or electron microscopy (Muirden & Peace 1969). Oedema is the first disturbance due to the disease which can be recognized microscopically (Movat 1966). Mast cells are the first to appear (Gryfe, Sanders & Gardner 1971) and (shortly preceding clinical arthritis) perivascular accumulation of mononuclear cells occurs in the synovium. This infiltration progresses, becomes more diffuse, and monocytic or histiocytic elements predominate. Secondarily, fibroblasts and synoviocytes proliferate in the eroding pannus. Polymorphonuclear leucocytes are not apparent in early histopathogenesis, are not a constant

finding later, and never predominate in the diseased synovium nor the synovial effusion (Hadler 1976). It was reported (Gryfe, Sanders & Gardner 1971) that polymorphonuclear leucocytes predominated in an inflammatory joint exudate (which appeared only after synovitis was established), while mononuclear cells predominated in the tissues.

The severity of the disease may be modified by steroidal and non-steroidal anti-inflammatory drugs (Pearson & Wood 1959; Newbould 1963). It may be prevented by cytotoxic-immunosuppressive drugs such as cyclophosphamide (Brown et al.1970) and methotrexate (Ward et al. 1964). Reports on the effects of the so-called anti-rheumatic drugs, chloroquine and gold preparations, on the rat adjuvant arthritis model have not been consistent. No drug has been found that will cure the established disease in this model.

Rat adjuvant arthritis has been described as the best available model (i.e. closest in character) to human rheumatoid arthritis (Walz, Di Martino & Misher 1971; Glenn & Kooyers 1966). The syndrome is similar to both rheumatoid arthritis and to Reiter's syndrome in humans (Pearson & Wood 1963; Pearson 1963).

Circulating reactants associated with naturally occurring disease (such as the rheumatoid factor) have not been found in adjuvant arthritis (Lowe 1964) and (also unlike the human disease) there are few polymorphonuclear leucocytes present at the affected joints (Pearson 1963). A further difference is that there is no local synthesis of immunoglobulins by plasma cells (Ibid). Nothing comparable to rheumatoid nodules develops in this model: in fact, certain other tissue lesions such as balanitis, uveitis or a spondylitis may in some respects relate the model more closely to human Reiter's syndrome (Ibid).

Rat adjuvant arthritis is probably more important for understanding the aetiology and pathogenesis of the human condition than for selecting new drugs for human therapeutic use. This is because (with the exception of most immmunosuppressive-cytotoxic agents) it does not appear to select drugs that are any different from those detected by other anti-inflammatory models.

1.3.3 Rabbit monoarticular arthritis

Dumonde and Glynn (1962) first reported the induction of arthritis in rabbits by an antigen. They used the insoluble antigen, heterologous fibrin, to induce the disease, but the chronic synovitis which is a feature of this model can also be induced with soluble antigens such as ovalbumin (Consden et al. 1971) and bovine serum albumin (Cooke & Jasin 1972).

Rabbit monoarticular arthritis induced by an antigen has certain important features in common with human rheumatoid arthritis. These are: the disease appears to be immmunologically induced; the synovitis is associated with a pronounced local response in which lymphocyte and plasma cell infiltration is prominent; there is a prolonged local synthesis of immunoglobulins which takes place in the plasma cells of the inflamed synovium; and furthermore the condition is chronic, often lasting many months in the animal after a single injection of antigen into the joint (Glynn 1968,1969). These similar characteristics suggest that this induced animal disease may be a satisfactory model for human rheumatoid arthritis.

In this model the rabbits are sensitized with an emulsion of the antigen and Freund's complete adjuvant, which is injected intradermally at the back of the animal. Three weeks later the animals are skin tested by injecting a small amount of the antigen intradermally and measuring the increased skin thickness 24 hours later. The skin test gives an indication of the delayed cell-mediated response to the antigen. A good correlation has in fact been observed between the joint swelling and the increase in skin thickness produced by the intradermal injection of antigen (Blackham & Radziwonik 1977).

Following a satisfactory skin test, the antigen is then injected into the joint. Chronic synovitis then develops, which in histopathological terms closely resembles rheumatoid arthritis in man. This is shown by features such as: hypertrophy and hyperplasia of the synovial lining cells; villous hyperplasia; pannus formation with erosion of articular cartilage and adjacent bone; a chronic inflammation of the synovial membrane characterized by aggregations of lymphocytes into follicles, with germinal centres, diffuse infiltration with plasma cells; and new bone formation under the periosteum of the metaphysis (Dumonde & Glynn 1962).

The joint swelling is triphasic: an initial acute swelling appears and peaks 2 to 4 days after challenge and then decreases, followed by a more prolonged secondary increase in joint size starting approximately 1 to 2 weeks after challenge. In the case of Old English strain rabbits, the joint diameter increases by about 5 mm. There is a species difference in response to the antigen challenge - for example, in rabbits sensitized to ovalbumin and challenged by intra-articular injection, the Old English strain produced more severe monoarticular arthritis than did either New Zealand White or Dutch rabbits (Blackham et al. 1974).

Several possible mechanisms have been considered to explain the chronic nature of the arthritis in this model. Dumonde & Glynn (1962) proposed a delayed hypersensitivity to the antigen, since establishment of the chronic disease required previous immunization with Freund's complete adjuvant. Consden et al. (1971) have suggested that the local presence of persisting antigen (and hence local antibody synthesis) is responsible for the maintainance of inflammation for periods up to 6 months. However, it seems improbable that such a mechanism could be responsible for inflammation lasting more than a year, so there must be another explanation for the lesions that remain active beyond this time.

Another possibility was suggested by Phillips, Kaklamanis & Glynn (1966), who postulated the induction of an immune response to an auto-antigen, such as a by-product of the local inflammatory reaction.

The elimination of antigen from the normal joint has been shown to be a highly efficient process, which is moderately delayed in the presence of acute inflammation and somewhat more delayed in the presence of specific immunization, but only when this is performed with Freund's complete adjuvant (Consden et al. 1971).

It has been demonstrated that antigen is selectively retained within the rabbit joints. A radioactive tracer technique showed that up to 6 times more antigen was retained in the joints of rabbits with arthritis induced by that antigen than was retained by normal rabbits or by rabbits with arthritis induced by another antigen (Cooke & Jasin 1972; Jasin et al. 1973). The same tracer was used to monitor the elimination of antigen from the joint. There was an initial phase of rapid immune elimination (to less than 1% in 5 days), after which the remaining intra-articular antigen was then eliminated at a very slow rate, with a half-life of more than 20 days (Cooke & Jasin 1972).

The inducing antigen persists in the joint in the form of complexes with antibody and complement in a sequestered state on the articular surfaces of the collagenous tissues (Jasin et al. 1973), and at least a portion of the antigen retained in the rabbit synovium is bound to or associated with synovial macrophages (Webb et al. 1972). This indicates a dual role for the intra-articular antigen: as a stimulus for the prolonged local antibody synthesis and as a source of the antigen-antibody complexes mediating continuous inflammatory activity (Cooke & Jasin 1972).

Experiments by Consden et al. (1971) to determine the minimal dose of ovalbumin capable of exciting a chronic inflammatory reaction of at least 8 weeks' duration suggested that amounts of antigen under 10 μg are insufficient, while with a dose of 100 μg or more then almost all injected rabbits show a severe degree of arthritis. However, antigen retained in the joint may be more effective than the same amount injected, due to the rapid elimination of the latter. Webb, Ford & Glynn (1971) determined that the retained antigen is equivalent to 10 - 100 times the same amount freshly injected.

As a model of human rheumatoid arthritis, rabbit monoarticular arthritis will detect steroidal and non-steroidal conventional anti-inflammatory agents, but its ability to detect anti-rheumatic drugs is said to be uncertain (Blackham & Radziwonik 1977). The activity of these drugs was greater when used against the development phase of the disease than against established arthritis (Blackham 1977). The effects of non-steroidal anti-inflammatory drugs, steroids, immunosuppressants and gold on this model fairly accurately reflects their activities in clinical use.

1.4 Drugs used in the treatment of rheumatoid disease

1.4.1 Steroidal anti-inflammatory drugs

Glucocorticoids are well-known for their marked amelioration of many aspects of the acute inflammatory response. However, the molecular mechanism involved is not yet understood.

The current model for glucocorticoid action suggests that initially the steroid binds to a cytoplasmic receptor protein. The resulting complex undergoes modification and enters the cell nucleus. In the nucleus, the steroid (either associated with the cytoplasmic receptor or after interchange with a nuclear receptor) interacts with DNA. The net result of this interaction is an altered rate of synthesis of certain species of RNA. These alterations are reflected in altered rates of synthesis of specific proteins, which lead to expression of the phenotypic effect. (Baxter, Rousseau et al. 1972; Tsurufuji, Sugio & Takemasa 1979).

The overall synthesis of DNA is also markedly inhibited by glucocorticoids. Henderson & Loeb (1970) have shown that in the liver of young rats cortisone produced a rapid fall in DNA synthesis, this inhibition remaining as long as drug administration continued. The reduction was due to a fall in DNA polymerase activity.

Makman, Dvorkin & White (1968) found that high concentrations of glucocorticoids (10^{-3}molar) could suppress DNA, RNA and protein synthesis in rat and rabbit lymphoid cell cultures. Pharmacological doses of glucocorticoids have been found to inhibit rat liver protein synthesis in vitro and in vivo (Kim & Kim 1975).

Cortisone treatment can depress both the abundance and the phagocytic action of lymphocytes, macrophages and polymorphs (Crepea, Magnin & Seastone 1951; Werthamer, Hicks & Amaral 1969). The phagocytic reticulo-endothelial function is likewise depressed (Heller 1955; Gotjamanos 1970). Corticosteroids are not believed to influence the engulfing ability of PMNs (Hirsch & Church 1961; Allison & Adcock 1965; Dillard & Bodel 1970) but they may inhibit the cell's enzymatic activities, which correspond to its capacity for intracellular killing of bacteria (Alexander et al. 1968; Chretiens & Garagusi 1972).

Both naturally occurring and synthetic steroids have been shown to have a stabilizing effect on lysosomes. This was demonstrated by the decreased release of lysosomal enzymes in vitro (Symons, Lewis & Ancill 1969). On the other hand, high concentrations of glucocorticoids (10^{-3}molar) lead to a loss of stability of the lysosomal membrane, as was again demonstrated by increased release of lysosomal enzymes (Symons, Lewis & Ancill 1970).

Glucocorticoids have been observed to have a similar mode of action on mitochondrial structure and function (Symons, Lewis & Ancill 1974). Samples of human synovial fluid cells were found to concentrate steroids from solution and the amount taken up was related to the action of the drug (cortisol and prednisolone) on the stability of the lysosomal membrane present (Lewis & Day 1972). The lower concentrations of steroids again induced stabilization of lysosomes, but this effect diminished at higher concentrations (Lewis & Day 1972; Lewis & Day 1975).

Persellin & Ku (1974) reported that glucocorticoids did not protect lysosomes isolated from human PMNs against detergent lysis or heat incubation. However, Ignarro (1972) suggested that the differences in experimental procedure (for assessing membrane stability) may account for the conflicting results. Thompson & Lippman (1974) concluded that the case for direct lysosomal membrane stabilization by glucocorticoids is not convincing. They suggest that with present knowledge it seems that the lysosomes are affected more indirectly: the glucocorticoids probably act by binding to cytoplasmic receptors and inhibit certain cells by somehow interacting with their genes.

Wright& Malawista (1973) demonstrated that cortisol inhibited the normal mobilization and release of granular enzymes from phagocytosing human PMNs. It has also been suggested that cortisol may act to prevent the merging of lysosomes and hence the release of lysosomal enzymes (Weissmann 1971).

Corticosteroids also inhibit leucocyte migration in capillary tubes (Ketchel, Favour & Sturgis 1958) and at the site of inflammation (Boggs et al. 1964).

Corticosteroids (and some non-steroidal anti-inflammatory drugs) are known to inhibit the prostaglandin-mediated functional vasodilation (Sutton, Feldmann & Maibach 1971). The mechanism of action is, however, different for steroids and non-steroids. The non-steroids produce their effect by inhibition of prostaglandin synthesis (Vane 1971) whereas it has been suggested that corticosteroids inhibit prostaglandin release (Lewis & Piper 1975; Chang, Lewis & Piper 1977).

The appearance of prostaglandins in some inflammatory exudates is paralleled by infiltration of the inflamed area with PMNs. These are known to liberate phospholipase-A during phagocytosis (Authi & Traynor 1980). A close correlation between the release of lysosomal enzymes from these cells, and the appearance of prostaglandins led to the suggestion that lysosomal phospholipase-A causes the release of precursor fatty acids for prostaglandin production (Anderson, Brocklehurst & Willis 1971). The importance of phospholipase-A_2 in prostaglandin biosynthesis has been confirmed (Blackwell et al. 1978).

Nijkamp et al. (1976) showed that the release of prostaglandins is blocked by anti-inflammatory steroids and suggested that their activity might be related to a suppression of phospholipase activity.

Further evidence for this was given by Blackwell et al. (1978). The selective blocking of release reaction of prostaglandins (from guinea-pig perfused lungs) by anti-inflammatory steroids was shown to be effected by preventing activation of phospholipase-A_2. The steroid did not block phospholipase activity in cell-free homogenates, but only in intact cells. This suggests that these drugs act on the enzyme directly, perhaps by modifying the micro-environment of the enzyme.

Flower & Blackwell (1979) showed that (in guinea-pig perfused lungs) steroids induce the synthesis of a factor which blocks phospholipase-A_2, and that this factor can be transferred from one perfused lung to another. This steroid-induced anti-phospholipase factor is probably a peptide or protein, as its biosynthesis is prevented by cycloheximide.

Tsurufuji, Sugio & Takemasa (1979) have shown that the anti-inflammatory effects of dexamethasone are mediated by RNA and protein, both of which are synthesized following binding of the steroid to the glucocorticoid receptor and induction of gene expression. They found that the anti-inflammatory effect of dexamethasone was completely blocked by actinomycin-D or by cycloheximide, and that both new RNA and protein synthesis appears to be essential for manifestation of the anti-inflammatory activity.

It has been suggested that the biosynthesis of these anti-inflammatory mediators requires the polyamine putrescine (Bartholeyns, Fozard & Prakash 1981). One site where this or other polyamines might be involved in the mechanism of the action of dexamethasone is the liver. Putrescine formation from ornithine is catalysed by ornithine decarboxylase (ODC) (Tabor & Tabor 1976). Dexamethasone induces a rise in hepatic ODC activity and hence a rise in putrescine concentration.

The anti-inflammatory effects of dexamethasone were studied by Bartholeyns, Fozard & Prakash (1981). They found that the anti-inflammatory effect was inhibited by α-difluoromethylornithine (DMFO) which is an irreversible inhibitor of ODC. By contrast, DMFO did not inhibit the effect of indomethacin. Pretreatment with aminoguanidine sulphate (an inhibitor of putrescine catabolism) prevented the blocking effect of DMFO.

In summary, compounds which irreversibly inhibit ODC reduce the anti-inflammatory effects of dexamethasone (but not the non-steroid indomethacin) in carrageenan paw oedema in the rat. Since the effect is prevented by an inhibitor of putrescine catabolism, it is suggested that de novo synthesis of putrescine is an essential factor in the induction of new protein synthesis which is known to be involved in the anti-inflammatory action of dexamethasone.

The properties of glucocorticoids described may be beneficial as anti-inflammatory factors, but they also increase susceptibility to infections by altering the body's defence against infection (e.g. inhibition of lysosomal enzyme release, decreased phagocytosis by the reticulo endothelial system, leucocyte migration inhibition etc.).

Amongst other unwanted side-effects of glucocorticoid treatment are ulceration of the gastro-intestinal tract, loss of calcium from bones (causing osteoporosis), damage to blood cells, the retention of sodium and the water in the body and sodium loss.

Synthetic steroids, as well as being more potent, cause fewer side-effects, but still may have serious side-effects, particularly after extended use.

In blood, glucocorticoids exist in at least three forms: (1) unbound, or native (2) albumin complexed - in varying amounts (3) complexed with globulin.

The binding of these hormones and proteins diminishes or abolishes their biological activity in vivo (Slaunwhite et al. 1962; Kawai & Yates 1966; Heller, Richardson & Yates 1969) and in vitro (Matsui & Plager 1966; Blecher 1966).

Cortisol is largely transported in human plasma by a specific globulin, called transcortin or corticosteroid binding globulin (Sandberg & Slaunwhite 1963). Less than 10 % of cortisol is free at physiological concentrations, and the transcortin-bound complex is essentially inert, not representing a ready availability for catabolism (Rosenthal, Slaunwhite & Sandberg 1969). On the other hand, the synthetic steroids used are not bound in plasma to transcortin or albumin, and therefore provide a higher concentration of available drug for the same dose (Blecher 1966).

1.4.2 Non-steroidal anti-inflammatory drugs (NSAIDs)

Some of the most widely used drugs in the treatment of rheumatoid arthritis are the non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, salicylic acid, indomethacin, phenylbutazone, oxyphenbutazone, ibuprufen, flufenamic acid, mefenamic acid, chloroquine, penicillamine and gold salts.

To varying degrees all of these compounds reduce the pain, stiffness and fever, as well as inflammation in rheumatoid diseases. NSAIDs are not free of undesirable side-effects, and can produce nausea, giddiness, severe stomach upsets or even bleeding and ulceration in the gastrointestinal tract (Weiner & Piliero 1970).

NSAIDs are versatile inhibitors of many enzyme activities in vitro. These include dehydrogenases, decarboxylases, aminotransferases, ribonucleic polymerases, proteases and others (Smith & Dawkins 1971).

The basis of the pharmacological action of NSAIDs has been related to the property of many of them to uncouple oxidative phosphorylation (Whitehouse & Haslem 1963; Lewis, Capstick & Ancill 1972). It seems, however, that the concentration required for these activities in vivo would be well above the toxic dose and therefore these mechanisms are not important in their anti-inflammatory and related physiological effects.

Many NSAIDs have been found to stabilize plasma proteins against thermal denaturation by binding to the protein (Piliero & Colombo 1967). It was suggested that the stabilizing action of some NSAIDs on human erythrocytes (protection against thermal and hypotonic rupture) was due to their stabilizing certain membrane proteins (Inglot & Wolna 1968; Mizushima, Sakai & Yamaura 1970). Binding of NSAID to the erythrocyte membrane was found to correlate with the influence of the drug on erythrocyte stability in hypotonic solutions (Robak, Paczenko & Gryglewski 1975). Similarly, inhibition of heat-induced lysis by some NSAIDs was correlated with their anti-inflammatory activity by Mizushima, Ishii & Masumoto (1975).

The ability of NSAIDs to stabilize lysosomes may also contribute to their anti-inflammatory activity. As with the erythrocytes, the stabilization of the lysosomal membrane proteins has been suggested as the mode of action of NSAIDs on lysosomes (Mizushima, Sakai & Yamaura 1970; Lewis & Krygier 1973).

Some NSAIDs were found to inhibit the release of lysosomal enzymes during phagocytosis of starch particles by rabbit PMN leucocytes (Chang 1972) and during phagocytosis of zymosan particles by cultured mouse peritoneal macrophages (Ringrose, Parr & McLaren 1975).

Many NSAIDs were found to have a stabilizing action on isolated lysosomes over a wide concentration range, but a lytic action at concentrations above 10^{-3} molar (Lewis, Capstick & Ancill 1971). Although such high concentrations are not usually of physiological significance, it is possible that local high concentrations of the drug may occur after oral administration. Lewis (1970) suggests that gastro-intestinal ulceration - a side effect of these drugs - may be associated (at least in part) with lysosomal damage induced by local high concentrations. Additional evidence consistent with this suggestion was obtained by whole animal experiments reported by Lewis, Capstick & Ancill (1971).

A similar effect on lysosome stability was found with indomethacin (Lewis & Krygier 1977), with the new anti-rheumatic drug azapropazone (Lewis & Capstick 1977) and the anti-leprotic and anti-malarial drug dapsone (Williams, Capstick, Lewis & Best 1976).

Some conflicting evidence on the stabilization and labilization of lysosomes by certain NSAIDs has been presented (Brown & Schwartz 1969; Pollock & Brown 1971). However, Ignarro (1972) has suggested that the different methods of assessment of lysosome stability (i.e. by either hypotonic lysis or thermal lysis) may account for the conflicting results.

Brodie (1965) suggested a possible mode of action of NSAIDs via protein binding. He proposed that the drugs could displace cortisol from albumin and/or transcortin binding sites and thus increase the levels of "free" cortisol. However, no significant increase in plasma levels of "free" cortisol during incubation with NSAIDs was reported by Stenlake et al. (1971) nor Parrot & Lewis (1975).

While the details are not yet fully established, it is clear that the NSAIDs share certain biochemical properties, such as protein stabilization, uncoupling of oxidative phosphorylation, and stabilization of lysosomes. Some or all of these properties may well be involved in at least part of the anti-inflammatory action of NSAIDs.

The inhibition of prostaglandin synthesis has been proposed by Vane (1971) as a mechanism for the anti-inflammatory action of aspirin, salicylic acid and indomethacin. These drugs prevent prostaglandin generation by directly inhibiting the cyclo-oxygenase enzyme responsible for prostaglandin biosynthesis (Vane 1971). Many of the NSAIDs have now been demonstrated to inhibit prostaglandin synthesis in a wide variety of human and animal tissues.

The anti-inflammatory potency of the drugs in the carrageenan-induced oedema was correlated with their relative potency as inhibitors of prostaglandin synthetase (Paulus & Whitehouse 1973). Indomethacin at the equivalent of human therapeutic doses was found to almost completely inhibit prostaglandin production in the synovial fluid of rabbits with monoarticular arthritis. This was accompanied by a fall in joint temperature and a moderate reduction in joint swelling (Blackham et al. 1974). Potent prostaglandin synthetase inhibitors (such as ketoprofen, fenoprofen and indomethacin) were found to inhibit cell infiltration during the late phase of rat pleurisy by reduction of chemotactic prostaglandins (Blackham & Owen 1975).

1.5 Endogenous anti-inflammatory substances

Inflammation is the most common characteristic of rheumatoid diseases. A major part of the treatment of such diseases is therefore control of inflammation with suitable drugs. Unfortunately, the adverse side-effects of available anti-inflammatory drugs often limits their use, especially in the long term therapy required for chronic rheumatic disease.

It has long been known that natural remissions of the disease do occur, and that certain other medical conditions may result in remission, notably pregnancy (Hench et al. 1949) and viral hepatitis (Hench 1933). This suggests the existence of some natural defence to the disease, and the possibility of stimulating this natural mechanism has been explored by some workers. The biochemical events in the spontaneous remissions are unknown, but the role of endogenous anti-inflammatory proteins is currently being examined in efforts to elucidate the molecular mechanisms involved.

1.5.1 Anti-inflammatory proteins

Endogenous anti-inflammatory proteins can be defined as proteins which can mediate against the biochemical mechanisms induced by inflammation. Such naturally occurring anti-inflammatory substances have been found in the inflammatory exudates of laboratory animals (Rindani 1956) and in humans (Billingham, Robinson & Robson 1969a).

These substances have been found to inhibit the deposition of granulation tissue (Di Pasquale et al. 1963), carrageenan induced oedema (Billingham, Robinson & Robson 1969a; Billingham, Robinson & Robson 1969b) and delayed hypersensitivity reactions (Gaugas, Billingham & Rees 1970).

It has been suggested that the liver is the site of the synthesis of anti-inflammatory proteins, since in rats injured by sponge implantations, proteins with anti-inflammatory activity have been isolated from the plasma (Billingham, Gordon & Robinson 1971). They have also been found in the inflammatory exudate and in perfusates of livers in the same animals (Ibid). The synthesis of the anti-inflammatory proteins was blocked by treating the rats with actinomycin-D, which inhibits the synthesis of RNA. The anti-inflammatory fraction

was separated from the irritant substances present in the exudate. After further purification it was found to migrate electrophoretically between α-glycoproteins and transferrin (Billingham & Robinson 1972).

These observations suggest that the liver is somehow stimulated by the inflammatory process to synthesize anti-inflammatory proteins, which are carried by the blood to the site of the disease or injury. However, it appears that this response of the liver is not immediate. The anti-inflammatory proteins were found in the liver perfusates of injured rats only several hours after sponge implantation (Billingham, Gordon & Robinson 1971) and similarly in plasma and exudates some 3 to 4 days after the original injury (Robinson & Robson 1966). It was shown that the anti-inflammatory activity was not due to endogenous corticosteroids: this was done both by monitoring corticosteroid levels and by experiments with adrenalectomized rats (Billingham, Robinson & Robson 1969b).

A protein fraction of high molecular weight (> 150 000 daltons) was obtained from the livers of rats that had been previously treated with dimethylnitrosamine, being isolated by column chromatography on saline extracts of the liver. This fraction was found to have anti-inflammatory activity against carrageenan-induced paw oedema in the rat (Lewis et al. 1979). The protein fraction was active both when injected intra-peritoneally and also when implanted subcutaneously inside a small dialysis sac. Evidence was presented that the anti-inflammatory activity may be due to a protein fragment produced by proteolysis of a larger molecule (Ibid).

Although anti-inflammatory substances mainly arise as a result of inflammatory injury, there is evidence that such substances may be present in the plasma of normal rats, although at lower concentrations. Plasma from adrenalectomized rats was found to have some

anti-inflammatory activity in the carrageenan rat model (Atkinson & Hicks 1975). A high molecular weight protein fraction isolated from normal Wistar rat plasma has also shown anti-inflammatory activity in the carrageenan and adjuvant arthritic rat models (Lewis, Capstick & Best 1976).

Normal serum or plasma has also been found to have anti-inflammatory properties as demonstrated by the stabilization of lysosomes. Lowe & Turner (1973) found that plasma from normal rats could stabilize PMN leucocyte lysosomes in vitro against lysis by the detergent Triton-X-100. Human sera has also been shown to inhibit lysis of rabbit PMN leucocytes by Triton-X-100 (Hempel, Fernandez & Persellin 1970). This activity increased during pregnancy and was not due to increased corticosteroid levels.

Lysosomal stabilization (as well as reaction with rat erythrocytes and rat liver granules) has been further demonstrated by a protein which appeared in adjuvant arthritic rat plasma (Persellin 1972). This protein migrated electrophoretically as an α-globulin, and had a molecular weight greater than 170 000 daltons.

Synovial fluid aspirated from patients with rheumatoid disease can also stabilize rat liver lysosomes in vitro (Capstick, Lewis & Cosh 1975). This stabilizing action was abolished by pre-treatment of the fluid with trypsin, and it was shown that proteins in the synovial fluid were responsible for the action. It has been suggested (Lewis 1976) that the responsible proteins in the synovial fluid are in fact normal plasma proteins. In particular, a high molecular weight protein that migrates electrophoretically in the α / β globulin region was demonstrated (Ibid).

It should be noted, however, that although proteins stabilizing lysosomal membranes have certain properties which suggest they might be anti-inflammatory, there is no direct evidence that they have anti-inflammatory action against the usual animal models.

It is likely that there are other factors in human blood apart from proteins or hormones which have some anti-inflammatory activity. For example, a substance was discovered in normal human plasma which showed anti-inflammatory properties in the carrageenan rat model (Ford-Hutchinson et al. 1973). This substance had a molecular weight below 1 000 daltons, was resistant to acid and proteolytic digestion, and could be extracted by solvents (all uncharacteristic of proteins). Further

work by Badcock et al. (1975) has shown that the anti-inflammatory substance has a molecular weight below 500 daltons and that it can also inhibit the release of chemotactic and anaphylactic factors from rat and guinea-pig serum: similar action was demonstrated in human serum (Walker et al. 1975).

1.5.2 Anti-proteases

Anti-proteases (i.e. protease inhibitors) are a group of peptides and proteins characterized by their ability to block the catalytic site of proteolytic enzymes (i.e. proteases). Since some proteases are known to be directly involved in inflammation and tissue destruction, anti-proteases have an obvious potential to counter these conditions. They are found to be generally present in living organisms, in all groups from bacteria to plants and animals. In mammals they are distributed in different concentrations in the various tissues.

Direct evidence of anti-inflammatory activity has been found with anti-proteases. The inflammatory symptoms of kaolin arthritis in the rat were found to be strongly suppressed by intra-peritoneal administration of the soybean inhibitor (Hladovec, Mansfeld & Horakova 1958) and by intravenous administration of the potato inhibitor (Hladovec, Horakova & Mansfeld 1960). The bovine inhibitor known as aprotinin (Trasylol)has been used experimentally in the treatment of rheumatoid arthritis, where its anti-inflammatory activity was similar to that of the corticosteroids (Bruckner & Eisen 1969). Trasylol is also anti-inflammatory in the rat

oedema models (Kaller, Hoffmeister & Kronenberg 1966), in the adjuvant arthritis rat model and in the dextran rat model (Foerster 1969). Trasylol was found to reduce the proteolytic activity of PMN lysates at both acid and neutral pH.

A preparation of rat plasma protein that was found anti-inflammatory against carrageenan and adjuvant arthritic rat models also inhibited trypsin, and it was found that 20 mg of the preparation contained the same activity as 5 ml of rat blood (Lewis, Capstick & Best 1976).

The best-known anti-proteases are those found in plasma. Since these are polyvalent in their action, much interest has been shown in the possible role of plasma anti-proteases in inflammatory disease.

Human plasma can be shown by electrophoresis and immunological methods to contain at least seven distinct anti-proteases (Schwick, Heimberger & Haupt 1966; Wooley, Roberts & Evanson 1976). They have been designated according to their electrophoretic mobility and with respect to their most important physiological function known at the time of isolation.

The human plasma anti-proteases are:
α_1-antitrypsin, α_1-antichymotrypsin,
inter-α-trypsin inhibitor, antithrombin-III,
C1-inactivator, α_2-macroglobulin,
serum β_1-protein inhibitor.

The last-named is of relatively recent discovery (Wooley, Roberts & Evanson 1976). Of the seven, α_1-antitrypsin and α_2-macroglobulin predominate in plasma; the others are only present in trace amounts.

1.5.3 α_1-Antitrypsin

α_1-Antitrypsin (α_1AT) is the most abundant anti-protease in human blood (2 grams per litre), accounting for about 90 % of the trypsin-inhibiting capacity of plasma (Ganrot 1972).

It is an α-glycoprotein with a molecular weight reported to be in the range 47 500 to 55 000 daltons, and it migrates electrophoretically with the α-globulins. Its half-life in plasma is 2 to 4 days.

α_1AT was first isolated by Schultze et al. (1955) as α_1-3.5-glycoprotein. Its identity as the main plasma inhibitor was recognized by Schultze, Heide & Haupt (1962) who also gave it its present name.

α_1AT is polyvalent in its anti-proteolytic actions. As well as binding trypsin, it also binds proteases such as chymotrypsin, plasmin, thrombin (Rimon, Shamash & Shapiro 1966) kallikrein (Habermann 1968), elastase (Kaplan, Kuhn & Pierce 1973), granulocytal elastase (Ohlsson 1971), granulocytal collagenase (Harris, Di Bona & Krane 1969) and both skin and synovial collagenases (Tokoro, Eisen & Jeffrey 1972; Harris, Di Bona & Krane 1969).

However, not all collagenases are bound by α_1AT. A neutral collagenase isolated from human gastric mucosa is not inhibited by α_1AT (Woolley, Tucker, Green & Evanson 1976) and neither are the acid proteases (Kopitar & Lebez 1978). The main anti-collagenolytic activity of plasma is usually attributed to α_2-macroglobulin (Evanson, Jeffrey & Krane 1968).

α_1AT appears to bind with proteases on a 1 : 1 molar ratio in every case (Ohlsson 1975). Although the protease / anti-protease complexes are tightly bound, the type of inhibition is probably competitive (Cohen 1975). The mechanism of action of α_1AT was studied by Cohen (1973) who proposed that α_1AT has two overlapping active sites. One site contains an arginyl residue that binds serum proteases which cleave peptides at arginyl residues. The other site contains an aromatic amino acid or leucyl residue that binds those proteases which cleave at these types of residue.

The designation α_1-antitrypsin by no means indicates a unique substance. Different mammalian species have distinguishable differences in α_1AT (though function and activity are much the same). Even in human beings, more than 20 genetic variations have been recognized (Fagerhol & Laurell 1967).

A naming system for these variations has been adopted, based on the bands which appear when α_1AT is separated electrophoretically on an acid starch gel (Fagerhol 1968). The system refers to the "Pi" (standing for protease inhibitor) allele (i.e. relative position on chromosome), each allele having a letter of the alphabet (based on the relative positions on the electrophoretic pattern). The so called normal form (i.e. the commonest - over 90 %) is Pi M. Variants such as Pi Z are associated with severe deficiency diseases. A good discussion of these genetic variants is given by Kueppers(1975). Since both parents contribute, any individual person will be classified on this system as Pi MM, Pi ES etc.

There is an association between α_1AT and rheumatoid arthritis (Cox & Huber 1976). It is found that the heterozygotes Pi MZ and Pi SZ have a deficiency in α_1AT and are at 3 to 4 times the risk of the average adult for contracting classical rheumatoid arthritis. The homozygotes Pi ZZ are severely deficient in α_1AT. However, the deficiency is so severe that the individuals do not survive long enough to manifest rheumatoid arthritis. This severe deficiency is predisposing to pulmonary emphysema (Laurell & Eriksson 1963) and to infantile cirrhosis (Sharp et al. 1969).

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α_1AT is a so-called 'acute phase reactant' material; its concentration in the blood rises with certain acute and some chronic clinical states (Laurell 1972; Werner & Odenthal 1967). Plasma levels of α_1AT rise during pregnancy to double the normal values (Fagerhol & Laurell 1970) and in hepatitis (Kindmark & Laurell 1972). It is known that remissions of rheumatoid disease can occur in both conditions.

In rheumatoid disease the levels of α_1AT in blood and synovial fluid are significantly higher than normal (Swedlund, Hunder & Gleich 1974), which is suggested to be a response to the increased release of lysosomal enzymes during inflammation (Ibid). Lewis & Capstick (1976) found that the amount of active α_1AT present in rheumatoid synovial fluid varied inversely with the neutral protease activity of the fluid (against hide powder azure).

Since the rat is commonly used for animal models of rheumatoid arthritis, its anti-protease activity should be considered. In fact (like humans) the major plasma anti-protease in the rat is α_1AT. Rat α_1AT has similar biochemical characteristics and properties to its human equivalent, and is likely to inhibit a similar range of serum proteases. However, it is immunologically distinct from human α_1AT (Huttunen & Korhonen 1973; Blackwood et al. 1974).

In the rat adjuvant arthritis model, protease activity and α_1AT levels in serum(and plasma) are inversely related (Parrott & Lewis 1977), which may be compared with the previously mentioned relationship in rheumatic synovial fluid in humans (Lewis & Capstick 1976). The levels of α_1AT in plasma fell as the rat arthritis developed. At the same time proteolytic activity of the blood increased, possibly by leakage into the circulation from inflamed sites (Parrott & Lewis 1977).

The anti-inflammatory action of dimethylnitrosamine on rat adjuvant arthritis and on rat carrageenan oedema models was found to be accompanied by elevated plasma α_1AT levels. Further, dialysed saline extracts prepared from the livers of rats treated five days previously with dimethylnitrosamine were also found to be anti-inflammatory against these models (Lewis, Parrott, Bird & Best 1979). The same authors found that the extracts contained significant amounts of α_1AT (Ibid, unpublished).

Daily administration of cortisol acetate (5 mg kg^{-1}, subcutaneously) to normal rats and to adjuvant arthritic rats caused elevation of α_1AT levels to above normal values, which may reflect the

anti-inflammatory action of this drug (Parrott & Lewis 1977). It has also been found that elevation of α_1AT levels by various anti-inflammatory corticosteroids was dose responsive (Pitt & Lewis 1979). It has been suggested that the additional α_1AT synthesis was due to the direct action of the drug on the liver, and not due to liver damage by the steroid (Ibid).

1.5.4 α_2-Macroglobulin

α_2-Macroglobulin (α_2M) is the other major anti-protease found in human plasma. Its concentration varies from about 4.5 g l^{-1} at the age of 1 to 3 years, to between 2 and 4 g l^{-1} in adults. Women have about 20 % higher plasma concentration than do men (Ganrot & Schersten 1967).

α_2M is reponsible for nearly 10 % of the trypsin inhibitory capacity of plasma (Ibid). Its molecular weight has been estimated to be in the range from 725 000 to 820 000 daltons (Schonenberger, Schmidtberger & Schultze 1958; Roberts, Riesen & Hall 1974). The quaternary structure is unknown, although electron microscope studies have shown an image similar to two beans facing each other (Bloth, Chesebro & Svehag 1968). Several degradation studies suggest an eight chain subunit structure with dimers of these chains forming higher subunits with a molecular weight of about 196 000 daltons. Four of these latter subunits may form an α_2M molecule (Jones, Creeth & Kekwick 1972).

α_2M displays a broader spectrum of activity than α_1AT, and can inhibit most of the proteases associated with inflammation, by irreversibly binding to them.

These proteases which are inhibited include: trypsin, chymotrypsin, plasmin, thrombin, kallikrein, granulocyte elastase, granulocyte collagenase, cathepsin B_1, cathepsin D_1, thermolysin, and clostridiopeptidase A (Barrett & Starkey 1973).

Opinions vary as to whether α_2M is monovalent or bivalent in its action with proteases. In one study two binding sites were reported (Ganrot 1966) although other workers could only find one per molecule (Barrett & Starkey 1973; Ohlsson 1975).

Barrett & Starkey (1973) have proposed a model for the interaction of α_2M with proteases. They suggest that the approaching protease attacks a peptide bond in the α_2M molecule. This acts like triggering a snare, causing a conformational change in the α_2M molecule which entraps the protease. This mode of binding would be expected to be essentially irreversible, as is generally found. However, Baumstark (1970) reported that during prolonged incubation, elastase can digest the α_2M from within and thereby escape.

Levels of α_2M in the plasma are elevated during pregnancy (Schumacher & Schlumberger 1963).

Synovial fluid from patients with rheumatoid disease was found to contain α_2M (Schtacher, Maayan & Feinstein 1973). However, in about 25% of patients the α_2M was found to be inactive in the synovial fluid, although it was active in the patient's serum (Ibid). The same authors reported that in patients with osteoarthritis about 60% lacked the active α_2M in their synovial fluids. Such absences of active anti-protease may suggest that the supply has been exhausted by excessive proteolytic activity. Inactivation of anti-proteases may be a cause of enhanced enzymatic degradation of articular cartilage in these diseases (Ibid).

Ohlsson (1975) used elastase radioactively labelled with ^{125}I to demonstrate the removal of protease-anti-protease complexes from human circulation. The α_2M-protease complexes were rapidly removed with a half-life of 12 minutes, being broken down by the Kuppfer cells. By contrast, the α_1AT-protease complexes were removed more slowly - approximately 50% in an hour. At least some of the labelled protease appeared to be transferred from α_1AT to α_2M, whereupon it was rapidly removed and broken down by the kuppfer cells. These results suggest that α_2M is not just a minor equivalent to α_1AT, but plays an important role in the removal of proteases from circulation.

In the plasma of normal rats there is little or no α_2M present, but its role appears to be taken by α_1-macroglobulin (Ganrot,K., 1973). In rats with inflammation, however, a high molecular weight α_2-acute phase globulin appears in large concentrations, and is found to be immunologically similar to human α_2M (Heim 1968). The inhibition of trypsin and chymotrypsin by both these proteases is similar to human α_2M (Ganrot, K., 1973; Huttunen & Korhonen 1973).

During adjuvant induced arthritis in the rat, the trypsin protein esterase activity in plasma due to the two macroglobulin anti-proteases rises in response to inflammation (Lewis & Parrott 1976). The administration of cortisol was found to lower the levels of activity towards normal values, which was interpreted as being dueto the anti-inflammatory action of the steroid, as the drug has no effect on α_1-macroglobulin levels in the normal rat (Lewis & Parrott 1976).

1.5.5 Serum β_1-Protein Inhibitor

This protease inhibitor in human plasma has only recently been discovered (Woolley, Roberts & Evanson 1976). The discoverers suggest it may have an important function in rheumatoid disease.

It has a molecular weight of about 40 000 daltons, migrates electrophoretically as β_1-globulin, and specifically inhibits neutral collagenases (Ibid). Hence the name β_1-anticollagenase has been suggested for this inhibitor.

Previously, only α_2M was known to normally inhibit neutral collagenases. However, β_1-anticollagenase has a smaller molecule and is therefore more likely to diffuse through tissues than the much larger α_2M. This would allow it to reach inflammatory sites more easily than would α_2M.

1.6 Drug encapsulation

The use of carriers for certain drugs, enzymes and other substances has been developed for use in situations where the use of the substance in the free state is not suitable (or less suitable). These situations include, for example, the need to protect the material from premature degradation, inactivation or excretion. Alternatively, it may be necessary to protect the host from unwanted immunological or pharmacological effects, or the carrier may be intended to deliver the drug or enzyme more specifically to its intended target.

Several approaches have been tried to make successful drug carriers. These have included the use of synthetic materials as well as natural macromolecules, such as albumin conjugates, antibodies, lecithins, glycoproteins, DNA complexes and dextran.

A possible (and favoured) form of such a carrier is a natural or synthetic cell, vesicle or capsule, in which a membrane encloses a space containing the material to be carried. The material may be associated with the membrane substance, or the membrane may simply act as a barrier (of varying degrees of effectiveness) to the passage of molecules and ions in or out of the cell.

Included in this category are microcapsules whose membrane is composed of synthetic substances such as nylon (Chang 1972a),liposomes which have a synthetic membrane modelled after natural membranes (Gregoriadis 1976), vesicles formed of natural membranes (Steck 1974), and actual cells such as erythrocytes (Ihler, Glew & Schnure 1973).

1.6.1 Microcapsules

Microcapsules are made by a process whereby small discrete solid particles or liquid droplets are completely surrounded and enclosed by an intact shell.

Most microcapsules fall between 5 and 500 μ in diameter, but they can be made below 1 μ and up to 5000 μ. The final preparations take the form of free-flowing powders, dry aggregates or suspensions in a suitable liquid vehicle.

Numerous methods and combinations of techniques can be used, such as coacervation (phase separation of a liquid or solid from a solution), polymerization, spray drying, frozen liquid coating, extrusion coating, or electrostatic coating (Luzzi 1976). Representative examples of wall materials are polyamide (nylon), polyurea, polyurethane, epoxy resin, ethyl cellulose, polystyrene, silicone rubber, and gelatin (Chang 1972a, 1977).

A recent technological field of encapsulation by polymerization is the partition of drug molecules into ultrafine compartments of a few nanometers in diameter, hence known as 'nanocapsules'. Such encapsulation is performed with colloidal or micellar suspensions - thus called micellar polymerization (Speiser 1976).

An attempt has been made to model some of the simpler properties of biological cells in the production of so-called artificial cells for drug and enzyme carrying. In these, the use of ultrathin synthetic membranes to form spheroidal enclosures of cellular dimensions creates a system where there is a separation of internal and external environments, but certain permeant substances can enter or leave the cell. Variations in the properties and permeability of the synthetic membrane permits selective permeation of different types of molecules (Chang 1977).

Natural materials can also be used to make the ultrathin membranes, giving the advantage in certain circumstances of biodegradability. For example, cross-linked protein has been used (Chang 1972a, 1977). Stabilized phospholipids have a wide range of application. The resulting artificial cells are known as liposomes, and will be separately discussed. Certain synthetic polymers, such as polylactic acid, also give membranes which are biodegradable (Chang 1976a).

The main uses of microcapsules in the biomedical field are for protection of sensitive materials against the environment, masking unpalatable tastes, separation of incompatibles, converting liquids into solid preparations, and for controlled release. The process has also been used to decrease the risk to workers handling very toxic substances such as pesticides (Calanchi1976; Luzzi 1976).

Since microcapsules are normally prepared using a solvent, they are unsuitable for the entrapment of enzymes, which become deactivated (Kondo & Muramatsu 1976).

Drug-containing nanocapsules (micelles) may open up new fields of parenteral medication. As an example, Speiser (1976) noted the adjuvant effect of nanoencapsulated immunoantigens, which gave permanent stimulation of the RES in guinea-pigs, causing quantitatively raised antigen activity (detected by a higher and longer lasting antibody titre in the serum). This effect permits reduction of the dose and reduces the need for repeated 'booster' immunizations.

Artificial cells provide a versatile means of enclosing a wide variety of substances. They have been applied to single enzyme systems, combinations of enzymes and proteins, multienzyme systems, cell extracts, cell contents, drugs, vaccines, antigens, hormones, and other materials (Chang 1977). Biospecific adsorbents have been enclosed in artificial cells to form the basic units of a detoxifying system for use in artificial liver, artificial kidney and drug detoxification work (Chang 1972a, 1976b).

Artificial cells have been used for local implants, intravenous injection and subsequent localization, extracorporeal perfusion, oral administration and local lesions application. The main problem in intravenous usage is that the cells tend to be less deformable than natural ones, and can cause blockages in the capillary circulation. Biodegradable membranes partly reduce this problem (Kondo, Arakawa & Tamamushi 1976).

Artificial cells containing enzymes or proteins have been investigated for red blood cell substitutes (Ibid), enzyme replacement in hereditary enzyme deficiency conditions, enzyme therapy for substrate-dependent tumors, removal of waste metabolites in organ failure, as immunoadsorbents, and other purposes (Chang 1972a; 1977).

1.6.2 Liposomes

Liposomes are microscopic spheres, the surface being a membrane of bilayered lipids which totally encloses the interior.

They are prepared by dissolving appropriate concentrations of phospholipids in an organic solvent, evaporating off the solvent, and dispersing the resultant dry layer of lipid into excess water or buffer solution. This leads to the spontaneous formation of multilamellar liposomes of heterogeneous size. Each lamella (membrane) in these is a bimolecular sheet of lipid molecules, and is separated from neighbouring lamellae by water. The application of sonic energy to this heterogeneous dispersion leads to the formation of smaller liposomes of more uniform size, many of which are unilamellar. These may be as small as 25 nm in diameter, while the multilamellar units can be several μm across.

The stability (i.e. rigidity and permeability) of the phospholipid bilayers can be improved by the inclusion of other lipid soluble compounds (e.g. sterol) into the liposomal structure.

A wide variety of substances can be entrapped into liposomes. Lipophilic substances may be dissolved in the organic solvent and thus become incorporated in the lipid membrane on evaporation. On the other hand, water-soluble substances can be added to the aqueous solution into which the lipid membrane is dispersed, and end up in the interiors of the spheres (passive encapsulation). Macromolecules with both hydrophilic and hydrophobic regions can accordingly occupy both the aqueous and the lipid phase of the liposomes.

Separation of liposomes and their contents from unencapsulated solutes is carried out by a variety of methods, which depend on the physical characteristics (e.g. size, charge) of both liposomes and solutes. These methods include dialysis, centrifugation and molecular sieve chromatography.

New techniques have been developed for the preparation of large unilamellar or oligolamellar liposomes, of the order of microns in dimension, without the use of sonic energy (which can denature or deactivate certain proteins) and in the presence or absence of organic solvents, to give a high efficiency of entrapment (Szoka & Papahadjopoulos 1978).

The first experiments involving deliberate injection of liposomes into animals was intended to investigate this carrier as a means of introducing enzymes into the cell's lysosomes (Gregoriadis & Ryman 1971; 1972b).

The behaviour of liposomes and their contents in vivo is dependent on the route of administration. Owing to their special properties, liposomes can associate with cells in a variety of ways. Endocytosis has been shown to occur both in vitro (Weissmann et al. 1975) and in vivo (Rahman & Wright 1975). Fusion and adsorption is another possible manner of incorporation into cells, although there is no convincing evidence for these two mechanisms. However, active agents which cannot reach specific intracellular sites can be transported into inaccessible cellular regions by endocytosis.

When liposomes are administered intravenously, their size and surface charge appear to control their rate of elimination from the blood. Large liposomes are removed more rapidly than small ones, and this is reflected in the biphasic clearance of liposomal preparations of mixed sizes (Gregoriadis & Neerunjun 1974). The tissues mainly responsible for removal of liposomes from the blood are liver and spleen; Kupffer cells probably attract large liposomes rapidly, while smaller ones (< 100 nm) reach the hepatic parenchymal cells at a

slower rate. This feature can be utilized in the targetting of liposome preparations. The stability of liposomes in the presence of blood is related to their lipid composition, the presence of sterol giving a more stable liposome.

In general, by 24 to 48 hours after injection, blood is almost completely free of liposomes (Gregoriadis, Neerunjun & Hunt 1977).

Intraperitoneally injected liposomes are transported mainly into the liver and spleen (Dapergolas, Neerunjun & Gregoriadis 1976) the route being most likely via the lymphatic pathways. After local injection of liposomes, large liposomes are retained and disintegrated in the site of injection, while smaller vesicles enter the circulation and reach the liver and spleen (Ibid).

The main potential application of liposomes is as drug carriers in therapeutic and preventative medicine. It was shown in vivo that the liposomal carrier can under certain conditions direct entrapped enzymes to specific intracellular sites, demonstrating that liposomes can introduce drugs into cells and thereby modify the cell metabolism (Gregoriadis & Ryman 1971; 1972b).

The two most promising fields for medical application of liposomes are enzyme replacement therapy and cancer chemotherapy.

A recognized approach for inherited enzyme deficiencies is replacement therapy with enzymes isolated from human or foreign sources. However, such use of enzymes is associated with many problems such as antibody response and immunological reactions which would both inactivate the given enzyme and endanger the patient's life. The enzyme may also act in unwanted sites with harmful metabolic effects or may be inactivated in the blood. Encapsulation into liposomes enables the enzyme to be protected until it reaches the intended site.

Apart from successful experiments with animal models, there has been successful treatment of humans with glycogen storage disease (Pompe's disease) by liposomes containing amyloglucosidase (Roerdink et al. 1976) and with deficiency of the glucocerebroside β-glucosidase (Gaucher's disease) with liposomes containing the enzyme β-glucosidase (Belchetz et al. 1977).

In cancer chemotherapy, liposomes can influence the rate of clearance of entrapped drugs from the circulation and protect the drugs (such as methotrexate) from inactivation. Liposomes also determine to some extent the distribution of the drugs into different tissues: liver and spleen take up doses comparable to that of the liposomal carrier uptake. On the other hand, tissues such as intestinal mucosa, skeletal and cardiac muscle, lungs and kidney take up less than when an equivalent dose is given in the free form (Gregoriadis 1973; Juliano & Stamp 1978). However, correct estimates of tissue uptake are difficult to achieve because of drug loss from the carrier during its circulation in the blood. Such problems may be overcome by incorporation of the drug in the lipid phase of the liposome, or by the use of a second carrier within the liposome.

An example of the use of cytotoxic drugs with liposome carriers is therapy with asparaginase, which has a therapeutic action based on the dependance of certain malignant cells on an exogenous supply of L-asparagine. Long-term treatment with the free enzyme is accompanied by toxicity problems and development of antibodies to asparaginase, which inactivate the enzyme and produce allergic reactions. Neerunjun & Gregoriadis (1976) used free and liposome entrapped Erwinia L-asparaginase

injected by various routes. Higher doses of the enzyme were needed to produce a cure when encapsulated in liposomes, compared with the free enzyme. However, some advantages were shown. Liposomal asparaginase was cleared from the blood very rapidly, but the livers of mice treated with liposomal asparaginase contained much more of the enzyme than the livers of mice treated with free asparaginase. The liposome carrier prevented immune reactions to the asparaginase and reduced its overall toxicity.

Other potential uses of liposomes are as carriers of anti-microbial agents, in treating metal storage disease, in use as immunological adjuvants, and in local administration of drugs into body joints. Dingle et al. (1978) showed that cortisol palmitate anchored onto the membrane of large multilamellar liposomes and given intra-articularly had anti-inflammatory action at doses much lower than the free steroid, and this action was prolonged.

Liposomes may also be used as drug carriers in oral treatments, both for reasons of convenience and as a method of introducing the drug into the system via the portal circulation. An example is an oral administration of insulin, where reduction of blood

glucose in rats was achieved (Dapergolas, Neerunjun & Gregoriadis 1976) and some effect in reduction of blood glucose in diabetic rats was shown (Patel & Ryman 1977).

Administered liposomes can themselves be toxic. For example, adverse reactions could appear as a result of their size: large (unsonicated) liposomes may block lung capillaries. Certain lipid components may be directly toxic or may induce harmful metabolic changes. Toxicity can also be induced as a result of an altered drug distribution or action when delivered by liposomes.

1.7 Encapsulation into erythrocytes

1.7.1 The erythrocyte structure and function

The mature mammalian erythrocyte is a highly specialized but fairly simple cell, whose primary function is to serve as a carrier for oxygen bound to haemoglobin.

Haemoglobin quantitatively accounts for almost all the protein of the cell (more than 95%), although there are numerous other important enzymes and structural proteins. The erythrocyte lacks both nucleus and ribosomes and so it has lost the capability to synthesize new proteins. It cannot synthesize new lipids, but its membrane constituents are renewed and modified by exchange with plasma. The erythrocyte does not contain mitochondria and its energy production is independent of oxygen. Energy and reducing power are supplied by glucose via the anaerobic glycolytic and pentose phosphate pathways.

The normal shape of the cell in circulation is a biconcave disc. This shape is optimal for maintaining a large surface area to volume ratio, and ensures that

most of the haemoglobin molecules are close to the surface - both properties are important for the rapid diffusion of gases. The biconcave disc shape also allows the cell to deform easily.

The maintainance of this complex and unusual shape is not clearly understood, but the membrane is generally considered to be responsible for the biconcavity (Weed, Reed & Berg 1963), although many believe that beneath the erythrocyte membrane is a layer of ATP-dependent contractile protein called elinin, which is in fact responsible for the shape of the cell (Nakao, Nakao & Yamazoe 1960).

There are numerous types of abnormal erythrocyte shapes. The simplest of these is the spherocyte in which the depressions are lost and the cell becomes spherical. The echinocyte is a sphere covered with many projections called spicules. It may be induced by chemical agents such as lysolecithin or bile acids and by depletion of cellular ATP. These abnormal shapes are potentially reversible. Many other erythrocyte shapes are known to occur associated with disease or produced in the laboratory by various agents.

The erythrocyte membrane contains about equal weights of protein and lipid. The bimolecular layer forms the basis of the erythrocyte membrane structure: the two arrays of molecules of lipid are arranged with their non-polar (hydrophobic) chains towards the centre of the membrane, and the hydrophilic heads are in contact with the extracellular and intracellular environment. Lipids have high rates of lateral diffusion within one layer but exchange very slowly (hours) between the two layers (Kornberg & McConnell 1971). Proteins are arranged at the surface of the erythrocyte membrane; some of these are responsible for passive or active transport into the erythrocytes.

The ionic composition of the human red cell (like that of many other cells of the body) is noticeably different from that of plasma. Human erythrocytes have high internal potassium concentrations (142 m mol l^{-1}) and low internal sodium concentrations (6 m mol l^{-1}). This difference is reversed in plasma (K = 4 m mol l^{-1}, Na = 141 m mol l^{-1}). The ionic assymmmetry is maintained in the presence of passive diffusion of these cations by active transport of sodium out of the cell, coupled with transport of potassium into the cell. The transport is

not equal for the two ions: in fact 3 sodium ions are transferred out for every 2 potassium ions brought in. Thus there is an active movement of solute and water across the membrane to balance out the net passive diffusion into the cell, and the erythrocyte remains at equilibrium with the extracellular fluid. Thus there is no osmotic gradient across the erythrocyte membrane under normal circumstances.

If the extracellular fluid (plasma) is made hypertonic, the cell loses water by osmosis and shrinks. Conversely, if the surrounding medium is made hypotonic, the cell gains water, swells and ultimately undergoes osmotic lysis.

The ion pump is important in the control of cell volume, and if pumping is interrupted, the erythrocyte begins to accumulate sodium (which is accompanied by an uptake of water to maintain the osmotic equilibrium) until a critical volume is reached and eventually haemolysis results.

The transport is ATP-dependent and utilizes part of the ATP generated through glycolysis; hence glucose metabolism is important to the maintainance of the normal relationship between Na^+ and K^+ in cell and plasma.

If erythrocytes are incubated in the absence of any glucose, thereby depleting the energy supply, they gradually swell to form spheroids as the ATP level falls. This event may be related to the breakdown of the ATP-dependent sodium pump, which would allow leakage of sodium and water into the cells. Alternatively, it could be due to more direct changes in membrane permeability. When the supply of energy is reintroduced, the ATP level rises and the cells return to their normal shape.

The principal anions in the erythrocyte are chloride and bicarbonate, and these are apparently distributed in a passive manner. The erythrocyte membrane is highly permeable to these ions (compared to Na^+ and K^+). The membrane is permeable to some other ions, notably phosphate, sulphate and some organic anions.

Anion permeability is mediated by a membrane protein of size 95 000 daltons. There are estimated to be 300 000 anion transport sites per erythrocyte (Rothstein, Cabantckik & Knauf 1976).

Passive transport of water into erythrocytes is very rapid, with a half-time of 5 ms. Glucose can also be transported into the cell more rapidly than it can be metabolized, so rate of entry does not limit the rate of glycolysis (Ihler 1979).

The movement of water and hydrophilic molecules between the cell interior and the surrounding medium conceivably proceeds in two different ways: (a) the molecules dissolve in the membrane phase and diffuse under their activity gradient from one side of the membrane to the other; (b) the molecules penetrate by diffusion or by convection through channels, the "pores" which connect extra- and intra-cellular fluids.

According to present thinking the anatomic routes whereby H_2O and Na^+ and K^+ ions move across the cell membrane are pores or channels penetrating the membranes. These are about 0.35 nm in radius (Goldstein & Solomon 1960; Solomon 1958; Solomon 1960; Passow 1963). To determine the pore dimensions the penetration rates of molecules of various sizes have been measured by the rate of ion exchange (Giehel & Passow 1960) or by the osmotic pressure changes (Solomon 1960). Some of the pores are apparently filled with H_2O molecules, while others contain some concentration of positive charges (probably Ca^{++} within the membrane) which forms an effective barrier to the passage of Na^+ and K^+ ions. K^+ enters by a different route than does H_2O , and also exits by a pathway different from its entrance. Motion of Na^+ and K^+ through the pores is probably not a direct result of the active cation pump.

The surface of the human red cell under the electron microscope appears to be covered by a layer of circular plaque-like structures approximately 10 to 50 nm in diameter and 3 nm thick (Hillier & Hoffman 1953). The interstices of the plaques are thought to represent pores or channels that allow for the entry and exit of water and electrolytes etc. The composition of these plaques is not certain, but it is proposed that they are made of lipid - carbohydrate - protein complexes (Ibid).

There are various ways in which these plaques have been incorporated into models of the red cell membrane (Whittam 1958; Kavanau 1966). Kavanau (1966) proposes a model giving the flexibility and dynamic nature which is essential for the observed functions of the red cell. It is based on the proposal that the lipid phase exists in the form of globular micelles structured between the inner and outer layer of protein (Lucy & Glauert 1964; Lucy 1968). The globular micelles are dynamic, and transform between pillars (which would leave open pores) and discs (which would allow closed pores) (Kavanau 1966). This type of dynamic structure would permit and facilitate many membrane functions, including diffusion, active transport, contraction and expansion, coalescence, and fragmentation.

When erythrocytes are suspended in media of varying tonicity, their volumes will change accordingly, water being transported across the cell membrane, so that the cells effectively behave like an osmometer (Guest 1948). In hypertonic media the cells will shrink, water being lost to the media, whereas in hypotonic media water is taken up by the cells. If the medium is sufficiently hypotonic then haemolysis will occur, with liberation of haemoglobin into the medium. Haemolysis normally begins for the most susceptible cells at about 0.5% NaCl and is complete for the most resistant cells in 0.35% NaCl. This spread of suceptibility is due in part to the variation in ages of a sample of erythrocytes, since the young cells are more resistant than older ones to this type of haemolysis.

Water enters the cell more rapidly than ions leave, and this is associated with a shape change. The cell alters from a biconcave disc to a spherocyte, with the ratio of surface area to volume thereby decreased. The sphere gives the maximum volume which can be contained by a fixed surface area. The additional volume which can be contained by this shape change is 154 to 175% of the initial cell volume (Hoffman et al. 1958). It is thought that there is no stretching of the membrane up to this point, the critical haemolytic volume.

Beyond the critical haemolytic volume, the cell has no more " excess surface area" and is unable to expand further, hence the internal pressure rises rapidly, the membrane ruptures to give holes or tears. Rand (1964) considers there may be some stretching between the so-called critical haemolytic volume and actual haemolysis but the amount is negligible and may only apply to cells with thicker than average membranes.

1.7.2 Aging of erythrocytes

The erythrocyte has a finite life span and is normally removed from the circulation after about 120 days in humans (rat: 55 - 65; guinea-pig: 65 - 80; rabbit: 50 - 65 days). In fact, the measured span depends to some extent on the analytical method used (Berlin 1964).

The erythrocyte aging process involves changes in the structural components as well as chemical changes. Although the final result is the destruction of the cell, the critical reactions that actually determine the life span are not known.

Erythrocytes can be destroyed in the body in several ways. The normal aged red cell is destroyed in the bone marrow or spleen by phagocytosis. The mechanism for the selective destruction of erythrocytes in the spleen may be related to the capacity of the spleen to trap them for an extended time under conditions of low pH and low glucose concentration. These conditions apparently unmask the limited metabolic reserve capabilities of senescent erythrocytes, leading to damage and subsequent uptake by macrophages.

If erythrocytes are minimally altered (e.g. by low amounts of antibody, mild heating or mild chemical modification) they are taken up mainly by the spleen. This is a reflection of the spleen's ability to detect mild amounts of damage or loss of function in these cells. More seriously damaged cells are taken up throughout the reticuloendothelial system. Quantitatively, it appears that most of the heavily damaged erythrocytes are removed by the liver, since blood flow through this organ is very large and its Küpffer cells are very active (Wagner et al. 1962).

Erythrocytes may also undergo intravascular haemolysis and perhaps some destruction of normal erythrocytes occurs with release of haemoglobin (Ganzoni, Oakes & Hillman 1971). Alternatively, lysis may be due to membrane leakiness, ion pump breakdown or complement-induced lysis. Intravascular haemolysis is undesirable since haemoglobin can be toxic to the kidney.

1.7.3 Preservation of erythrocytes

An understanding of the conditions which lead to damage and destruction of the erythrocyte is important for determining suitable conditions for storage and preservation to ensure good survival of the cells after transfusion.

Two general approaches have been pursued to improve cellular viability in vivo after storage and transfusion. One is to arrest cell metabolism by freezing and preserving the cells at very low temperatures. Cell preservation at $-80^{\circ}C$ or lower can be carried out for prolonged periods (months or years) without evidence of metabolic deterioration (Derrick, Lind & Rowe 1969), or decreased post-transfusion survival times (Strumia, Colwell & Strumia 1960b). Blood can be frozen rapidly by releasing small drops mixed with glycerol or sucrose into liquid nitrogen ($-196^{\circ}C$). In a related technique, the blood is mixed with more concentrated glycerol (or some other endocellular cryoprotective agent) and frozen more slowly at $-80^{\circ}C$ or lower. The glycerol is distributed between the extracellular and intracellular compartments, where it binds water thus preventing the formation of large ice crystals which would damage the cell. (Rapid freezing results in the formation of

many tiny crystals rather than permitting larger crystals of ice to grow.) At the time of use, the blood is thawed by dilution at 40°C and washed with large volumes of low ionic strength wash fluid.

This procedure is not yet in general use because of the impracticalities of the freezing and thawing steps. Its main advantage may be the accumulation of rare type erythrocytes which can be frozen until needed.

The other approach to storage of erythrocytes is to attempt to control the metabolic changes that occur under normal refrigeration at 4°C in a solution of acid citrate, sodium citrate and dextrose. The original studies indicated that the metabolic changes were not primarily due to factors external to the erythrocyte (such as the storage medium, presence or age of plasma) but were due to intrinsic derangement.

It was found that post-transfusion viability is closely associated with erythrocyte ATP content. Thus when erythrocytes become depleted of ATP (as occurs upon prolonged storage) they lose their biconcave shape and become spherical (Nakao et al. 1960) with associated increased osmotic and mechanical fragility, and are rapidly destroyed in vivo. (This fragility is sometimes referred to as 'storage lesions'.) Addition of glucose to the medium gave elevated ATP and improved storage (Maizels 1941).

It has been determined that the addition of purine nucleosides such as adenosine, inosine and guanosine to the storage medium allows erythrocytes to regenerate ATP (Lowy & Williams 1966). (Once ATP is regenerated, anaerobic glycolysis can occur.) Supplementation of the storage medium with small amounts of adenine alone results in slightly higher intracellular ATP levels, better glycolytic capacity and a viability of greater than 70% for human cells stored for 5 weeks (Simon 1967, Valeri 1972). These processes are known as rejuvenation.

The acid citrate, sodium citrate and dextrose preparation presently used supports a minimum red cell survival 24 hours after infusion of 85% viability after 2 weeks at $4^{\circ}C$, and 70% after 3 weeks. After longer periods of storage the post-transfusion survival is usually so limited that the blood is unsuitable for clinical use.

1.7.4 The use of erythrocytes as carriers

Erythrocytes have many properties of the ideal carrier for enzymes and drugs. Since the patient's own erythrocytes may be used, there is no danger from any adverse effects from foreign substances used to make the carrier membrane. The erythrocyte carrier is "ready made", easily available, and is not only biodegradable, but degradation is accomplished by the same cells and in the same manner as for normal erythrocytes.

The majority of the research into erythrocytes as carriers has utilised erythrocyte ghosts prepared by osmotic haemolysis. Generally in this method the red blood cells are spun down from heparinized whole blood and washed several times. Lysis of the cells is carried out with several volumes of distilled water containing the substance to be entrapped. Resealing of the lysed ghosts is accomplished by the addition of the appropriate volume of NaCl solution of high molarity so as to restore the cells normal osmotic condition. Incubation at 37 ^{o}C may be used to aid the resealing process ("annealing"). The resealed cells thereby have their membrane components return to the normal configuration. The cellular preparation is washed prior to use or assay. (Method of Ihler, Glew & Schnure 1973.)

There is some controversy as to whether the cell membrane actually suffers local tears or if transient pores open up. This may in fact depend on the method of haemolysis. If the rise in pressure is rapid (e.g. when the cells are diluted in hypotonic medium) there may be a sudden release of cellular material through a hole which opens up at one point, this initial violent discharge being followed by gradual diffusion out of the erythrocyte (Baker & Gillis 1969; Danon 1961; Heedman 1958). Conversely, if the change in osmotic pressure is less abrupt (e.g. when the erythrocytes are slowly dialysed against hypotonic medium) it appears that a number of pores open up on each cell, through which haemoglobin is released uniformly (Danon 1961). Such pores in membranes of haemolysed cells have in fact been shown by the electron microscope (Seeman 1967).

After release of haemoglobin, which becomes distributed equally between the ghosts and the supernatant fluid (Hoffman 1958), the suspension becomes more transparent, due to the refractive index of the cell contents becoming more like the surrounding medium. The cells likewise become comparatively less dense, and are more difficult to centrifuge down at low speeds.

If salt is added to the haemolysed suspension to restore normal tonicity, the mixture becomes turbid, indicating that the membrane holes have closed and the erythrocytes are now intact 'ghost' cells. Entry or exit of macromolecules is then no longer possible, though such resealed cells are permeable to small molecules or ions.

Bodemann & Passow (1972) distinguished three types of resealed cell in a ghost population. Type I ghosts reseal spontaneously immediately after haemolysis under hypotonic conditions. These ghosts were incapable of incorporating alkali ions added after haemolysis. If haemolysis is carried out at 0 °C, formation of Type I ghosts is minimized.

Type II ghosts reseal after the addition of salt. Alkali ions added to the haemolysate becomes trapped inside these ghosts in the course of the resealing process at temperatures above 0 °C.

Type III ghosts reseal after the addition of salt, but remain leaky for ions but not for large molecules. The type II ghost is probably derived from the type III, this conversion being most efficient after incubation at 37 °C for about an hour.

It was noted that at 37 °C the resealing process is fast, while at 0 °C it is very slow. Johnson (1975) observed a transition in resealing rates at 20 °C and suggested that resealing rates are a measure of membrane fluidity.

The events occurring in human erythrocyte membrane during hypotonic haemolysis were presented by Seeman (1967) with electron micrograph eveidence: ferritin and colloidal gold were found to permeate human erythrocytes during rapid or gradual (by dialysis) hypotonic haemolysis. Only cells which have undergone haemolysis contained these particles: intact cells did not. The cells were found to be transiently permeable to these particles, and for the majority of the cells the permeable state (defined as the interval between the time of development and closure of membrane holes) existed only from about 15 to 25 seconds after the onset of haemolysis. (If particles were added 3 minutes after the onset of hypotonic haemolysis, they did not permeate the cells.)

The transient holes could be fixed in the open position by the addition of glutaraldehyde between 10 and 20 sec. after the onset of haemolysis. The existence of such fixed holes was confirmed by the entry of ferritin and gold added to the cell suspension. Electron micrographs of these pre-fixed cells showed membrane discontinuities 20 to 50 nm wide, which were not present in cells which had not undergone haemolysis but had been treated with glutaraldehyde alone.

The discontinuities (holes) were not found all around the perimeter of the cell profile, but were clustered in one zone (about 1 μm long) for any particular cell profile. The size of the holes is sufficiently large to allow the passage of macromolecules such as haemoglobin and even larger in both directions. (Ferritin has relative molecular mass about 10^6 dalton, diameter about 12 nm. Colloidal gold is about 2.5 to 30 nm in diameter.) In fact the experiments showed that ferritin was not uniformly distributed throughout the cell, but was located near the zone of holes and that its presence in the cell was in most cases an all-or-none phenomenon. It was also observed that there is an area of bulge in the membrane prior to haemolysis which may be a precursor state of the membrane before the discontinuities appear, since there was only one such bulge per cell profile and since these cells with only a bulge never contained ferritin. The experiments suggest that the appearance of transient holes is associated with the haemolytic process. It is likely that haemoglobin has been released through the observed defects (holes) which apparently admit the ferritin particles.

Baker and Gillis (1969) noted in a study of osmotic haemolysis of human erythrocytes that there was a mass of precipitated haemoglobin (crown) around a single membrane break, which was up to 2 μm in diameter. Ghosts with larger holes were not seen, possibly due to membrane repair.

Similar observations to Seeman's were made by Baker (1967) using human erythrocyte ghosts produced by hypotonic haemolysis. Ferritin was found to diffuse across the membrane at the time of haemolysis, and could be seen inside the ghosts under the electron microscope. (This indicates a lower limit of pore size of at least 11 nm to accommodate the ferritin.)

However, in contrast to Seeman's findings, Baker observed that colloidal gold (particle size ~25 nm) did not enter the cells. This suggested either that the pore size was less than 25 nm or that the presence/absence of a protein coat on the probing particle is crucial to its being permitted entry to the cell. The experiments also suggest that some rate-limiting factor is in effect for the entry of ferritin molecules, since varying the concentration of extra-cellular ferritin had little effect on the number of ferritin molecules seen inside the cells.

At present it is not at all certain whether the haemoglobin release and the entry of extra-cellular material proceeds through pre-existing pores which have been stretched. If this is the case, it is not known if they are the same pores which regulate water and small ion transport.

The pore size for water transport in the normal cell was measured indirectly as 0.4 nm (Goldstein & Solomon 1980) and the total pore area was calculated to correspond to 0.01% - 1.0% of the cell surface area. Thus there must be 10^5 to 10^8 pores per cell. A single red cell contains about 10^8 molecules of haemoglobin, and so complete haemolysis must involve the passage of between 1 and 1000 molecules per pore. The diffusion radius of haemoglobin is not less than 3.2 nm (Perutz 1960) and so the pores in the slightly stretched cell membrane of the haemolysing cell must be at least 6.4 nm in diameter during release of haemoglobin (Baker 1967).

Erythrocyte ghosts have been most widely used as carriers for enzymes which might be used as therapy in some enzyme deficiencies. Many diseases are known which are either caused or have been associated with elevated plasma levels of various small molecules. Potentially, suitable enzymes released from a carrier might be able to control plasma levels of the unwanted biochemical by converting it to a less toxic or more easily removed compound. As mentioned before, erythrocytes have some advantages for carrying enzymes, preventing premature inactivation, immunological response or poor targeting.

For some purposes it is desirable to prepare ghosts that are as free as possible of haemoglobin. This is basically accomplished by diluting and washing the cells in large volumes of hypotonic medium or distilled water. These ghosts can be as much as 99.5% free of haemoglobin, but contain the original amount of membrane cholesterol and phospholipid, retain ATP activity and are still biconcave (Weed, Reed & Berg 1963). Most of the cytoplasmic enzyme activity is however lost.

Using such a technique, Ihler, Glew & Schnure (1973) have demonstrated that β-glucosidase and β-galactosidase can be trapped inside erythrocytes by rapid haemolysis of the cells in the presence of these enzymes, and that they remain active after the cells are lysed. More than 99% of the enzyme activity was recovered when the enzyme loaded cells were lysed by distilled water. Entry of the β-glucosidase was shown to parallel the escape of haemoglobin, optimum enzyme uptake occurring within 60 s.

Smaller proteins were found to be preferentially trapped by the erythrocytes, supporting the report on the molecular sieve properties of the membrane pores by Marsden & Ostling (1959), who were using dextrans of various molecular sizes. They found the distribution of size of the entrapped dextrans markedly different

from the original material, more of the smaller units (< 50 000 daltons) being incorporated. However, enzymes of size at least 180 000 daltons can be readily trapped by erythrocytes. These findings provide a useful approach to the problem of enzyme replacement in such disorders as Gaucher's disease in which catabolic enzyme deficiencies exist.

For other purposes, such as to maximize cell survival, it is desirable to prepare loaded cells that are as similar as possible to normal cells. Ihler et al (1975) accomplished this by modifying the original method of cell dilution by 10 volumes of enzyme in water solution (Ihler, Glew & Schnure 1973) to a minimum dilution possible (about 1:3 to 1:5 dilution). The use of the large hypotonic dilution to encapsulate enzymes resulted in a relatively low maximum encapsulation (less than 1 %) whereas the 1:3 and 1:5 hypotonic dilutions resulted in 2 to 5 % efficiency of loading with the enzyme uricase. Another modification of the cell dilution step is dialysing concentrated erythrocytes (haematocrit of 80 %) against hypotonic medium. In this way the change in osmotic pressure is gradual as is the membrane stretching, until the pores open. Although dialysable molecules are lost, the concentration of macromolecules remains virtually unaltered (Ihler et al 1975).

Deloach & Ihler (1977) have subsequently developed a dialysis procedure for hypotonic haemolysis in which erythrocytes can be loaded with water-soluble enzymes, detergent-solubilized enzymes (glucocerebrosidase) and detergent-dispersed glycolipid (glucocerebroside). The procedure allows approximately 40 - 50 % of the added enzyme or glycolipid to be encapsulated. This is achieved by reducing the salt concentration by dialysis, so the dilution step is avoided. There is thus full equilibration between intracellular and extracellular material at a relatively high concentration of the material to be encapsulated. An important benefit is that erythrocyte enzymes and haemoglobin are not lost from the cell interior to the same extent as is the case with hypotonic dilution, which aids the survival of the laoded erythrocyte in the circulation. The use of detergent deoxycholate at doses sufficiently low not to lyse erythrocytes allows the otherwise water-insoluble enzyme and substrate to be encapsulated to the same extent as water-soluble enzymes (25 μg glucocerebroside per 10^8 erythrocytes). The loaded cells can be ingested by macrophages in vitro and the glucocerebroside is partially degraded by lysosomal glucocerebrosidase. Some similar procedure may conceivably be useful for introducing substances into the lysosomes of macrophages in vivo,

which is suggested by the authors as a potential therapy for Gaucher's disease.

In fact, patients with Gaucher's disease have been given preparations of glucocerebrosidase encapsulated in human erythrocyte ghosts (Beutler, Dale & Kuhl 1977). Both the patient's own cells and the compatible donor cells have been used via intravenous administration, with no reported adverse reactions. Studies with Cr^{51} isotopically labelled erythrocytes showed a half-life in the circulation for loaded ghosts ranging from 14 hours to 10 days.

Dale, Willacarte & Beutler (1971) have also achieved a high yield entrapment of proteins in resealed human erythrocytes. Their technique relies on lysis and resealing of erythrocytes in dialysis sacs to maximize the ratio of intracellular to extracellular volume. By this means, up to 40% of exogenous protein could be incorporated into the cells. Loaded cells of this nature might be of potential value in the treatment of lysosomal storage diseases.

Based on the dialysis procedure for hypotonic haemolysis, DeLoach, Harris & Ihler (1980) have fabricated an erythrocyte encapsulator dialyser which can dialyse 150 cm^3 of erythrocytes for encapsulation in less than 25 minutes. (The whole process of mixing with substances

to be encapsulated and the annealing takes a further 60 minutes). An efficiency of 30 % encapsulation of the pesticide diflubenzuron was achieved with bovine erythrocytes for veterinary use. The authors also noted a species difference in the optimum temperatures used for dialysis and annealing.

The loss of haemoglobin from the cells after hypotonic haemolysis can be controlled by the addition of extracellular high molecular weight substances (e.g. the dextrans) which suggests it may be possible to prepare loaded erythrocytes that will survive in the circulation (Marsden & Ostling 1959).

It is possible to allow the ions and small molecules of the cell to be reconstituted (Ihler 1979). In this procedure, the erythrocytes are first sonicated and then dialysed. The low molecular weight substances which escape through the dialysis bag are collected. The water is removed from this solution by rotary evaporator, and the substances reconstituted with distilled water to give the original intracellular concentration (osmotically equivalent to 0.9 % NaCl). This solution may be used for resealing the membranes and so provide the lost substances.

The kinetics of the passage of substances across the erythrocyte membrane are important in many ways. For example, if the rate of entry of a substance is low during encapsulation, it will not be possible to entrap much of it, no matter how concentrated the extracellular volume. Thus a much larger number of erythrocytes will be required to contain the desired dose.

On the other hand, if the rate of release of an encapsulated substance from the cell is too slow, then that substance may not become available in the circulation as required. This method of release has been tested for encapsulated asparaginase by Updike, Wakamiya & Lightfoot (1976) for treatment of plasma asparagine levels. It was found that direct injection of free asparaginase maintained low plasma levels of asparagine for 10 - 12 days after treatment: while the enzyme encapsulated in monkey erythrocytes (with a circulation half-life of 8 days) maintained low plasma levels for at least 20 days.

Another application of erythrocyte loading has been in the study of models of haemoglobin function. Whittaker et al (1974) and May & Heuheus (1975) have both incorporated foreign haemoglobins into erythrocytes with a view to studying haemoglobinopathies in vitro.

The fate of encapsulated therapeutic agents in resealed erythrocyte ghosts has been investigated in vivo by Tyrrell & Ryman (1976). The enzyme β-fructofuranosidase is well known for its behaviour when administered to rats in liposomes (Gregoriadis & Ryman 1972) or as the free agent (Jacques & Bruns 1965). Tyrrell & Ryman (1976) entrapped this enzyme in rat erythrocyte ghosts and injected the suspension intravenously into rats. Assays and radioactive tracer studies showed extensive localization of the entrapped enzyme in the liver and the spleen 30 minutes after injection. This level remained unchanged after 24 hours. A crude subfractionation of the liver showed that most of the enzyme activity was in the lysosomal-mitochondrial fraction. Similar results have been reported for β-glucuronidase encapsulated in mouse erythrocyte ghosts when injected into other mice (Fiddler et al 1974). Thus it seems that the process of lysis and resealing damages the erythrocyte and it is rapidly removed from the circulation after injection. The liver removes the greater part of the injected material, but the spleen is also implicated in this process.

It therefore seems that although the resealed erythrocyte ghosts may have some therapeutic value as drug delivery systems, the major applications would be limited to conditions affecting the liver and spleen, and perhaps malignant diseases in which erythrocytes are taken up by the malignant cells. For example, Ihler and Glew (1977)

reported substantial uptake by a transplantable hepatoma. It may be possible, as for liposomes (Gregoriadis 1975), that suitable surface modification of the resealed ghost might lead to a degree of target specificity for the preparation.

Some degree of targetting of enzyme-loaded erythrocytes has been reported (DeLoach et al 1977) using glutaraldehyde. This chemical treatment renders the cell more resistant to lysis without inactivating the encapsulated enzyme. Glutaraldehyde does not prevent the ingestion of the loaded erythrocytes by macrophages in vitro, hence these treated cells can be used to deliver enzymes to lysosomes. In vivo the glutaraldehyde treated cells are quickly removed from circulation by the reticuloendothelial system of the spleen and liver. The degree of glutaraldehyde treatment allows the erythrocyte to be targetted either to the liver or the spleen. Low concentrations $\sim 0.05\%$ favour the spleen; higher concentrations $\sim 0.5\%$ favour the liver. Relatively small but significant amounts of the encapsulated material are found in other organs such as the lungs and kidneys.

Another method of introducing substances into the erythrocytes has been the use of cell lysis under intense electric fields (dielectric breakdown). Aqueous pores can be introduced into erythrocyte membranes when an isotonic suspension of red cells is exposed to an electric field of a few kV cm^{-1} for a pulse duration in the μs range. These pores are formed when the transmembrane potential induced by the externally applied field exceeds a critical value of 1 V, which causes the destruction of the permeability barrier and results in osmotic imbalance.

Such a method of electrical haemolysis has been used to prepare erythrocyte ghosts loaded with enzymes (Zimmermann, Riemann & Pilwat 1976). Haemolysis induced by dielectric breakdown (field 12 kV cm^{-1}, pulse 40 μs) was used as a rapid method for the preparation of electrically homogeneous ghost cells from human erythrocytes in large quantities with high efficiency. The ghosts were fairly impermeable to entrapped ^{14}C sucrose and behaved like osmometers. Their haemoglobin content was 5 % of the original. It was demonstrated that the ghosts can be loaded with enzymes such as urease, which has a possible clinical application for the continuous hydrolysis of urea in the treatment of kidney failure.

The same method was also used (Zimmermann & Pilwat 1976) to load tritium-labelled methotrexate into mouse erythrocytes, which were then re-injected. It was shown that with this delivery system, more than 50 % of the drug (after 10 minutes) was accumulated in the liver and and that high activity level could be sustained in this organ for more than 3 hours. Free drug injections led to only 25 % accumulation of methotrexate (after 10 min) in the liver, and the drug was excreted completely after 1 to 2 hours. The authors propose to load erythrocytes simultaneously with para- or ferro-magnetic substances to obtain organ-specificity for any selected site in the body.

Kinosita and Tsong (1977; 1978) have reported that a procedure involving voltage-induced permeability changes can be used to introduce molecules the size of sucrose (i.e. several hundred daltons) into erythrocytes without involving gross haemolysis of the cell. With an electric field of 2 to 4 kV cm^{-1} and pulse length 20 to 80 μs, the effective radius of the aqueous pores formed is of the order of 0.1 nm or so, and can be varied by adjustment of the field intensity, pulse duration, and the ionic strength of the medium. The pores remain open at low temperatures, but close completely on incubation at 37°C (larger pores are difficult to reseal). In a suitable medium, the

resealing of perforated cells takes place without haemolysis (stchyose is added to the medium to prevent haemolysis) resulting in a preparation of erythrocytes (not ghosts) of altered intra-cellular composition.

The resealed erythrocytes with incorporated foreign molecules, such as sucrose, are apparently intact in terms of cell volume, cell shape, glucose transport and Na-K pump activity. Mouse erythrocytes loaded with ^{14}C sucrose were demonstrated to survive in the circulation with a lifetime almost indistinguishable from that of normal cells, while the sucrose remained entrapped within the cells. (The half-life of sucrose levels corresponded to the half-life of the erythrocytes.) This technique might offer a means of sustaining a low plasma level for a long period of time for drugs that slowly permeate the erythrocyte membrane, and this would be an advantage for some clinical and other situations.

A different approach to the entrapment of substances into erythrocytes was described by Kitao & Hattori (1980), who encapsulated daunomycin in human and mouse erythrocytes with the aid of exogenous amphotericin-B, which is a polyene antifungal antibiotic that binds to cholesterol in the plasma membrane of eukaryotic cells. Cells that have been treated with amphotericin-B have an enhanced uptake of ions (Deuticke, Kim & Zöllner 1973) as well as

small and large molecules, including DNA and even latex beads 1.1 μm in diameter (Kumar et al. 1974).

An antileukemic drug such as daunomycin added exogenously is unable to penetrate the erythrocyte membrane. However, when the cells were incubated with a low concentration of amphotericin-B, daunomycin was entrapped into the erythrocytes without haemolysis or alteration in the chemical parameters of the erythrocytes. It seems possible that the low concentration of amphotericin-B might cause transient membrane lesions that enhance uptake of daunomycin. This process is reversible and can be controlled because the increased transport stops when the cells are washed free of amphotericin-B. A short exposure time to amphotericin-B is not toxic to cells (Kumar et al. 1974).

The erythrocytes with entrapped daunomycin may act as a time release system so that cells are exposed to the drug as they enter DNA synthesis. Here the erythrocyte has been used as a carrier vehicle to enhance the cytotoxic activity of daunomycin against certain leukemic cells in the mouse, where the preparation prolonged survival of the animal.

1.7.5 Encapsulation in erythrocytes by the minimum damage system to produce viable loaded cells

The drawback of the majority of encapsulation systems is their non-viability in the circulation. When adminstered intravenously, most encapsulated preparations are removed from the circulation by the reticuloendothelial system, often very rapidly. As a consequence, the full potential as a prolonged release method has not been realized in practice.

In the present study, materials have been encapsulated in erythrocytes by a method which does not involve a very large change in the constitution of the cells. Thus the finished product is an intact live cell, not a grey ghost.

In developing the encapsulating procedure, we wished to give the materials to be encapsulated as free access as possible to the cell interior, while minimizing permanent damage to the erythrocyte. In particular, we attempted to avoid seriously depleting the erythrocyte of any of its usual constituents.

For a cell to remain viable, it is most important that it retains its metabolic constitution and also its deformability, this latter requirement being both necessary in the body and an indicator of the good general condition of the erythrocyte. It is clear that the good functional condition of the membrane and the good

metabolic state of the cell interior are interdependent, and in fact it appears that the shape and deformability are closely linked to the energy status of the cell (Haradin 1967). Therefore to obtain viable cells it is necessary to ensure both that the membrane is intact and also that the cell energy supply is maintained.

Although our procedure involves exposure to hypotonic solutions, we have tried to produce cells whose final constitution is much the same as before treatment. That is, we have tried to maintain or restore both the internal levels of ions and metabolites of small molecular weight as well as the levels of the internal and external complement of macromolecules and lipids.

Preliminary experiments (Jenner & Offord, unpublished; Jenner 1976) indicated that to achieve this "minimum damage system" of loading erythrocytes, several criteria should be followed:

(1) The hypotonic solution should have as nearly as possible the same materials in the same proportions as the normal cell interior, plus the material to be encapsulated.

(2) The cells should be taken rather gently to the point of haemolysis.

(3) The very minimum hypotonic stress should be used, consistent with the majority of the cells becoming permeable to the material to be encapsulated.

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(2) The cells should be taken rather gently to the point of haemolysis.

(3) The very minimum hypotonic stress should be used, consistent with the majority of the cells becoming permeable to the material to be encapsulated.

(4) The cells should be exposed to the minimum external volume at the point of haemolysis.

Criterion (1) would be best achieved by using a mechanical haemolysate of erythrocytes, i.e. actual cell contents diluted to the desired tonicity. However, such a lysate is optically dense, making it difficult to observe the changes necessary to judge and hence control the loading procedure. The alternative we used was a K^+ reversed Hanks solution (Hanks 1948) which mimics the ion concentrations in erythrocytes fairly accurately. It is particularly useful in preventing the loss of potassium from the cell interiors during the pre-swelling stage, when the membrane is more than usually permeable to cations. Loss of potassium would adversely affect viability.

Control of ions does not of course prevent loss of lipid and protein, which is one of the most common forms of erythrocyte injury caused by in vitro handling (e.g. by cell washing or incubation). Weed (1966) reported that the lost lipid is in the form of fragments of the membrane rather than dissolved or leached away. We therefore took most particular care to be as gentle as possible in handling and transfer to avoid mechanical shear forces on the cells which could cause physical disruption.

Weed later (1969) suggested that ATP depletion was in some cases associated with lipid loss in erythrocytes - i.e. loss is more likely when the cell has been deprived of an energy source for some time. The technique we used supplied some glucose in the K^+ reversed Hanks solution, and did not in any case take long enough for ATP depletion to become likely.

Loss of lipid would be particularly disadvantageous for encapsulating, since it increases the fragility of the membrane and also (by reducing the available area of membrane for stretching) make the cell more vulnerable to haemolysis.

Unlike many other body cells, the erythrocyte does not have the ability to synthesize new lipids. However, it does have some capacity for turnover and replacement of membrane lipids by using plasma lipids. Cholesterol and other lipids can be acquired from or lost to the plasma: for example erythrocyte cholesterol has been shown to be in equilibrium with plasma cholesterol (Murphy 1965). In vitro experiments with abnormal erythrocytes, which lose lipid easily and are consequently more fragile than normal (hereditary spherocytosis) have shown that the addition of extraneous lipids - e.g. linolenyl sorbitol- can effect some improvement in the membrane, making it more resistant to haemolysis (Livine 1973).

For the above mentioned reasons, our minimum damage technique utilised a small volume of erythrocytes additional to those to be loaded. This additional volume was totally haemolysed with an equal volume of distilled water. The lysate was added during the procedure with two intentions. Firstly to provide membrane material for any repair which might take place. Secondly to reduce the gradient of intracellular components during equilibration with the extracellular medium, and thus reduce the rate of loss of these components during this time.

The point of haemolysis was reached by gradual additions of several small volumes of water or drug solution to the cells, in each case followed by very gentle mixing to allow gradual equilibration. It is thought that the means of haemolysis determines whether the erythrocyte membrane ruptures, leaving a tear (as in rapid one-step dilution into hypotonic medium) or whether membrane pores open (such as occurs when the erythrocytes are slowly dialyzed against hypotonic medium) (Danon 1961).

The erythrocytes were exposed to the minimum hypotonic stress which allowed the cells to become permeable to the substance to be encapsulated. To achieve this, the cells were allowed to reach the point of haemolysis, but no further hypotonic dilution was made beyond this point. Therefore the recognition of the point at which the

majority of the cells are haemolysed is vital to avoid unnecessary cell damage by over-dilution.

To prevent loss of intracellular components by simple dilution, the technique utilized the minimum practical volume of liquid in contact with the cells in a permeable state. The onset of permeability is in fact at a degree of swelling something less than the point of haemolysis (MacGregor & Tobias 1972). Thus the cells are partially swollen to a sub-permeable state with hypotonic Hanks solution, and the supernatant liquor removed before completing the haemolysis and resealing.

Jenner (1976) used this minimum damage system to load erythrocytes with different materials. However, the in vivo survival of the preparations was disappointing. An investigation of the fate of asparaginase encapsulated in rabbit erythrocytes indicated very rapid destruction after re-injection: the bulk of the injected enzyme appeared to have been released into the plasma after one hour. Similar results were found after encapsulation carried out in the presence of ^{51}Cr labelled haemoglobin. (No other material was encapsulated.) The erythrocyte membrane is normally impermeable to haemoglobin unless haemolysis takes place, hence any labelled cell must have been to the point of haemolysis. It was found that the majority of the labelled cells had been removed from the circulation only 10 minutes after injection.

Jenner (1976) obtained a somewhat different result by putting rabbit erythrocytes through the minimum damage loading technique but in the absence of any material to be loaded. The resealed cells were then tagged with labelled sodium chromate. Measurements were taken over 14 days, and extrapolated to give a mean lifetime for the tagged cells of 58 days, i.e. the normal lifespan for rabbit erythrocytes.

This would suggest that the minimum damage loading procedure does not in itself significantly damage the cells. However, it is pointed out that this result is valid only if it was assumed that all the erythrocytes actually haemolysed in the process and that all the cells taking up the radioactive label were cells which had haemolysed. For this reason, the encapsulation of labelled haemoglobin probably gave the more valid result.

Although Jenner did not find a definite answer in determining whether it is possible to load erythrocytes with therapeutic agents in such a way that they remain viable on retransfusion, his investigation was invaluable in defining parameters for the present study.

The present study utilized the minimum damage system of encapsulation with some modifications. The results of the animal experiments showed that, in contrast to previously reported work, cells loaded with a marker substance could be made with an essentially normal lifespan. It was also demonstrated that erythrocytes can be a useful vehicle for the administration of anti-inflammatory steroids intravenously, resulting in a more prolonged and effective treatment of adjuvant induced arthritis in the rat.

If the method proves applicable to man, erythrocytes may realize their potential in delivering drugs for a wide variety of inflammatory and other chronic diseases.

1.8 Aims of research

The work involved the development of several lines of research which showed potential for new therapies. As might be anticipated from such a combination of relatively new techniques, the results were not entirely as expected, and the aims of the project were modified accordingly. However, overall the aims may be summarized as follows:

(1) To develop the work of Lewis & Parrot on the anti-inflammatory action of α_1AT. To isolate, purify and characterize a substantial amount of α_1AT for experimental therapeutic use in animal models.

(2) To investigate the effect of corticosteroids on the biosynthesis of α_1AT in vivo, as a possible mechanism of action of these drugs.

(3) To test the therapeutic value of α_1AT as an anti-rheumatic agent when administered by direct intra-articular injection for the treatment of mono-articular arthritis in the rabbit.

(4) To develop the work of Offord and Jenner on the encapsulation of material into erythrocytes. A later aim was to produce preparations which would be viable in the circulation.

(5) To test the therapeutic value of cortisol phosphate when administered as a preparation encapsulated in erythrocytes by direct intra-articular injection for the treatment of monoarticular arthritis in the rabbit.

(6) To test the therapeutic value of α_1AT when administered as a preparation encapsulated in erythrocytes by direct intra-articular injection for the treatment of monoarticular arthritis in the rabbit.

(7) To test the therapeutic value of a combined preparation of α_1AT and cortisol phosphate encapsulated in erythrocytes when administered by direct intra-articular injection for the treatment of monoarticular arthritis in the rabbit.

(8) To test the therapeutic value of encapsulated anti-inflammatory corticosteroids administered intravenously as compared with non-encapsulated preparations of the same drugs administered by conventional injection regimes in the treatment of rat adjuvant arthritis.

(9) To make an in vitro evaluation of preparations encapsulated in erythrocytes.

(10) To examine the survival of loaded erythrocytes in the circulation of experimental animals.

2 MATERIALS AND METHODS

2.1 Animals

The rats were male Wistar strain (Bantin & Kingman, Hull) young adults of 200 to 300 g body weight. They were caged in groups of up to 5, and allowed food and water ad libitum. They were divided into experimental groups on the basis of individual weights so that the total weight of each group of 7 was the same $\pm$ 5 g. The nominal body weight is stated for the experiments.

The guinea-pigs were male Dunkin-Hartley strain (Bantin & Kingman, Hull) young adults of 300 to 350 g body weight. They were caged in groups of up to 5, and allowed food and water ad libitum. They were put into experimental groups at random.

The rabbits were both male and female Old English strain (Fisons Pharmacology Laboratories, Loughborough) young adults of body weight 1.5 to 2.0 kg. Some were supplied in normal condition, some sensitized to ovalbumin (see Section 2.7). They were caged singly and allowed food and water ad libitum. Experimental groups were chosen to give a sex balance and (for the sensitized groups) an even distribution of response to the skin test for sensitization.

2.2 Anaesthesia

The animals were put under total anaesthesia by an inhaled gas mixture dispensed by a Boyle's veterinary anaesthetic apparatus (British Oxygen Co.).

Rats received a mixture of 3 % halothane in oxygen (300 cm^3 min^{-1}) and nitrous oxide (1250 cm^3 min^{-1}).

Guinea-pigs received the same mixture.

Rabbits received a mixture of 3 % halothane in oxygen (400 cm^3 min^{-1}) and nitrous oxide (1500 cm^3 min^{-1}).

2.3 The purification of human α_1-antitrypsin from plasma

The method of Laurell, Pierce, Persson and Thulin (1975) was used to prepare a protein fraction rich in α_1AT using disulphide exchange chromatography.

The initial protein fraction containing α_1AT was precipitated out by ammonium sulphate at 50 to 72 % saturation at room temperature and pH 6 . Mercaptoethanol 0.005 M was added to reduce plasma protein disulphides. The precipitate was collected by centrifugation at 20 000 g for 30 minutes in a high speed MSE-18 centrifuge. It was dissolved in distilled water and dialysed against distilled water for 48 hours at 4 ^{o}C, followed by dialysis against 0.1 M Tris-HCl buffer (pH 8.1) containing 0.005 M EDTA for 12 hours at 4 ^{o}C.

The next step utilized column chromatography. A 30 cm x 5 cm diameter column was packed with approx. 120 ml of activated thiol-sepharose 4B. The activated thiol-sepharose was obtained as a glutathione 2-pyridyl disulphide derivative of sepharose 4B (Pharmacia Fine Chemicals, Sweden). To activate it, it was taken up in distilled water and the 2-pyridyl mercaptan cleared by elution with 200 ml of 0.02 M 2-mercaptoethanol.

The column was washed with 300 ml 0.1 M Tris-HCl buffer (pH 8.1) containing 0.005 M EDTA. It was then treated with 250 ml of 0.0025 M Nbs_2 (5,5'-dithiobis-(2-nitrobenzene)) in Tris-HCl buffer. This formed Nbs derivatives on the terminal cysteine of the thiol-sepharose 4B. The initial dark yellow band of Nbs_2 was eluted and the Nbs_2-Tris-HCl buffer recirculated through the column for a minimum of one hour. The column was then washed with 300 ml of the Tris-HCl buffer alone.

The initial protein fraction (200 ml, containing up to 800 mg protein) was applied to the column and recirculated through it overnight at room temperature with a flow rate of about 1.5 ml min^{-1}. The column was then washed with 300 ml Tris-HCl buffer (pH 8.1) containing 0.5 M NaCl, 0.003M NaN_3 and 0.01 M EDTA, then finally washed with Tris-HCl buffer alone.

Part of the protein-bound fraction was displaced from the column by 25 ml Tris-HCl buffer (pH 8.1) containing 0.0025 M Nbs and 0.00126 M Nbs_2 . (This was obtained by dissolving 20 mg Nbs_2 in 25 ml Tris-HCl and partially reducing with 4 mg dithiothreitol.)

The remainder of the protein bound to the column was released with 100 ml 0.02 M 2-mercaptoethanol in Tris-HCl buffer, and the column washed out with 100 ml Tris-HCl buffer (pH 8.1) containing 0.005 M EDTA. The protein solutions thereby obtained were combined.

The free Nbs and Nbs_2 in the eluted solution were removed by dialysis and adsorbed by charcoal in the dialysate (0.5 mg ml^{-1}). The Nbs bound to protein was released by reduction with dithiothreitol. The protein fraction was finally cleaned by dialysis against distilled water for 48 hours at 4 °C.

The solid protein fraction was obtained by freeze drying, with a yield of 500 to 700 mg per litre of plasma. Electrophoresis of this material showed α_1AT as a single band, with no evidence of impurities. It was tested for trypsin inhibitory capacity (TIC) and found to be 50 % active. By comparison, the commercial 'pure' α_1AT (Sigma Chemical Co.) was found to be 33 %.

2.4 Assay procedures

2.4.1 α_1-antitrypsin (TIC)

The trypsin inhibitory capacity (TIC) was used as a measure of α_1AT activity. The method is described by Dietz, Rubinstein and Hodges (1974).

The assay uses the inhibitory action of the sample on the breakdown of α-N-benzoyl-DL-arganine-P-nitroanilide (BAPNA) (Sigma Chemical Co., U.S.A.) to P-nitroaniline by trypsin (from bovine pancreas, twice crystallized) (Sigma Chemical Co., U.S.A.).

A suitable sample (e.g. 10 µl of synovial wash-out) was added to 2 ml of trypsin solution (20 µg ml^{-1} in 0.1 M Tris-HCl buffer (pH 8.2) containing 0.02 M $CaCl_2$. A control mixture was prepared by substituting 10 µl of a 40 mg ml^{-1} bovine serum albumin (fraction V) solution (Sigma Chemical Co., U.S.A.). The solutions were then allowed to stand at room temperature for 10 to 15 minutes to activate the trypsin inhibitory proteins. 0.5 ml of the activated solution was then added to 2.5 ml BAPNA substrate (0.45 mg ml^{-1} in 0.1 M Tris-HCl buffer pH 8.2 containing 0.02 M $CaCl_2$) and the mixture incubated for 10 minutes in a shaking water bath at 37°C. The reaction was quenched by the addition

of 0.5 ml of 30% v/v aqueous acetic acid.

The optical absorption at 400 nm was measured for the samples versus a blank on a Pye Unicam SP500 spectrophotometer. The blanks were prepared by the same procedure and at the same time, but the α_1AT sample was omitted until after the acetic acid quench.

The absorbance was compared with a curve made from a set of known solutions of p-nitroaniline, and hence the TIC could be calculated.

2.4.2 α_2-macroglobulin (TPE)

The trypsin protein esterase (TPE) activity was used as a measure of α_2M , by the method of Ganrot (1966).

The principle is that α_2M can bind trypsin with the formation of an enzymatically active complex, TPE. The esterolytic activity of this complex can be determined by the amount of a substrate (BAPNA) it breaks down under controlled conditions. The amount of breakdown is determined by the photometric absorbance of the breakdown product p-nitroaniline as in section 2.4.1.

A suitable sample (e.g. 25 µl serum) was added to 0.5 ml trypsin solution (100 µg ml^{-1} in 0.1 M Tris-HCl buffer, pH 8.2, containing 0.02 M $CaCl_2$). After 5 to 10 seconds, 1.5 ml was added of a solution of BAPNA and soybean trypsin inhibitor (STI) (0.9 mg ml^{-1} BAPNA, 0.0334 mg ml^{-1} STI, 0.02 M $CaCl_2$, 0.1 M Tris-HCl buffer pH 8.2). (STI from Koch-Light Laboratories, Bucks.) The mixture was incubated for 10 minutes at 37°C in a shaking water bath then quenched by 0.5ml of 30% v/v aqueous acetic acid and the absorbance read versus a blank as in section 2.4.1. The blank was prepared by the same procedure but omitting the trypsin from the Tris-HCl buffer and then adding the trypsin after the acetic acid quench.

2.4.3 Bilirubin

This was assayed in plasma by the method described in Sigma Technical Bulletin No 605 (Sigma Chemical Co., 1977) using the commercial "Sigma kit for the quantitative colorimetric determination of bilirubin (total & direct) in serum and plasma".

In this method, bilirubin is coupled with diazotized sulphanilic acid to form the blue compound azobilirubin at alkaline pH. The colour density is measured on a spectrophotometer at 600 nm and converted to concentration of bilirubin with a specially prepared calibration curve.

2.4.4 Glutamic-oxalacetic transaminase (GOT)

This was assayed in plasma by the method described in Sigma Technical Bulletin No 505 (Sigma Chemical Co., 1977) using the commercial "Sigma kit for the quantitative colorimetric determination of GOT in serum or plasma".

The method relies on the fact that GOT catalyses the transfer of α-amino groups from specific amino acids to α-ketoglutaric acid, yielding glutamic acid and oxalacetic acid. These keto acids are then determined by their absorbance at 505 nm when coupled with 2,4-dinitrophenylhydrazine. The absorbance reading is converted to Sigma Frankel units (SF) of transaminase activity with a prepared calibration curve.

2.4.5 Total protein

This was assayed colorimetrically via the complex produced by Folin-Ciocalteu reagent on alkaline copper treated protein, as described by Lowry et al. (1951).

Stock solutions were as follows:

A	5% $CuSO_4.5H_2O$
B	10% Na tartrate
C	2% Na_2CO_3 in 0.1 M NaOH
D	1 ml A + 1 ml B, made up to 10 ml with distilled water.

The following were the reagents used:

E	1 ml D made up to 50 ml with C (made up fresh each day)
F	"Diluted Folin-Ciocalteu Reagent"as supplied

The procedure was as follows. 0.2 ml of 1000 x diluted synovial washout and 2 ml E were mixed well in a tube and left to stand at room temperature for 15 minutes. 0.2 ml of F was added rapidly, and the tube shaken on a whirl mixer. A blank solution with water instead of synovial fluid was simultaneously run. After 30 minutes the absorbance at 700 nm was read versus the blank on a Pye Unicam SP 500 spectrophotometer. Results were calculated from a curve of standard solutions of crystalline bovine albumin treated in the same way.

2.4.6 Acid phosphatase activity

This was determined by a method adapted from that of Symons, Lewis and Ancill (1969).

A suitable amount (e.g. 0.5 ml synovial washout, or 0.1 ml serum) was incubated 0.5 ml of 0.015 M p-nitrophenylphosphate (which acts as substrate for the phosphatase activity) and 0.5 ml of 0.09 M Na citrate buffer (pH 4.8) for 20 minutes at 37°C in a shaking water bath. The reaction was quenched by the addition of 5 ml of 0.1 M NaOH solution.

A blank was run simultaneously with the sample. In the blank the fluid (serum or synovial washout) was only added after the NaOH quench.

The p-nitrophenol released was determined by its absorbance at 410 nm versus a blank on a Pye Unicam SP 500 spectrophotometer, using a curve prepared from known solutions of p-nitrophenol.

2.4.7 Protease activity

This was measured by a method similar to that of Rinderknecht et al. (1968).

0.5 ml of synovial washout and 2 ml of 0.05 M Tris-HCl buffer (pH 7.4) were incubated with 20 mg of 'azure blue impregnated hide powder' (Calibochem, U.S.A.) for 24 hours at 37°C in a shaking water bath. The solution was filtered to remove the remaining hide powder, and the blue dye released measured at 595 nm versus a blank on a Pye Unicam SP 500 spectrophotometer.

The blank solution was prepared by incubating the synovial washout separately, and adding it to the suspension of impregnated hide in buffer immediately before filtration.

The standard curve of absorbance was prepared from solutions of completely dissolved impregnated hide. (i.e. the dye colour is directly proportional to the amount of hide.)

2.5 The induction and measurement of carrageenan oedema in the rat

Carrageenan oedema was induced in 230 g male Wistar rats by the method of Winter, Risley and Nuss (1962).

A 2% w/v solution of λ-carrageenan (Sigma Chemical Co., U.S.A.) was prepared in sterile 0.9% saline the day before use, and stored under refrigeration overnight to permit bubbles to be eliminated.

With the rat under light anaesthetic, the plantar tissue of the left hind foot was carefully injected with 0.05 ml of the carrageenan solution, using a 25 gauge hypodermic needle entering from the heel towards the toes. Control animals were similarly injected with sterile 0.9% saline.

The inflammatory response to the carrageenan was estimated by measuring the increase in volume of the hind feet of the rat. The foot volume was measured by displacement of mercury in a modification of the method of Harris and Spencer (1962). In this, the foot was immersed to the hair line in a small mercury bath. The resulting rise in the bath level was measured by a (blood) pressure transducer linked to an oscillograph

recorder (Searle Bioscience Ltd, Kent). The output was so adjusted that a displacement of 1 ml of mercury produced a movement of 1 cm on the oscillograph. This relationship was periodically checked by immersion of a calibrated plunger into the mercury, during each series of measurements.

At each time of measurement, each hind foot was dipped twice, and the mean of the two results taken. The progress of the oedema was followed by measurements just prior to injection and 3 and 6 hours after the carrageenan administration.

2.5.1 The anti-inflammatory effect of α_1AT on the rat carrageenan oedema test: α_1AT injected into the foot pad together with the carrageenan

Two separate experiments were carried out on the possible anti-inflammatory effect of α_1AT on the carrageenan model. In the first, the α_1AT was mixed into the carrageenan solution prior to injection so that the α_1AT concentration was (a) 10 mg per rat, i.e. 45 mg per kg (b) 1 mg per rat, i.e.4.5 mg per kg. Control rats were given injections of carrageenan alone or α_1AT alone at both dose levels. The medium was 0.9 % saline and the total volume injected was 0.05 ml in every case.

Two sorts of α_1AT were compared by this procedure: that commercially available (Sigma Chemical Co., U.S.A.) and that prepared specially for this project.

There were 8 experimental groups, each of 6 rats. These are listed overleaf.

1. saline alone
2. carrageenan alone
3. α_1AT (Pitt) alone, 10 mg per rat
4. α_1AT (Pitt) alone, 1 mg per rat
5. carrageenan + α_1AT (Pitt), 10 mg per rat
6. carrageenan + α_1AT (Pitt), 1 mg per rat
7. α_1AT (Sigma) alone, 1 mg per rat
8. carrageenan + α_1AT (Sigma), 1 mg per rat

2.5.2 The anti-inflammatory effect of α_1AT on the rat carrageenan oedema test: α_1AT administered subcutaneously

In the second experiment α_1AT in 0.9% saline was injected subcutaneously, under the loose skin on the back of the rat's neck. One group received a single dose of 10 mg per rat 6 hours before carrageenan administration. Another group received the same dose in the 3 days preceding the carrageenan administration also, i.e. 4 daily doses. The control group received 4 daily doses of 0.9% saline prior to carrageenan administration in the same manner.

2.6 The induction and measurement of adjuvant arthritis in the rat

Adjuvant arthritis was induced in 200 g male Wistar rats by the method of Newbould (1963).

The adjuvant agent was heat-killed human strains of C, DT and N tubercle bacilli (Ministry of Agriculture Vetinary Labs., Weybridge). The supplied material was finely ground in the presence of a little liquid paraffin in a porcelain pestle and mortar. The powder was then suspended in liquid paraffin to a concentration of 5 mg ml^{-1}.

With the rat under light anaesthetic, the plantar tissue of the left hind foot was carefully injected with 0.05 ml of the adjuvant preparation, using a 25 gauge hypodermic needle entering from the heel towards the toes. Control animals were similarly injected with sterile 0.9% saline.

The inflammatory response to the adjuvant was estimated by measuring the increase in volume of the hind feet of the rat. This was by the method described in Section 2.5. Foot volume was measured just before injection and every 2 or 3 days during the experiment.

2.6.1 The anti-inflammatory effect of α_1AT on adjuvant arthritis in the rat

To test the effect of a higher than normal level of α_1AT, one group of 6 rats received a subcutaneous injection (to the skin at the back of the neck) of 10 mg α_1AT in 0.5 ml normal saline on the day before the administration of adjuvant. Further doses of α_1AT were given daily for 20 days. Allowing for the fact that the prepared α_1AT was only 50 to 75% active, this procedure would initially increase the level of α_1AT in the blood by about 25 %. The effect on the inflammation was monitored by foot volume.

A control group of rats received matching injections of normal saline only. A further group received matching injections of cortisol phosphate (which is a known anti-inflammatory agent) in therapeutic amounts, to act as a positive control. These injections were of 0.268 mg cortisol phosphate in 0.5 ml saline, equivalent to 1 mg of free cortisol per kg body weight.

2.7 The production of monoarticular arthritis in the rabbit

Male and female Old English rabbits were used, of approximate body weight 2 kg at the time of immunization, 3 kg during the experiments on arthritis. They were caged singly and allowed food and water ad libitum. Each experimental group consisted of 5 animals. Groups of 2 were used for the controls in which arthritis was not induced.

The following immune reagents were used:

5 x crystalline ovalbumin (Koch-Light Labs,)

Difco incomplete adjuvant (Difco Labs.)

Heat-killed mycobacterium tuberculosis, strains C, DT and N (Central Vetinary Lab., Surrey)

The method was similar to that described by Consden, Doble, Glynn & Nind (1971). First of all the rabbits were sensitized to the immune reagents at the laboratories of Fisons Ltd, Loughborough. This was done by injecting 1 ml of an emulsion of the three reagents in 0.9% sterile saline (containing 10 mg ovalbumin, 1 mg powdered mycobacterium, 0.5 ml Difco adjuvant) intradermally to 5 sites on the back of the neck of the animal (i.e. 0.2 ml at each site).

Three weeks later, the animals were skin tested with 0.1 ml of a sterile (Millipore filtered) solution of 10 μg ovalbumin in saline, which was injected intradermally into a shaved area on the back of the animals. A positive result showed as an increase in skin thickness (typically 200% increase) measured by pinching a fold with the electronic micrometer used for joint diameter measurements (Section 2.7.3).

When sensitization was confirmed, the knee joints were shaved of fur and washed with Hibitane in 70% alcohol. The rabbit was lightly anaesthetized and a sterile solution (Millipore filtered) of 5mg ovalbumin in 0.5 ml normal saline was injected through the the suprapatellar ligament into the right knee, using a 25 gauge injection needle. The left knee was similarly injected with 0.5 ml saline and acted as a control. See Fig 2.1.

To prevent the effects of possible acute anaphylaxis, each rabbit was given a subcutaneous injection of 2 mg Periactin (cyproheptadine hydrochloride) in 0.4 ml water, one hour prior to the above immune challenge.

Fig 2.1 Method of intra-articular injection into rabbit knee

2.7.1 The treatment of rabbit monoarticular arthritis by α_1AT and CP: experimental groups

There were 12 groups of 5 rabbits given arthritis and receiving various treatments and sham treatments. Additionally, 5 groups of non-arthritic rabbits (1 male and 1 female) received a selection of treatments. Thus several types of control comparisons were possible.

For convenience on graphs and in discussion, an abbreviation relating to the treatment is used to refer to the different experimental groups as follows:

Arthritic rabbits (5 per group)

C	control: saline injections instead of treatment (C-1 and C-2 are duplicates)
EOM	sham: encapsulated media only (mixed blood)
EOO	sham: encapsulated media only (own blood)
CPL	cortisol phosphate, low dose = 2.68 mg ml^{-1} (equivalent to 2 mg ml^{-1} cortisol)
CPH	cortisol phosphate, high dose = 6.7 mg ml^{-1}
ECPL	encapsulated cortisol phosphate, low dose = 2.68 mg ml^{-1}
ATL	$\alpha_1$antitrypsin, low dose = 8.4 mg ml^{-1}
ATH	$\alpha_1$antitrypsin, high dose = 16 mg ml^{-1}

EATL	encapsulated $\alpha_1$antitrypsin, 8.4 mg ml^{-1}
ATCPL	$\alpha_1$antitrypsin (8.4 mg ml^{-1}) + cortisol phosphate (2.68 mg ml^{-1})
EATCPL	encapsulated $\alpha_1$antitrypsin (8.4 mg ml^{-1}) + cortisol phosphate (2.68 mg ml^{-1})
ATLH	$\alpha_1$antitrypsin, late high dose = 100 mg ml^{-1}

Non-arthritic rabbits (2 per group, 1 m. 1 f.)

NA-EOM	sham: encapsulated media only (mixed blood)
NA-CPH	cortisol phosphate, high = 6.71 mg ml^{-1}
NA-ATH	$\alpha_1$antitrypsin, high = 16 mg ml^{-1}
NA-EATL	encapsulated $\alpha_1$antitrypsin, low = 8.4 mg ml^{-1}
NA-EATCPL	encapsulated $\alpha_1$antitrypsin (8.4 mg ml^{-1}) + cortisol phosphate (2.68 mg ml^{-1})

2.7.2 Administration of treatments as intra-articular injections

The treatments were introduced into the right knee joint in the same way as the challenging injection. A volume of 0.5 ml was used each time in the series of treatments. Control joints received 0.5 ml sterile saline at the same time.

All the treatments were administered roughly every two weeks (days 13, 35, 44 and 57 after initiation). The progress of the disease was followed by joint measurements etc up to 60 days, followed by pathological examination and biochemical tests.

For encapsulated preparations, 5 separate rabbits were used as donors of blood (by cardiac puncture under anaesthetic) 10 ml from each. The blood samples were pooled and used for encapsulation of the treating agent. 0.5 ml of the washed undiluted encapsulated preparation was then administered to each of the test group.

The sterility of the encapsulated preparation prior to injection was tested by aerobic and anaerobic cultures for 48 hours on various media (blood agar, cooked meat broth etc). No bacterial contamination was observed.

2.7.3 Joint diameter measurements

The swelling of the arthritic knee was well defined (see Fig 2.2). Both knee joints were shaved to permit more accurate measurements. The diameter across the latero-medial aspect of the articular junction was measured with an electronic micrometer manufactured according to the specifications of Dr A. Blackham, of the research laboratories of Fisons Ltd, Loughborough.

The electronic micrometer is mounted as a gun, with a handle and trigger. The joint or other item to be measured is lightly held between a fixed point and a spring-loaded barrel. The barrel is the moving part of a linear displacement transducer (type DLR/25 mm, Sangamo Weston Controls, Bognor Regis) which gives a linearly proportional direct current signal voltage for displacements up to 25 mm. This output was displayed on a digital voltmeter. The device is shown in use in Fig 2.3.

For all joint measurements, the animal was restrained on its back on a foam-padded board by two strips of strong cloth, secured by Velcro fastening strip. This left the animal relaxed with hind knees free for measurement by a single person. The same operator used the measuring devices throughout the experiments. The measurements were repeated every two or three days.

Fig 2.2 Arthritic knee and non-arthritic knee of rabbit, shaven for measurement

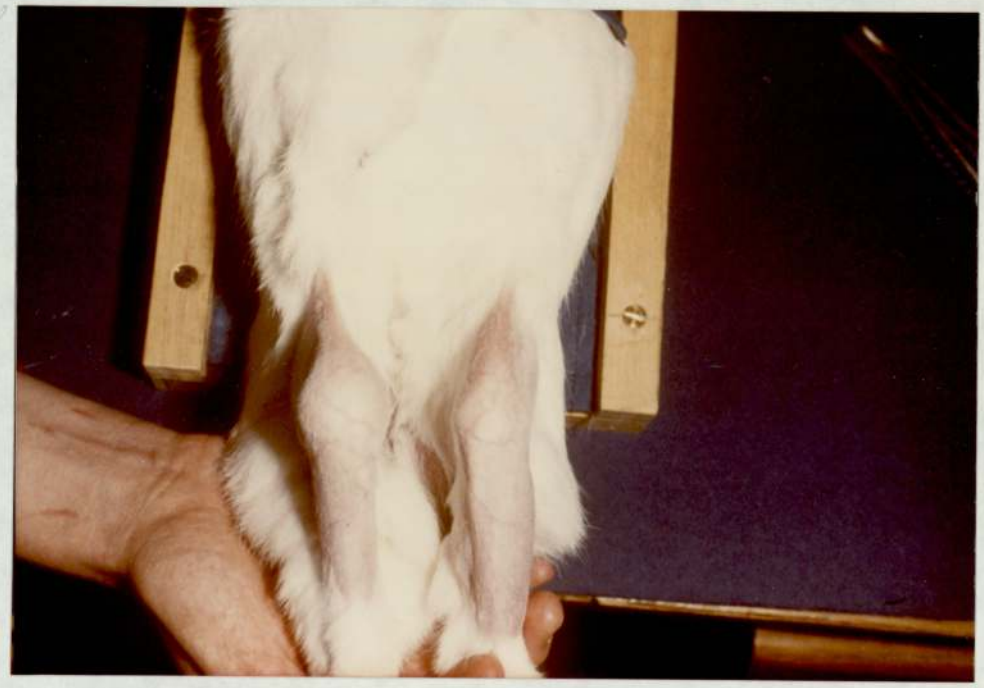
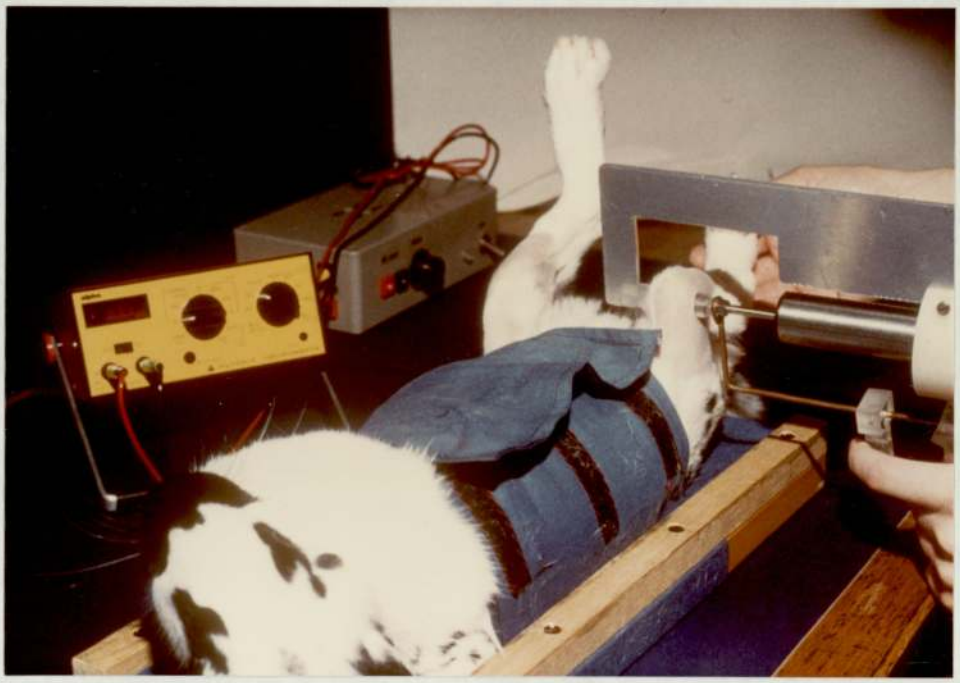


Fig 2.3 Measurement of joint diameter of rabbit knee using a specially made electronic caliper



2.7.4 Joint mobility measurements

The effect of arthritis on the knee was to reduce the angle between the femur and tibia when the leg was fully extended. This angle was measured using a simple goniometer consisting of a protractor to which had been attached a fixed arm and a moveable one. The use of the device is shown in Fig 2.4. The measurements were repeated every two or three days.

Fig 2.4 Measurement of maximum joint angle of a rabbit knee using a specially made goniometer

2.7.5 Joint dissection for macroscopic examination and synovial fluid collection

Just before the animal was killed, the knee joints (both challenged and control) were injected with 0.2 ml of 0.01 % heparinized normal saline to prevent blood clotting within the joint.

At the end of the experiment the rabbits were killed with an intravenous overdose of pentobarbitone sodium. The knee joints were then carefully dissected by cutting through the quadriceps tendon and then along the lateral medial aspects of the capsule as the flap of tissue was pulled forward.

2.7.6 Synovial fluid collection

During the course of the dissection, when the synovium was opened but the joint was not fully displayed, the synovial fluid was drawn up into a 1 ml disposable syringe fitted with a plastic pipette tip instead of a needle. Aliquots of 0.1 ml of heparinized normal saline were passed into the cavity to rinse out the remaining fluid, these washings being drawn up until a total of 1.2 ml (fluid + washings) had been collected.

The combined solution was centrifuged at 1200 g for 15 minutes and the cell-free supernatant was stored in stoppered plastic tubes at -20°C until required for biochemical assay. At this time they were made up to 5 ml and treated as described in the assay procedure.

2.7.7 Photography of dissected joints

The dissected joints (arthritic and non-arthritic) were examined for macroscopic clinical damage and then photographed in colour for a permanent record. Prints were made of approximately life-size. These prints were later used for macroscopic clinical assessment by independent observers. Examples are given in Figs 3.26 to 3.39.

2.7.8 Macroscopic examination of dissected joint by independent observers (clinical score)

Three people with experience of examining the effects of rheumatoid disease took part in a blind assessment of photographs of dissected joints. The photographs were assigned arbitrary numbers, and the assessors were not informed of any history or treatment of the joints under examination. They were asked to rate the following parameters on a scale of 0 to 3, where 0 represents negligible or nil effects of the disease, and 3 represents severe damage.

The factors considered were:

- patellar insertion erosion (not always well visible on the photographs, but noted at the time of dissection if such was the case)
- articular surface cartilage erosion
- femoral cartilage erosion
- synovial membrane attachment and obliteration
- synovial membrane thickening, adhesions and synovial space obliteration
- patellar appearance
- side tendon insertion erosion (after tendon removal)
- extension of fibro-cellular pannus
- fibrous capsule thickening
- fibrin and fibrous tissue deposition

2.7.9 Histological examination of synovial tissue

After dissection and photography of the joint, the synovial tissue was carefully excised and then immediately fixed in Bouin's Fixative.

The fixed tissue samples were embedded in paraffin wax, sectioned at 6 μm and stained with Ehrlich's haematoxylin and Eosin (which is a basic physiological structure stain) and Mallory's triple stain for fibrin. The histopathological changes in the synovial tissue were examined under a light microscope and graded by an overall subjective assessment. An independent expert in rheumatology histopathology separately assessed the samples, and agreement was reached about the relative order of severity of the effects observed.

The following factors were particularly examined:

- synovial intima: hypertrophy or hyperplasia; villi
- synovial connective tissue: oedema; infiltration by polymorphs, lymphocytes, plasma cells and macrophages; fibrin aggregates; fibrosis; pannus.

Examples are given in Figs 3.40 to 3.47.

2.7.10 Biochemical assays on synovial fluid

The following assays were carried out: total protein, acid phosphatase, protease activity, $\alpha_1$antitrypsin. The methods have been described in section 2.4.

2.8 The effect of various steroids on plasma α_1AT and α_2M levels and on the liver in the rat

Male Wistar rats were used of body weight about 200 g. In a dose response part of the experiment each rat was weighed at the start and at the end of the experiment (day 7).

Penicillamine was chosen as a non-steroidal (but anti-inflammatory) drug for comparison with the steroids. The steroidal drugs used were: cortisone, cortisol, dexamethasone, 17-β-oestradiol, corticosterone, triamcinolone acetonide, β-methasone alcohol, prednisolone and methyl prednisolone.

Blood samples were taken by cardiac puncture the day before the administration of the first dose of drug and the day after the last dose of the drug, and the plasma assayed for α_1AT and α_2M. At the end of the experiment the rats were killed and the liver removed. Its general appearance and histology were observed.

Dose levels were similar to those used in the experiments on arthritic animals.

2.8.1 Drug adminstration

For each drug, 50 mg was dissolved in 1 ml DMSO (dimethyl sulphoxide) and made up to 10 ml with arachis oil to give a suspension. A dose of 0.2 ml of the suspension was thus equivalent to 1 mg of the drug, i.e. 5 mg per kg body weight, or about twice the typical therapeutic dose for humans. Similar suspensions were made up pro rata for the second (dose response) tests.

In the first experiment, the rats received 0.2 ml of drug suspension by sub-cutaneous injection to the skin on the back of the neck, every day for 10 days. A control group received similar injections of the DMSO / oil vehicle.

In the second experiment, to look at dose response, three drugs only were used: cortisol, prednisolone and dexamethasone. They were administered daily for 7 days as above to separate groups at different doses, equivalent to 0.25, 1.25 and 2.5 mg drug per kg weight. A control group likewise received the vehicle only.

2.8.2 Blood sampling

Blood samples were taken by cardiac puncture under anaesthesia. For the 10 day experiment all rats had 1 ml of blood thus removed one day before the first drug dose and one day after the last dose.

For the 7 day experiment, much larger volumes (5 ml each) were required, so an additional control group was sacrificed to provide the blood samples at the start of the experiment. All the other rats were killed following removal of 5 ml of blood at the end of the experiment.

Plasma was separated from the heparinized blood samples by centrifuging at 2000 g for 10 minutes in a M.S.E. bench centrifuge. The plasma was stored in stoppered plastic tubes at -20°C until assayed, taking the precautions specified in the assay kit instructions (detergent-free, darkness, time of storage, haemolysis).

2.8.3 Histological examination of the liver

At the end of the dose response experiment, the livers were dissected out from the 3 groups of rats which had received dexamethasone at the different dose levels. Livers were also removed from the control group which had received the DMSO / oil vehicle only, and from a group which had received no treatment at all.

The general appearance of the liver was noted - e.g. a paleness would suggest accumulation of fat. Small cubes (~ 5 mm) of the liver were rapidly frozen on a mounting block in liquid nitrogen. The frozen tissue was then sliced on a freezing microtome to 5 μm thickness.

The sections were stained with Haematoxylin and Eosin stains, and also with Sudan Black, which is a specific stain for lipids. The sections were examined under the microscope and black-and-white micrographs taken of a representative section of each of the treatments.

2.9 The method of encapsulation of agents into RBCs

The loading procedure was carried out under aseptic conditions at room temperature. Particular care was taken throughout to avoid physical damage to the cells: movements were gentle and transfers by pipette were done slowly.

Blood was obtained from the animals under anaesthetic by cardiac puncture, being drawn into a heparinized syringe and used for encapsulation promptly after. The procedure was as follows.

(1) Erythrocytes were sedimented from whole blood by centrifugation at 600 g for 7 minutes. The plasma and white blood cells were discarded.

(2) A 1 ml portion of the packed erythrocytes was transferred to a test tube. 4 ml of swelling solution was then added. This solution consisted of a modified K^+-reversed Hanks' solution (see Section 2.9.1) diluted with distilled water so that its tonicity was 0.66 of that of the cell contents. The cells and the solution were mixed by gently inverting the test tube several times.

(3) The suspension was centrifuged at 600 g for 5 minutes and the supernatant removed, leaving a noticeably larger volume of swollen cells. (This increase in volume can be calculated and measured by haematocrit - about 13 %.)

(4) Meanwhile, another sample of the packed original erythrocytes had been lysed by dilution with an equal volume of distilled water. A 0.2 ml portion of this haemolysate was gently layered on top of the swollen cells, by running it slowly round the inside perimeter of the test tube from a pipette.

The object of this step was to provide a barrier between the cells and the aqueous material to be encapsulated so that transfer could be accomplished with the minimum chemical shock to the system. The haemolysate of course provides an environment containing all the various intra-cellular and cell surface materials which might otherwise be lost from the cell during encapsulation.

(5) An aqueous solution of the material to be encapsulated was prepared. A 0.2ml portion was gently layered on top of the haemolysate layer. The test tube was gently inverted several times to mix the contents, and then centrifuged at 500 g for 3 minutes.

(6) The supernatant was left in place to act as a barrier, and the next 0.2 ml portion of the drug solution was layered on. The test tube was mixed and centrifuged as before.

(7) Successive additions of 0.2 ml portions of the drug solution followed the same procedure until the swollen erythrocytes reached the point of haemolysis. This point was apparent from a sudden increase in the transparancy of the suspension, darkening of the supernatant layer, and a poorer separation on centrifuging, with the appearance of a few ghost cells.

It was occasionally convenient to estimate the point of haemolysis with a trial experiment. In general, for 1 ml of packed erythrocytes, 3 to 5 portions of 0.2 ml drug solution could be added without causing haemolysis. This of course depended on the drug and its concentration.

(8) After addition of the final portion of drug solution, the erythrocytes were resealed by restoration of their normal tonicity. This was done by the rapid addition and immediate shaking of the calculated amount of modified K^+- reversed Hanks' medium at 10 times the eutonic strength. In practice, the volume required was approximately one-tenth of the volume of the total added water to the original unswollen cells.

(9) The resealed cells were centrifuged at 500 g for 5 minutes and the supernatant discarded. The cells were then washed by suspending them in about 10 ml of eutonic modified K^+-reversed Hanks' medium and again centrifuging at 500 g for 5 minutes.

(10) The supernatant was discarded and the washing procedure repeated three times to recover packed erythrocytes encapsulating the chosen drug.

The whole process of encapsulation takes about two hours. The encapsulated preparation was used as soon as possible, and in any case within 6 hours. For intravenous administration, the packed cells were suspended in an equal volume of B.P. normal saline for injections.

Encapsulation into rabbit erythrocytes was carried out in the same way, except that a pooled blood sample from 5 animals was used, giving a packed volume of erythrocytes of 5 ml. All addition volumes were therefore increased proportionately. To minimize the volume injected, administration of encapsulated preparations into rabbit joints used undiluted packed cells.

2.9.1 Modified K^+-reversed Hanks' physiological medium

The swelling solution used was based on that originally specified by Hanks (1948) for tissue culture. The K^+-reversed version of such media means that the molar concentrations of the Na^+ and K^+ ions are reversed. This more closely matches the ion concentrations found in the interiors of cells.

Hanks specified the inclusion of the indicator phenol red (phenolsulphonephthalein) to warn of pH changes. This was not relevant to this work and a possible complication, so was omitted.

Problems were found with precipitation, which was most probably due to the relative insolubility of calcium and magnesium carbonates. (It has been suggested that the phenol red may in fact act as a chelating agent to prevent this precipitation). Thus after some preliminary tests, $CaCl_2$ and $MgSO_4.7H_2O$ were omitted from the composition. No disadvantages in the method and use of the preparation were noticed as a result.

The modified medium was therefore:
KCl (10.18 g l^{-1}); KH_2PO_4 (0.1 g l^{-1}); $NaHCO_3$ (1.273 g l^{-1}); NaCl (0.316 g l^{-1}); $Na_2HPO_4.2H_2O$ (0.1 g l^{-1}); glucose (2.0 g l^{-1}).

2.10 In vivo survival of erythrocytes after encapsulation

The ability of the preparation to survive in the circulation was tested by intravenous administration to the rat, the guinea-pig and the rabbit. The cells used were labelled with a fluorescing agent, FITC (fluorescein isothiocyanate). Blood samples were removed at intervals and examined under an ultra-violet microscope (Zeiss Universal with tungsten and deuterium lamps) where the labelled cells could be clearly discriminated by virtue of their fluorescence.

The red blood cells were counted using a haemacytometer and the labelled cells expressed as a percentage of the total red blood cell count.

Two methods of fluorescent labelling were used. In one, the encapsulation procedure was performed with a solution of FITC in place of the drug. In the other method, FITC was attached to the (outer surface of) the cells after they had encapsulated media only.

2.10.1 Encapsulation of FITC using rat's own RBC

1 ml of blood was removed from the anaesthetized rat by cardiac puncture. The erythrocytes were collected and put through the encapsulation procedure as described in Section 2.9. However, since FITC is relatively insoluble in water it could not be made up as in (5). It is more soluble in physiological media, so instead a saturated solution of FITC was made up in 0.66 eutonic modified K^+- reversed Hanks' medium. A single application of 0.2 ml of this was used in stage (5) of the procedure. To compensate for the additional salts thereby added, a single application of 0.2 ml distilled water was made in place of further aliquots of drug solution.

The erythrocytes were resealed and washed as normal. The packed cells were taken up in an equal volume of normal saline BP and returned to the rat by injection into the femoral vein.

2.10.2 FITC labelling after encapsulation, using rat's own RBCs

The blood sample was obtained as usual, and the erythrocytes were put through the encapsulation process in the absence of any drug.

The resealed erythrocytes were incubated with an equal volume of eutonic modified K^+ reversed Hanks' medium saturated with FITC, for one hour at room temperature. The cells were then washed three times in eutonic modified Hanks' medium, diluted with an equal volume of normal saline BP, and injected into the animal's femoral vein as before.

2.10.3 In vivo survival of FITC labelled rat RBCs after encapsulation using mixed donor blood

In a preliminary experiment, heparinized whole blood samples from 5 rats were pooled and centrifuged to separate out the erythrocytes. These mixed normal RBCs were labelled by incubation with FITC-saturated eutonic modified Hanks' medium for one hour at room temperature, as in Section 2.10.2.

The labelled RBCs were returned to the donor rats' circulations via the femoral vein as before. Blood samples (1 drop from the tail) were taken from each rat every 10 days for 50 days. Counts were made of labelled and unlabelled RBCs as described. No effect from the use of mixed donor blood rather than the rat's own could be detected.

This experiment was repeated with another group of 5 rats, except that the encapsulation procedure in the absence of any drug was carried out on the RBCs before labelling and return to the circulation.

In a further version of the experiment, a group of 3 rats were used, but cortisol phosphate was encapsulated (at the dose used in the adjuvant arthritis tests) prior to labelling and return to the circulation.

2.10.4 Encapsulation and FITC labelling of rabbits' own RBCs and of guinea-pigs' own RBCs

Trial encapsulations were carried out to observe any differences in the behaviour of rabbit and guinea-pig RBCs compared with rat RBCs. The procedure was followed as for encapsulation of media only, with water dilution proceeding until the point of haemolysis was reached and passed.

Guinea-pig cells appeared more fragile than rat's, in that they accepted about one-third less water dilution before haemolysing. Rabbit cells accepted even less - about half the dilution tolerated by rat erythrocytes.

The different points of haemolysis were taken into account for encapsulations involving rabbit or guinea-pig RBCs, but the method was otherwise the same as described for rat RBCs (Section 2.9). FITC labelling was likewise as for rat RBCs.

2.10.5 Intravenous injections of encapsulated and labelled preparations in rats and guinea-pigs

The animal was placed under light anaesthetic (3 % halothane in O_2 + N_2O). The inner half of the hind leg was shaved and swabbed with Hibitane in 70 % alcohol. The femoral vein was exposed by an incision in the skin and removal of connective tissue. The vein was made more prominent by pressure congestion and the syringe needle was inserted (bevel up) into the vein. The 1 ml volume of erythrocytes in saline was injected slowly over about half a minute.

A cotton wool swab was held over the vein during needle withdrawal and held on with slight pressure for about a minute. The vein was therby resealed and no blood loss usually appeared from the injection site. The surrounding skin was then cleaned with the Hibitane solution and the skin incision closed with metal clips. No post-operative difficulties were experienced after this procedure. The clips could be recovered (if wished) after about 3 days, with the site of incision well healed.

A particular procedure was developed for filling the syringe with the encapsulated preparations to avoid the risk of air embolism in the animal. Plastic syringes are especially prone to retain air bubbles, and the high viscosity of the preparation means that cavities may be formed in the needle space and elsewhere. The opacity of the preparation means that air bubbles are almost impossible to notice. Moreover, because the cells are subject to physical damage, vigorous expelling and refilling is not an adviseable method of clearing bubbles.

A 1 ml tuberculin syringe was first filled with sterile saline, and all air bubbbles expelled by as much vigorous plunger action as was necessary. The syringe was then slowly emptied, so that the neck and luer fitting remained filled with liquid. (This was a very small volume.) The syringe was then applied to the erythrocyte preparation and very slightly more than 1 ml drawn up. A 25 gauge needle was filled (including the luer adapter) with sterile saline and was then fitted to the syringe. The syringe contents was then adjusted to 1 ml by gentle expulsion, and the injection was then given.

2.10.6 Intravenous injections of encapsulated and labelled preparations in rabbits

The animal was placed under light anaesthetic (3% halothane in O_2 + N_2O). The hair over a section of an ear vein was removed by plucking, and the area was swabbed with Hibitane in 70% alcohol. The vein was made more prominent by pressure congestion and the syringe needle was inserted (bevel up) into the vein. The 1 ml volume of erythrocytes in saline was injected slowly over about half a minute.

A cotton wool swab was held over the vein during needle withdrawal and held on with slight pressure for about a minute. The vein was thereby resealed and no blood loss usually appeared from the injection site.

The particular method adopted for filling the syringe to avoid air bubbles is described in Section 2.10.5.

2.10.7 Method of taking blood samples for erythrocyte counts from the rat

The animal was placed under light anaesthetic, and the very tip of the tail cleaned with Hibitane in 70 % alcohol. The extreme fleshy tip of the tail was then cut off. The tail was gently massaged to obtain a few drops of blood into a small tube. The correct dilution was obtained by use of a haemocytometer pipette and heparinized saline or eutonic modified Hanks' medium.

For taking relative counts of labelled cells, it was not necessary to have absolute concentrations, so that only approximate dilutions were required.

The cells were counted on an ultra-violet microscope (Zeiss Universal) using a standard haemocytometer grid. Firstly a count was made under normal (tungsten lamp) illumination to get the total count. Then the ultra-violet light (deuterium) was added and only the fluorescing cells counted, i.e. the labelled ones. Hence the percentage of labelled cells could be calculated. At least a thousand cells were counted for each total cell count.

The first sample was taken about 5 minutes after the preparation injection, then after 1 hour, 24 hours and daily thereafter.

2.10.8 Method of taking blood samples for erythrocyte counts from the rabbit and the guinea-pig

For the rabbit, it was necessary to pluck the hairs from a small area of an ear vein, then swab with Hibitane in 70% alcohol. For the guinea-pig, it was only necessary to swab. The selected ear vein was punctured with a lancet, and a few drops of blood collected into a small tube. The bleeding was readily stopped by the pressure of a cotton wool swab for a short time.

Dilution and cell counting was carried out as for the rat (Section 2.7.7). The procedure was carried out on the lightly anaesthetized animal, the first sample being taken about 5 minutes after the injection of the encapsulated preparation. Subsequent samples were taken at 1 hour, 24 hours and daily thereafter. In the case of the rabbit, the first sample was taken from the ear which had not received the injection.

2.10.9 In vivo survival of erythrocyte ghosts labelled by encapsulated FITC in the rat

FITC was encapsulated in resealed erythrocyte ghosts using the technique of Tyrrell & Ryman (1976)
The method was very slightly modified because FITC has very low solubility in water: it was therefore dissolved in eutonic modified K^+-reversed Hanks' medium and added to the cells prior to their complete haemolysis in water.

Rat erythrocytes were spun down from heparinized whole blood, as in 2.9(1), and washed several times in phosphate-buffered saline. To 1 ml of the packed cells was added 1 ml of eutonic modified K^+-reversed Hanks' medium, saturated with FITC.

Lysis of the cells was carried out at 4^oC by 4.5 ml distilled water for 1 minute (this dilution represents 25% of the normal tonicity of the cells, and results in complete rupture and loss of cell contents). The membranes were restored to the normal cellular configuration by the addition of 0.375 ml of 2 M NaCl and incubation at 37^oC for 15 minutes.

The resealed membranes (ghosts) were then washed several times in phosphate-buffered saline, re-suspended in an equal volume of normal saline, and returned to the circulation of the donor rat via the femoral vein, as in Section 2.10.5.

2.11 The effect of intravenous adminstration of corticosteroids encapsulated in intact RBCs on adjuvant arthritis in the rat

The pharmacological effectiveness of encapsulated steroid preparations was tested against appropriate controls on the adjuvant arthritic rat model. The steroids used were cortisol (as the phosphate) and prednisolone (as the sodium succinate).

The drug doses quoted throughout are based on Jenner's claim (Jenner, D.A., thesis, 1976) that the "incorporated drug concentration is roughly the same as the pre-hemolytic drug concentration in the suspending medium". However, some other experiments later showed (see Section 2.12) that the encapsulation process is less efficient than Jenner suggests. This means that the effects of the drug observed result from lower actual doses.

The intention was to encapsulate 10 times the daily therapeutic dose (or a factor thereof) and to monitor its effects on the arthritis for a period after. Rats own RBCs were initially used (2.11.1) but mixed blood was used for subsequent tests, since it was found they survived as well in circulation (Section 3.7.3).

2.11.1 Intraveous administration of encapsulated cortisol phosphate, compared with the same drug in solution administered intravenously or subcutaneously

Groups of 7 rats of 200 g bodyweight received the various treatments and controls. Subcutaneous administration was equivalent to 1 mg cortisol per kg bodyweight given daily in 0.5 ml saline. Intravenous treatments were 10 times this dose given as a single injection (1 ml volume) on the day of adjuvant challenge and repeated on the 10 th day after. Groups were as follows, with abbreviations.

C	control. 0.5 ml saline s.c. daily 20 days.
EO	sham. 0.5 ml RBC encapsulated media only, in 0.5 ml saline, i.v. days 0 and 10.
CP	0.268 mg cortisol phosphate in 0.5 ml saline, s.c. daily 20 days.
ECP	2.68 mg cortisol phosphate, encapsulated in 0.5 ml RBC, + 0.5 ml saline, i.v. days 0 and 10 (nominal drug concentration)
CPIV	2.68 mg cortisol phosphate in 1.0 ml saline, i.v. days 0 and 10

2.11.2 Comparison of three different doses of cortisol phosphate, encapsulated and given intravenously

Groups of 7 rats of 200 g bodyweight received the treatments and controls. The control animals received the adjuvant challenge and developed arthritis. They were killed after 14 days to reduce animal suffering, since it was judged that sufficient effects had been observed.

One group received an encapsulated preparation on day 0, nominally equivalent to 10 therapeutic daily doses of 1 mg cortisol per kg. A further treatment was given on day 10. The other groups received encapsulated preparations equivalent to 20 and to 30 days supply on day 0 only. (Some difficulty was experienced in encapsulating the higher doses, which were probably not actually 2 and 3 times the nominal 10-day dose.) Groups were as follows, with abbreviations.

C	control. No treatment of induced arthritis.
ECP-10	2.68 mg cortisol phosphate, encapsulated in 0.5 ml RBC, + 0.5 ml saline, days 0 & 10
ECP-20	5.36 mg cortisol phosphate, encapsulated in 0.5 ml RBC, + 0.5 ml saline, day 0 only
ECP-30	8.0 mg cortisol phosphate, encapsulated in 0.5 ml RBC, + 0.5 ml saline, day 0 only

2.11.3 Comparison of encapsulated cortisol phosphate with the same drug incubated with RBCs

Groups of 7 rats each of 200 g bodyweight were treated with cortisol phosphate at two different doses, in two different preparations with rat RBCs. Two groups received preparations encapsulated in the standard way. Two other groups received equivalent amounts of the drug with an equal amount of RBCs. However, in the latter cases, the RBCs had not been swollen, but merely allowed to remain in contact with the drug solution (i.e. incubate) at room temperature for the same time of contact as occurred in encapsulation. Groups were as follows, with abbreviations.

C	control. as in 2.11.2.
ECP-10	10 day dose of encapsulated drug as 2.11.2
ECP-5	5 day dose, ie half drug concentration of ECP-10
RBC+CP-10	as ECP-10, but incubated not encapsulated
RBC+CP-5	as ECP-5, but incubated not encapsulated

The preparations were administered intravenously on day 0 and again on day 10.

2.11.4 Comparison of free and encapsulated cortisol phosphate, injected either in the early (acute) or late (chronic) phase of adjuvant arthritis

Groups of 7 rats each of 200 g bodyweight were used. Encapsulated solution preparations equivalent to 7 days dose were administered either on day 0 (acute) or day 7 (chronic). For comparison, cortisol phosphate in solution was administered as a daily dose either from day 0 to day 6 (acute) or from day 7 to day 13 (chronic). 1 day's dose was 0.268 mg per animal, equivalent to 1 mg cortisol per kg bodyweight. Groups were as follows, with abbreviations.

C	Control. No treatment of induced arthritis.
CPacute	cortisol phosphate in 0.5 ml saline, daily day 0 to day 6.
ECPacute	encapsulated cortisol phosphate in 0.5 ml RBC, + 0.5 ml saline, day 0.
CPchronic	cortisol phosphate in 0.5 ml saline, daily day 7 to day 13.
ECPchronic	encapsulated cortisol phosphate in 0.5 ml RBC, + 0.5 ml saline, day 7.

2.11.5 Intravenous administration of encapsulated prednisolone sodium succinate, compared with the same drug administered in solution or after incubation with RBCs

The drug prednisolone (as the sodium succinate) was used as another example of an anti-inflammatory corticosteroid. It was administered to groups of 7 rats of bodyweight 200 g on a daily basis in saline solution, encapsulated (5 days' or 10 days' dose) and also after incubation with red blood cells (10 days' dose) as in 2.11.3. Prednisolone is about 5 times more potent in its action than cortisol, so the doses were scaled down accordingly. The daily dose was equivalent to 0.2 mg prednisolone per kg bodyweight, given as 0.054 mg per rat of the sodium succinate salt. Groups were as follows, with abbreviations.

C	control. No treatment of induced arthritis.
P	prednisolone as the salt in 0.5 ml saline, daily from day 0 to day 14 sub-cutaneously.
EP-10	encapsulated prednisolone. 10 days' dose in 0.5 ml RBC, + 0.5 ml saline, days 0 & 10.
EP-5	as EP-10, but half drug concentration
RBC+P-10	prednisolone, 10 days dose, incubated with 0.5 ml RBC, + 0.5 ml saline, days 0 & 10.

2.12 In vitro tests of encapsulated preparations

2.12.1 Test of the slow release of encapsulated drug in dialysis sacs

The membrane of a standard dialysis sac was used as an analogue of the erythrocyte membrane to see if slow release through the cell wall was a plausible mechanism for release of encapsulated material. Measurements were made of materials leaking out of the sacs into eutonic modified K^+-reversed Hanks' medium. Encapsulated materials were also put into dialysis sacs to observe the relative rates of diffusion through the two membranes.

The sacs were made from 100 mm lengths of 10 mm diameter dialysis tubing (Gallenkamp & Co, Birmingham) soaked in water and tied at one end. 0.5 ml of the prepared solution was then pipetted in, and the sac was suspended in 25 ml eutonic medium in a McCartney bottle, being immersed to a depth of 50 mm.

After 30 minutes the sacs were transferred to a fresh bottle of medium, and the contents of the first bottle measured. This was repeated to give six readings over 3 hours. Each preparation was tested in 5 replicate sacs.

A trial run was carried out with methylene blue as the material to be dialysed. This is a blue dye which was easy to see and measure, and has a molecular weight similar to that of a typical steroid (methylene blue mol. wt. = 374; cortisol = 362.5).

3.55 mg of the dye was placed in 0.5 ml of medium in each of the sacs, and the experiment carried out as above, except that readings were taken every 10 minutes for the first half hour, then every 30 minutes for the next two hours, after a further 60 minutes and 120 minutes. A final reading was taken after 18 hours. The amount of methylene blue was measured by its absorbance at 664 nm on a Pye Unicam SP 500 spectrophotometer, compared with a specially prepared calibration curve.

The materials in the sacs for the main experiment are as follows, with abbreviations:

ECP	encapsulated cortisol phosphate 1.34 mg
CP	free cortisol phosphate 3.55 mg
CP+RBC	cortisol phosphate 3.55 mg & RBCs
ECPAT	encapsulated CP 1.34 mg & α_1AT 4.2 mg
CPAT	free CP 3.55 mg & α_1AT 8 mg
CPAT+RBC	free CP 3.55 mg & α_1AT 8 mg & RBCs

Cortisol phosphate was measured by its absorbance at 240 nm.

2.12.2 The stabilizing effect of internal and external cortisol phosphate on the rabbit erythrocyte membrane

The effect of encapsulated cortisol phosphate on the stability of the cell to lysis was tested, as was the effect of cortisol phosphate in the medium outside the cell.

In initial experiments, different techniques were tried to give gentle lysing. The detergent Triton-X was too uncontrolled in its effect (invariably complete haemolysis). Measurement of acid phosphatase release was unsuccessful due to the interference and denaturation of haemoglobin. Eventually, it was discovered that simple warming to 37°C in a shaking water bath gave gentle partial lysis. The degree of lysis was readily measured by the amount of haemaglobin released, measured by its absorbance at 543 nm, once the cells had been spun down at 600 g for 10 minutes.

Solutions of cortisol phosphate were made up in normal saline, and 1 ml was mixed with 4 ml of a 1% v/v suspension of washed rabbit RBCs in saline, to give a final steroid concentration in the range 10^{-7} M to 10^{-2} M. Each drug concentration was set up in duplicate, along with controls involving no drugs.

The tubes were incubated at 37°C for 30 minutes in a shaking water bath. They were then centrifuged at 600 g for 10 minutes. The cell-free supernatants were then measured for haemoglobin concentration via its absorbance at 543 nm on a Pye Unicam SP 500 spectrophotometer against a blank, using the modified method of Williams, Capstick, Lewis & Best (1976).

Samples of encapsulated cortisol phosphate and encapsulated media only were set up in the same way. The original solution used for encapsulation was 16 mg ml^{-1}, of which 0.2 ml (= 3.2 mg) was added to 1 ml erythrocytes during the encapsulation procedure.

Samples of encapsulated cortisol phosphate and encapsulated mediaonly were also lysed by the addition of 50 µl of Triton-X, diluted 10 times, and the haemoglobin measured. This gave an indication of the amount of haemoglobin lost during encapsulation.

2.12.3 Investigation of erythrocyte membrane charge by microelectrophoresis before and after encapsulation, and in grey ghosts

The electrophoretic mobility (which is a function of surface charge) was measured for erythrocytes which had received various treatments, including encapsulation.

The apparatus used was a Mark II Microelectrophoresis Apparatus (Rank Bros, Cambridge) with a cylindrical cell and platinum black electrodes, as described by Seaman (1965). The system was illuminated with a quartz iodine lamp, and the scattered light observed via a camera fitted to a microscope fixed at the stationary level. Ten erythrocytes were timed electronically as they moved in the applied electric field over a fixed distance (set by an eyepiece graticule and displayed on a TV monitor). Cells were timed with the field in both polarities to minimize errors due to drift etc.

The cylindrical cell was filled with Sorenson's Buffer (pH 7.4) and the erythrocyte suspension (about 1% v/v in saline) was gradually introduced (displacing the buffer) to prevent bubble formation. The cell was flushed 3 times with buffer between samples.

2.12.4 Investigation by TLC of the contents of rabbit erythrocytes used to encapsulate cortisol phosphate

When cortisol phosphate is encapsulated, it is quite possible that some of it could be hydrolysed to free cortisol by the action of some components of the cell. To detect this, a technique of thin layer chromatography (TLC) was used in which cortisol phosphate remains at the spotting site, but cortisol is separated and carried up the plate.

The plate used was silica gel. The developing solvent was absolute alcohol (100% I.M.S.). The detecting agent was 50:50 v/v methanol: conc. H_2SO_4 and heat.

Standards of cortisol (5 mg ml^{-1} in acetone) and cortisol phosphate (5 mg ml^{-1} in water) were developed at the same time as duplicate samples of 10 μl of cytoplasm.

The cytoplasm was obtained by freezing and thawing the packed cells to rupture them, and centrifuging at 10 000 r.p.m. for 30 minutes in a MSC bench centrifuge to give a cell-free supernatant.

2.12.5 Investigation by TLC of the site of acid phosphatase activity in the rabbit erythrocyte

The membrane fraction of the cells was separated from the cytoplasm by freezing and thawing a sample of packed erythrocytes, and centrifuging down as in 2.12.4. The cell-free supernatant was removed and the membrane fraction washed 4 times with 0.9% saline (spinning down each time as before) to remove traces of cytoplasm.

Samples of the isolated cytoplasm and of the free membranes were incubated with aqueous solutions of cortisol phosphate (10 mg ml^{-1}) in a shaking water bath at 37°C for 1 hour. 10 μl aliquots of the incubated solution were then tested by TLC as described in 2.12.4 to see which fraction produced more hydrolysis.

2.12. 6 In vitro phagocytosis of encapsulated preparations

Polymorphonuclear leucocytosis is a common feature of many inflammatory conditions. Large numbers of PMNs are found in synovial effusions in rheumatoid arthritis. Thus the reaction of PMNs against our encapsulated preparations was tested in vitro. Such a reaction might be expected to occur after intra-articular injections.

The process was tested first with guinea-pig PMNs against rabbit erythrocyte encapsulated preparations. Later rabbit PMNs were used against rabbit erythrocyte encapsulated preparations. In both cases the PMNs were obtained from induced peritoneal exudate.

To get the exudate, guinea-pigs were primed with up to 100 ml 0.9% saline injected intraperitoneally. Six hours later, the PMNs were harvested by injecting a further volume of saline and allowing the peritoneal fluid (exudate) to drip through a 15 gauge needle into sterile plastic tubes. Rabbits were similarly treated with up to 500 ml saline. All the above procedures were with the animals under light anaesthesia.

The concentration of PMNs in the exudates was convenient for microscopic examination, so they could be used directly (without the need for culturing).
or concentrating

To observe phagocytosis, 30 drops of exudate were mixed with 10 drops of serum homologous with the PMN donor and 1 drop of 3 times diluted (in saline) rabbit erythrocyte preparation. A drop of this mixture was immediately placed on a slide and covered with a slip (supported on the sides with sellotape to form a cell). The process of phagocytosis was observed by light microscopy, using normal transmission and phase contrast lighting.

2.12.7 Estimation of encapsulated α_1AT by TIC assay (rabbit RBCs)

The assay procedure described in Section 2.4.1 suffers from interference by cell haemolysate when present in high concentrations (as is bound to be the case for encapsulated preparations). In effect, the haemolysate components compete with BAPNA for the role of trypsin substrate. A more detailed investigation is given in the M.Sc thesis of Jenner (1976).

To partially compensate for these effects, known amounts of α_1AT were made up in the presence of (but not encapsulated by) erythrocytes in the same amount as the encapsulated preparation. These standards were then used as a calibration of the assay under such conditions.

This method gave some estimate of α_1AT concentrations, but a technique of rocket electrophoresis was preferred. (See Section 2.12.8.)

2.12.8 Estimation of encapsulated α_1AT by electro-immuno assay (rabbit RBCs)

The method of Laurell (1966) commonly known as "rocket" electrophoresis was used.

A gel was prepared by heating 1 % w/v agarose in 0.07 M barbital buffer (pH 8.6) containing 0.002 M calcium lactate. The mixture was allowed to cool to 40°C and 2 % v/v antiserum mixed in. The antiserum was that to human $\alpha_1$antitrypsin (Dako-immunoglobulins, Denmark). The mixture was then poured into a mould to set, giving a gel slab 180 x 80 x 1 mm. Holes of 4 mm diameter were cut into the gel 10 mm apart along the edge.

The gel slab was placed in a standard electrophoresis tank and 8 µl portions of the solutions to be assayed were pipetted into the wells formed in the gel. (From preliminary tests the most suitable dilution was found - up to 300 times.) A d.c. voltage of 70 - 80 volts was applied across the slab by a Shandon power pack. The electrophoresis was allowed to proceed for 18 hours, by which time the height of the 'rockets' formed was constant.

The rocket heights were measured from the centres of the holes to the tip of the rocket, this height being proportional to the amount of antigen precipitated by the antiserum. They were compared with standards of known α_1AT concentration.

2.12.9 Fluorimetric assay of encapsulated cortisol phosphate (rabbit RBCs)

A method modified from those of Peterson et al. (1957) and Mattingly (1962) was used.

The cortisol phosphate was extracted from aqueous solution into dichloromethane (0.5 ml into 5 ml). A 2 ml portion of the dichloromethane phase was treated with 1 ml of fluorescence reagent (30 : 70 v/v absolute alcohol : conc. H_2SO_4). The acid phase was read with a spectrofluorimeter, with an excitation wavelength of 464 nm, giving an emission at 525 nm. Readings were taken after a minimum of 13 minutes, maximum 20 minutes from the addition of the fluorescence reagent.

Samples of encapsulation supernatant; lysed encapsulation cells (by freezing and thawing) and washings were assayed by this method, both directly and after hydrolysis (boiling with 1% HCl). The direct method would respond to free cortisol only, but after hydrolysis cortisol phosphate would be converted to cortisol also, and this would correspond to total cortisol. Standards of cortisol and cortisol phosphate were measured in a similar manner for calibration.

2.12.10 Fluorimetric assay of encapsulated cortisol phosphate after chloroform extraction (rat RBCs)

Encapsulation of 3 different doses of cortisol phosphate and of media only was carried out. Samples of the preparations were lysed by freezing and thawing, and solubilized by the method of Kitao and Hattori (1980). The solubilizing medium was 0.1 M Tris-HCl buffer (pH 7.6) containing 5 M urea, 3% sodium dodecyl sulphonate, 0.1% mercaptoethanol and 0.001 M EDTA.

The cortisol was extracted from the medium into chloroform, evaporated to dryness at room temperature and assayed as in section 2.12.9. Note that chloroform would not extract cortisol phosphate, only the portion of the salt which had been converted to free cortisol.

The procedure was repeated, except that the samples were incubated with alkaline phosphatase at 37°C, minutes before chloroform extraction. By this means the total cortisol could be measured, hence the efficiency of encapsulation and the degree of conversion of the phosphate to free cortisol.

As a check, the supernatant liquid after encapsulation was also assayed for total and free cortisol.

2.12.11 Assay of encapsulated cortisol phosphate in rat RBCs by measurement of inorganic phosphate after enzymatic hydrolysis

A standard method (Fiske & Subbarow, 1925) was used for the measurement of phosphate colorimetrically by the colour of its complex with ammonium molybdate. The procedure exactly followed that given in "Sigma Technical Bulletin No 670" (Sigma Chemical Co, 1976).

A preliminary experiment showed that 60 minutes was an optimum incubation period at 37°C for a 1% solution of alkaline phosphatase in Tris-HCl buffer (pH 9.0). 1 ml of sample (cells lysed by freezing and thawing) was mixed with 1 ml of pH 9.0 Tris-HCl buffer and 1 ml of the enzyme solution and incubated.

The resulting solution was made up to 20 ml, and treated with the complexing reagents and read at 670 nm on a Pye Unicam SP 500 spectrophotometer. The absorbance was converted to ppm phosphorus using a calibration curve made from standard solutions of inorganic phosphorus.

The procedure was also carried out on the supernatant liquid from encapsulation, and was repeated omitting the enzyme (using buffer in place of it).

2.12.12 Estimation of encapsulated cortisol phosphate by a 'Cortipac' commercial assay kit (rat RBC)

The Cortipac SC.6 kit (Radiochemical Centre, Amersham) operates on the principle of competitive binding onto a limited substrate by radio-actively labelled (Se-75) cortisol with that in the sample, the nonbound labelled cortisol being measured by its γ-emission. The instructions supplied with the kit were followed.

No satisfactory results could be obtained, probably because (a) the kit is specific for cortisol not cortisol phosphate (b) materials in the cell haemolysate interfere with the binding process.

It is possible a method could be developed (involving hydrolysis and extraction) but instead other methods of measurement were used.

3 RESULTS

3.1 The anti-inflammatory effect of α_1AT on the rat carrageenan oedema test

3.1.1 α_1AT injected into the foot pad together with the carrageenan

Results are shown in Fig. 3.1. The mean volume of the (control) foot before carrageenan injection was 1.88 ml.

It was found that α_1AT injected into the foot pad did not have any anti-inflammatory effect, but was in fact irritant. The addition of α_1AT to the carrageenan gave a larger oedema in every case (both commercial α_1AT and that prepared for this project, both high and low doses). For the high dose, the increase was significant.

The commercial (Sigma) α_1AT appeared slightly less irritant than Pitt α_1AT (but it is also less active: see section 2.3).

α_1AT injected alone appeared slightly irritant, but the effect was not significantly different from injections of saline alone, when these were injected into normal rats.

Table 3.1 Abbreviations used on graphs

(further details in appropriate text sections)

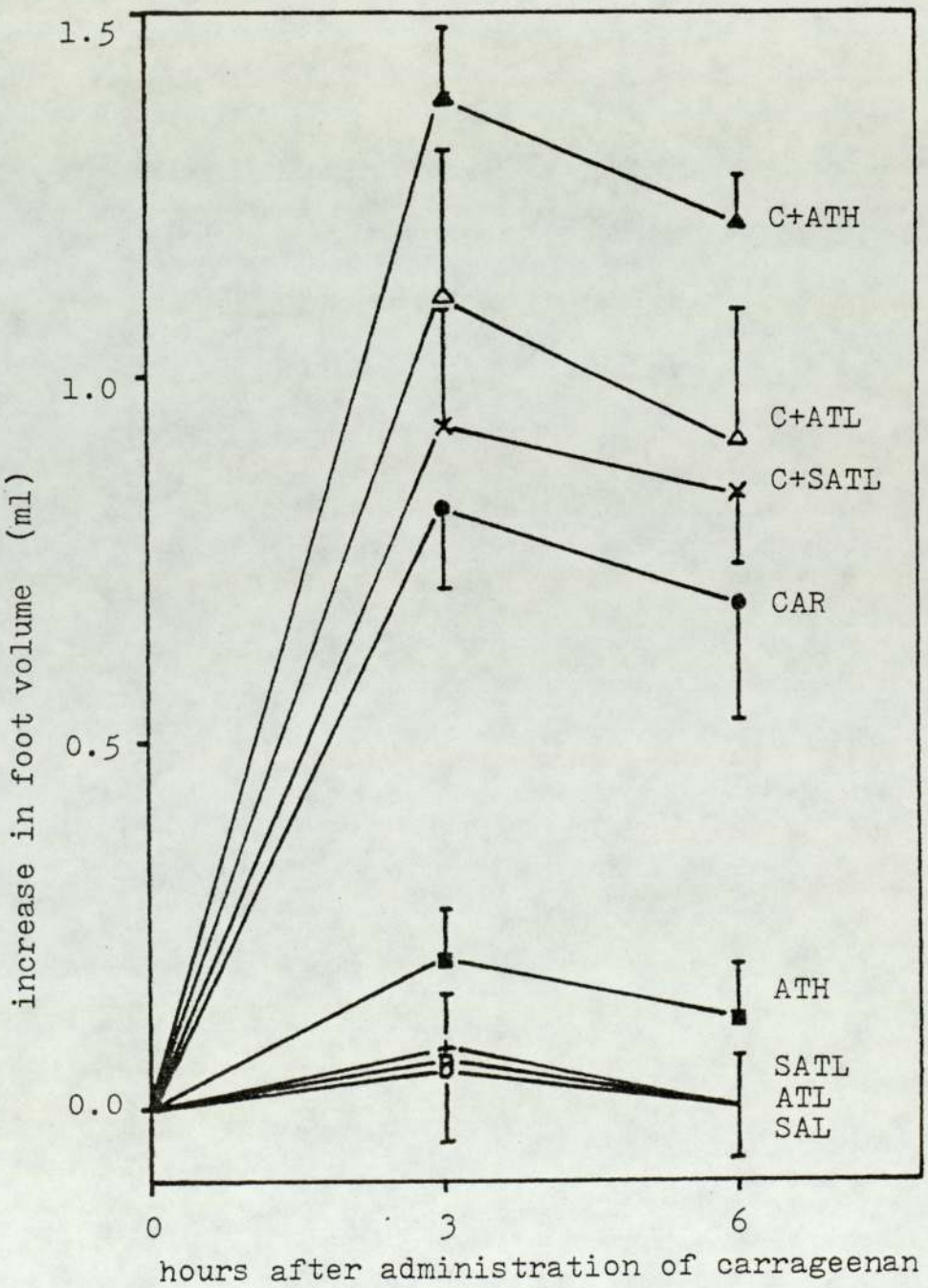
a	acute
c	chronic
AT	α_1AT
ATCPL	AT+CP low dose
ATH	AT high dose
ATL	AT low dose
ATLH	AT late high dose
C	carrageenan +
CAR	C alone
CP	cortisol phosphate
CPH	CP high dose
CPL	CP low dose
D	day
EAT	encapsulated AT
EATCPL	encapsulated AT+CP low dose
EATL	encapsulated AT low dose
ECPL	encapsulated CP low dose
EOM	encapsulated media (mixed blood)
EOO	encapsulated media (own blood)
SAL	saline
SAT	Sigma (commercial) AT
SATH	SAT high dose
SATL	SAT low dose

Asterisks or crosses denote statistically significant changes.

* = significant ($P < 0.05$), ** = highly significant ($P < 0.01$)

*** = very highly significant ($P < 0.001$) by Student's t-test.

Fig 3.1 Increase in foot volume of rats injected with α_1AT together with carrageenan into the foot pad



3.1.2 α_1AT administered subcutaneously

Results are shown in Fig. 3.2. The mean volume of the control foot before injection of carrageenan was 1.89 ml.

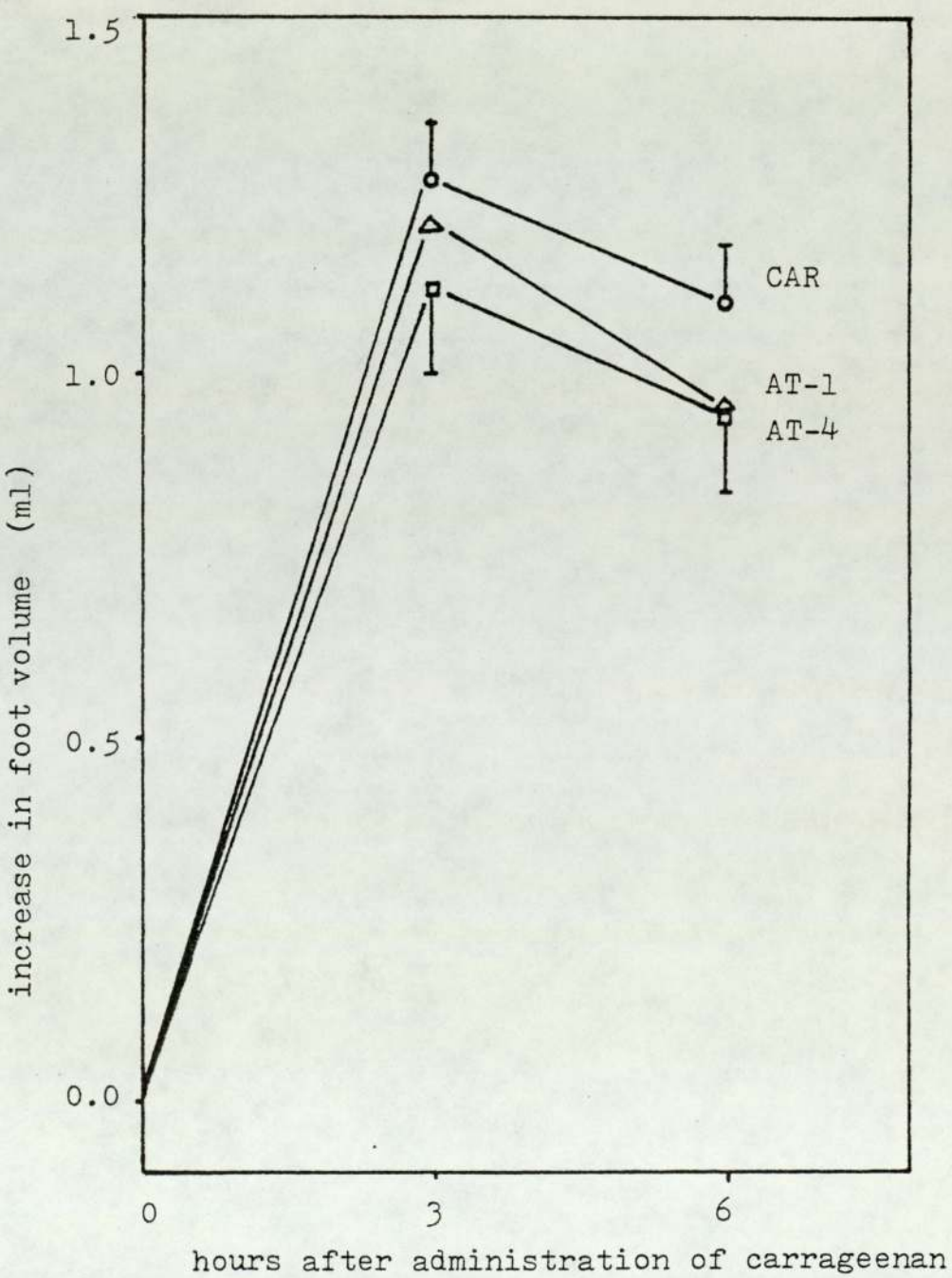
It was found that α_1AT by sub-cutaneous injection had no anti-inflammatory effect on this model, whether administered 6 hours before carrageenan injection or daily for 4 days prior to carrageenan. ($P > 0.05$)

CAR indicates carrageenan only (with subcutaneous injections of saline only on 4 days before)

AT-1 indicates a single dose of α_1AT at 6 hours before administration of carrageenan.

AT-4 indicates 4 daily doses of α_1AT prior to administration of carrageenan.

Fig 3.2 Increase in foot volume of rats injected with carrageenan into the foot pad, with and without prior subcutaneous injections of α_1AT



3.2 The anti-inflammatory effect of α_1AT on the rat adjuvant arthritis test

Results are shown in Fig. 3.3 (injected foot) and Fig. 3.4 (non-injected foot). The mean volume of the control foot before adjuvant injection was 1.62 ml.

It was found that daily injections of α_1AT (s.c., 10 mg per rat per day, starting the day before the adjuvant injection) had considerable anti-inflammatory action, both in the acute and chronic phases of the disease. The effect was comparable to a similar series of injections of cortisol phosphate (1 mg cortisol per rat per day).

The spread of the disease into the non-injected foot was also inhibited by α_1AT. In this case it was more effective than cortisol phosphate.

Fig 3.3 Increase in foot volume of rats with adjuvant arthritis, with and without daily subcutaneous doses of α_1AT or cortisol phosphate. (Injected foot)

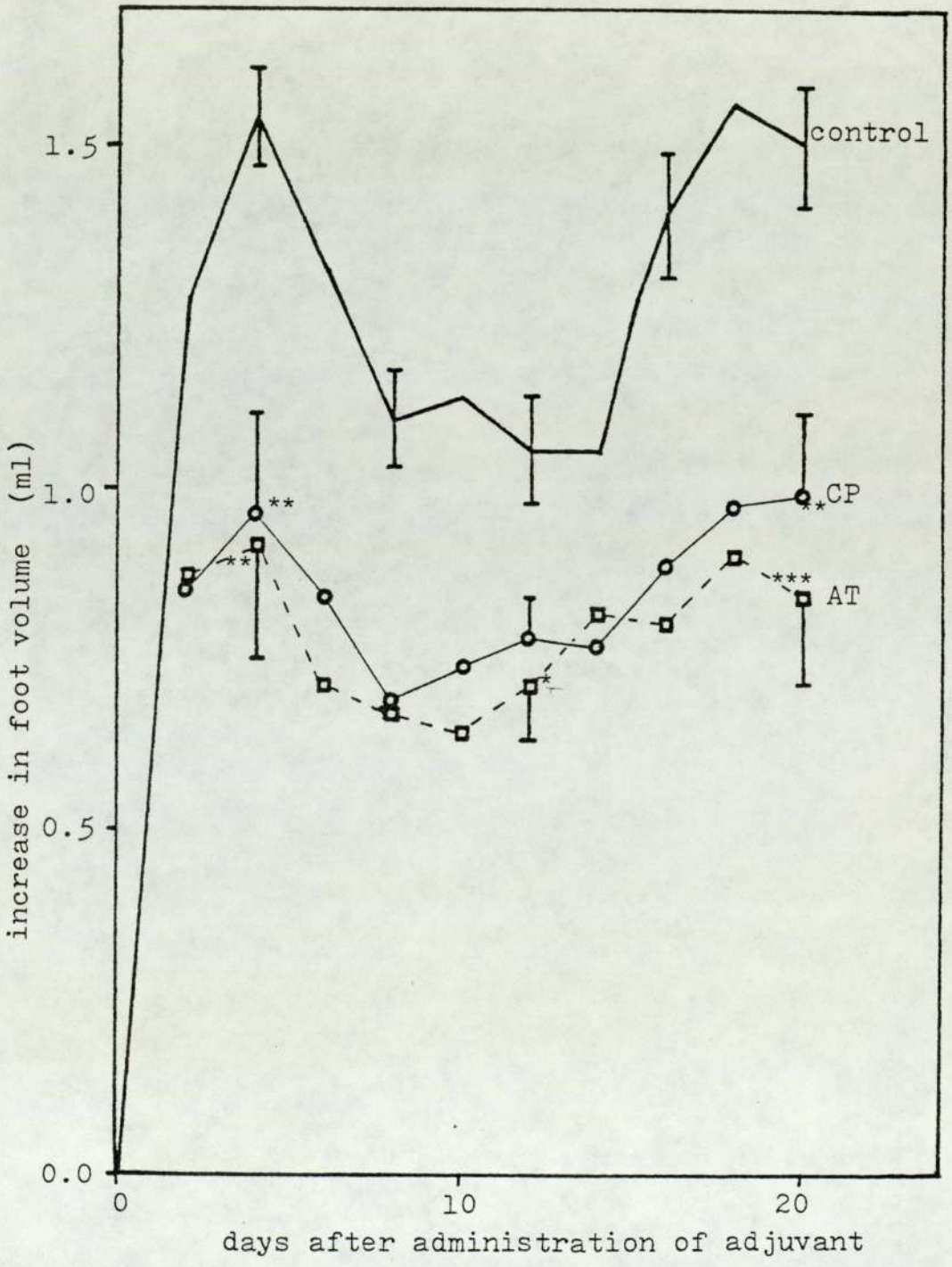
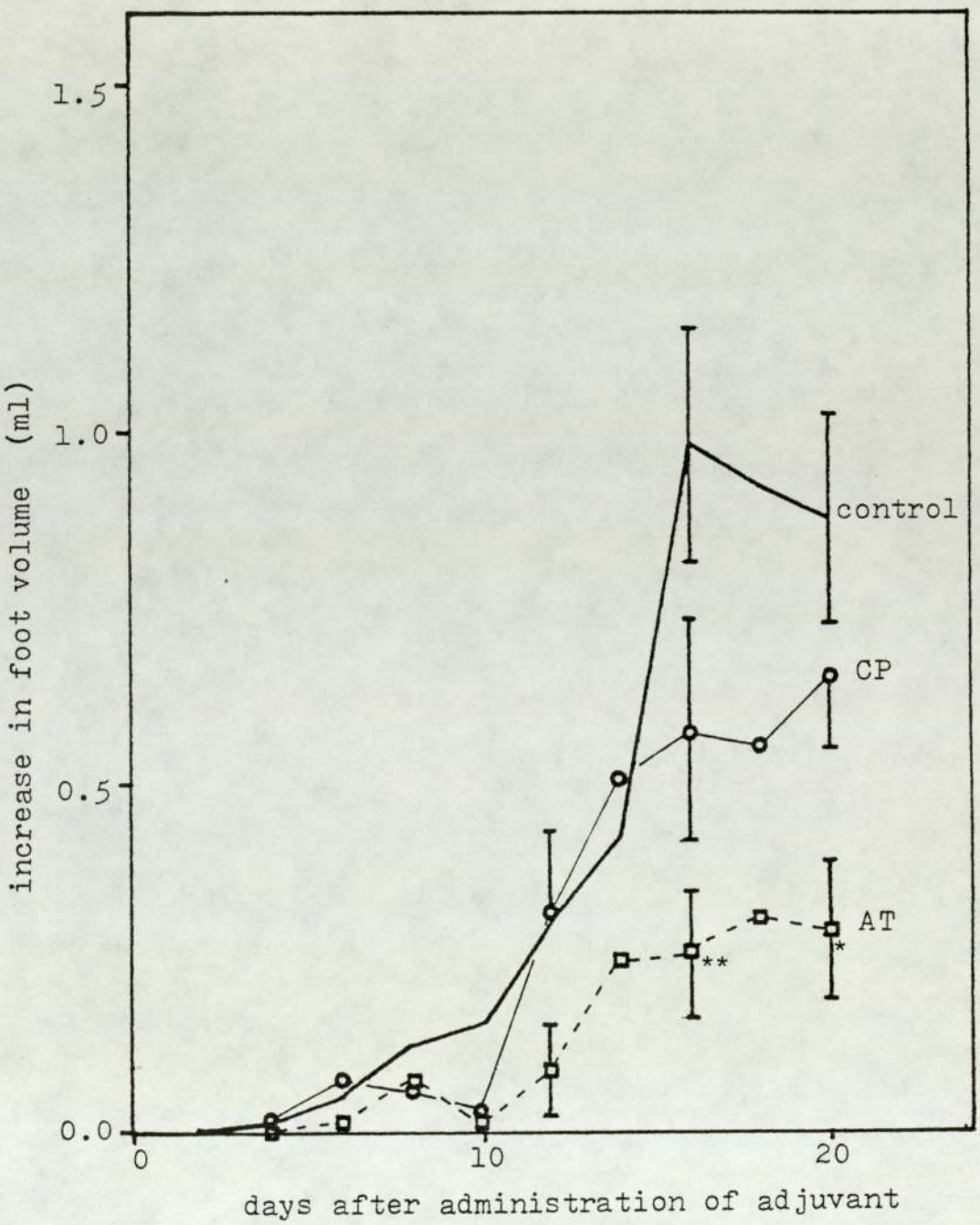


Fig 3.4 Increase in foot volume of rats with adjuvant arthritis, with and without daily subcutaneous doses of α_1AT or cortisol phosphate. (Non-injected foot)



3.3 The anti-inflammatory effect of α_1AT on rabbit monoarticular arthritis

The effect of intra-articular administration of α_1AT was investigated in conjunction with tests on the erythrocyte encapsulation system as an intra-articular means of administration. The results are most meaningful when viewed as part of the encapsulation programme, and are therefore given in section 3.6 and Fig. 3.17.

Briefly, α_1AT did not prove to have any anti-inflammatory properties when administered in this way, whether in solution or encapsulated. However the administration of an encapsulated mixture of cortisol phosphate and α_1AT proved more successful than the encapsulated cortisol phosphate preparation.

3.4 The stimulatory action of various steroidal anti-inflammatory drugs on plasma α_1AT and α_2M levels in the rat

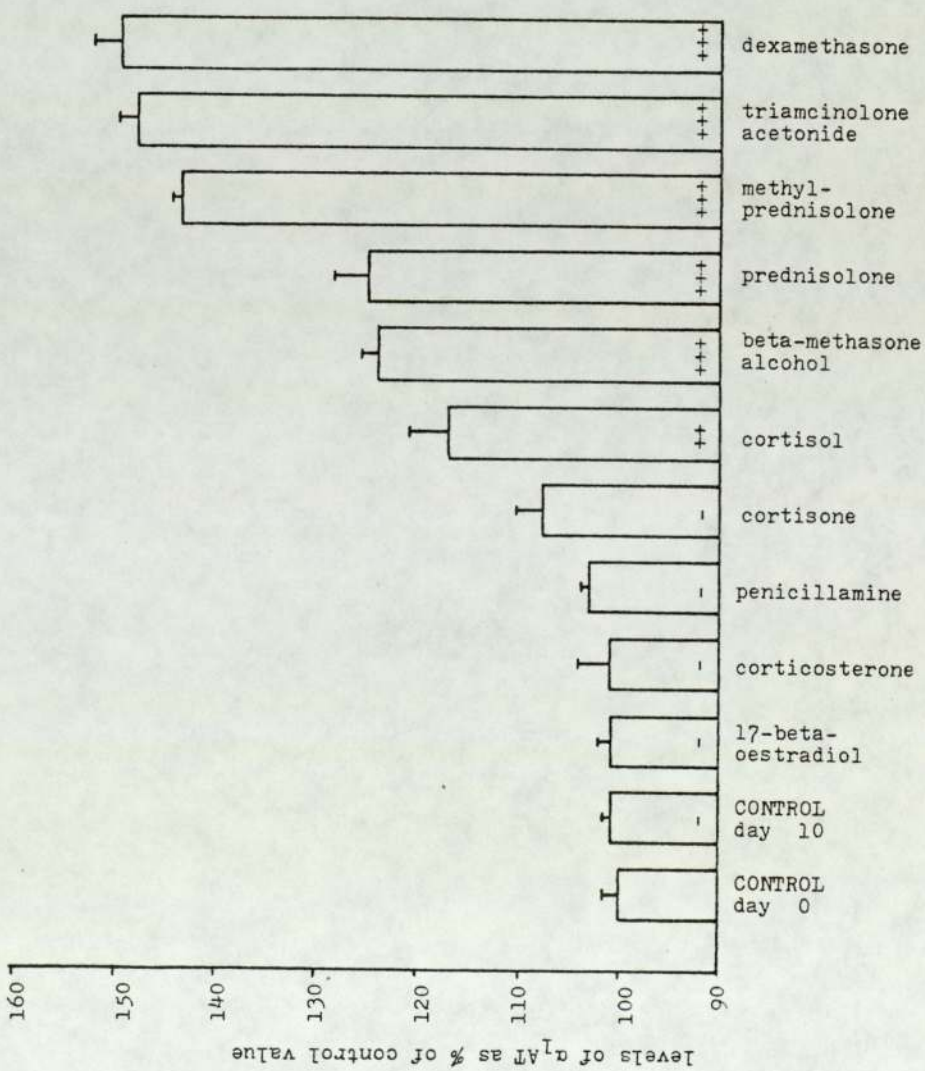
The drugs were administered daily for 10 days (1 mg per day per rat ≡ 5mg kg^{-1}) as described in Section 2.8.1.

3.4.1 Levels of α_1AT

Results are shown in Fig. 3.5. The mean TIC value for the control group at day 0 was 3.24 μ mol min^{-1} ml^{-1}.

The synthetic anti-inflammatory steroids produced the highest response. Levels of plasma α_1AT were elevated by a highly significant amount by the drugs hydrocortisone, betamethasone, prednisolone, methylprednisolone, triamcinolone and dexamethasone.

Fig 3.5 Levels of α_1AT in the plasma of rats after treatments with various steroids at a dose 5mg kg^{-1}

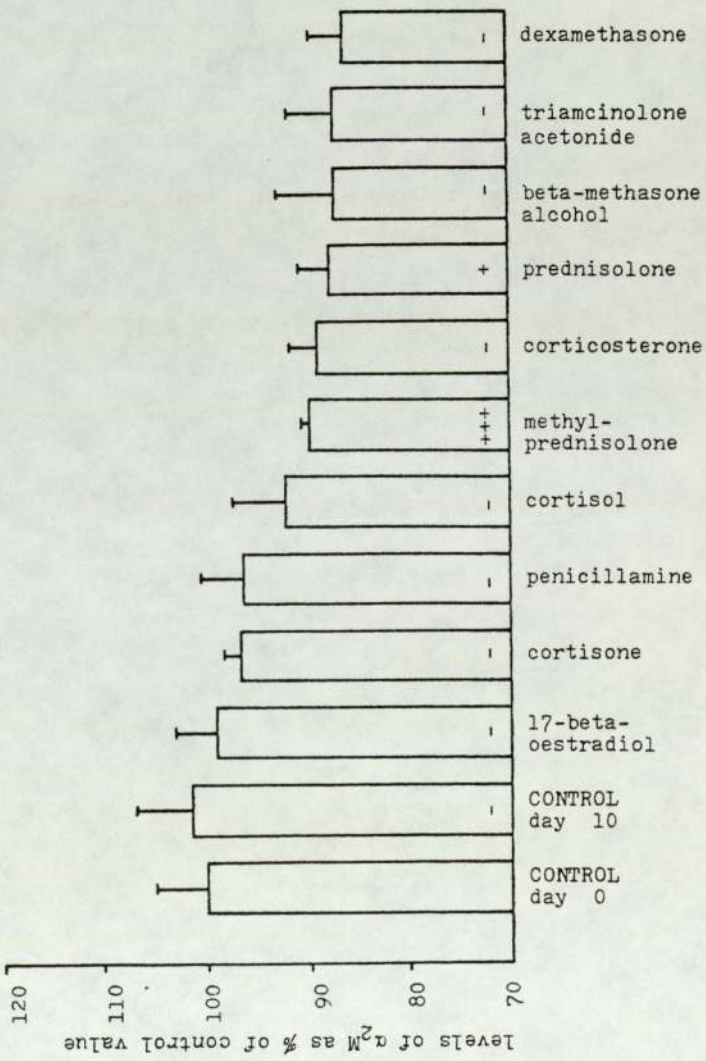


3.4.2 Levels of α_2M

Results are shown in Fig. 3.6. The mean TPE value for the control group at day 0 was equivalent to an absorbance of 0.274 at 400 nm.

There was a reduction in plasma α_2M in all the groups receiving drug treatment, the synthetic steroids producing the largest response. However, the change was significant only in the case of rats treated with prednisolone or methylprednisolone.

Fig 3.6 Levels of α_2M in the plasma of rats after treatments with various steroids at a dose 5mg kg^{-1}



3.5 The effect of verious steroids on plasma α_1AT levels and on the liver in the rat

In the first part of the experiment, groups received a variety of steroids at one dose level. Plasma α_1AT was measured.

As a result, three drugs were selected for further testing: cortisol, prednisolone and dexamethasone. They were administered at 3 different dose levels. α_1AT was again measured, as were the liver function tests of bilirubin, GOT and total protein.

The liver histology was examined for the group treated with dexamethasone.

3.5.1 Plasma levels of α_1AT

Results are shown in Fig. 3.7. The mean α_1AT level in the control group on day 0 was 3.463 μmolmin^{-1} ml^{-1} of trypsin inhibitory capacity.

A dose response was shown for all three steroids in elevating α_1AT levels. The elevation was significant for all dose levels of all drugs. Dexamethasone was the most potent in this respect.

3.5.2 Liver function tests

Results of measurements of plasma levels of bilirubin are shown in Fig. 3.8.

Results of measurements of plasma levels of GOT are shown in Fig. 3.9.

Results of measurements of plasma total protein are shown in Fig. 3.10.

These results suggest that the three steroids did affect the liver functions examined, and that these functions were also dose responsive.

Fig 3.7 Levels of α_1AT in the plasma of rats after 7 daily injections of steroids at 3 different doses

▲ = dose 5mg kg^{-1} from Fig 3.5

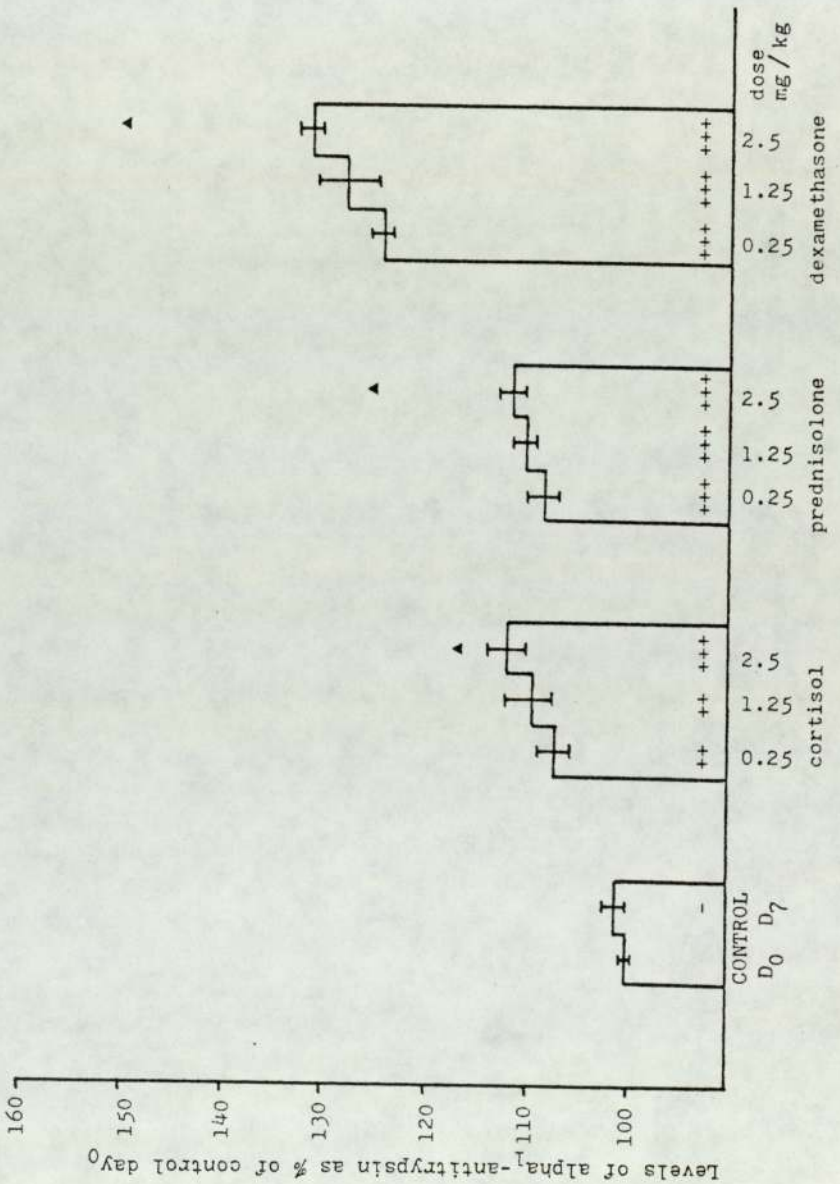


Fig 3.8 Levels of bilirubin in the plasma of rats after 7 daily injections of steroids at 3 different doses —— total bilirubin --- direct bilirubin

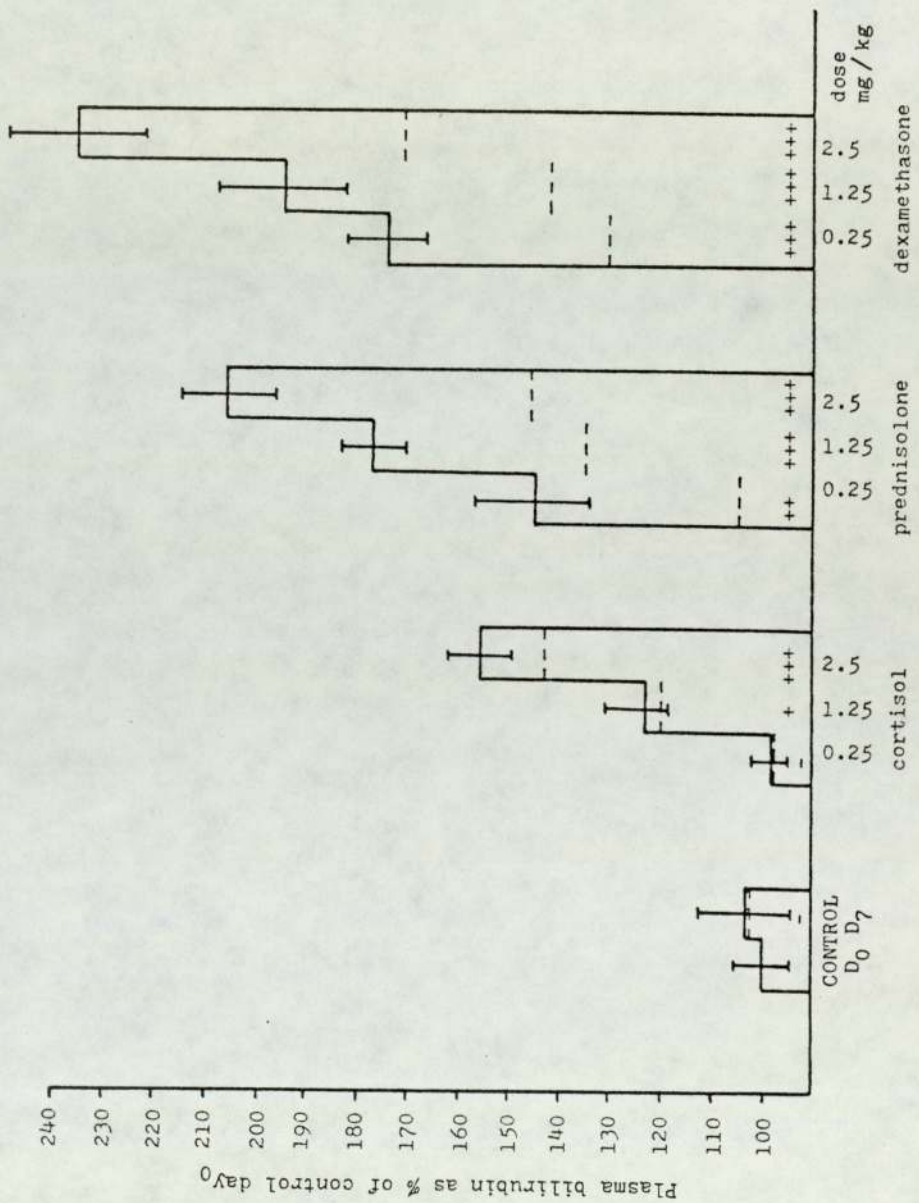


Fig 3.9 Levels of GOT in the plasma of rats after 7 daily injections of steroids at 3 different doses

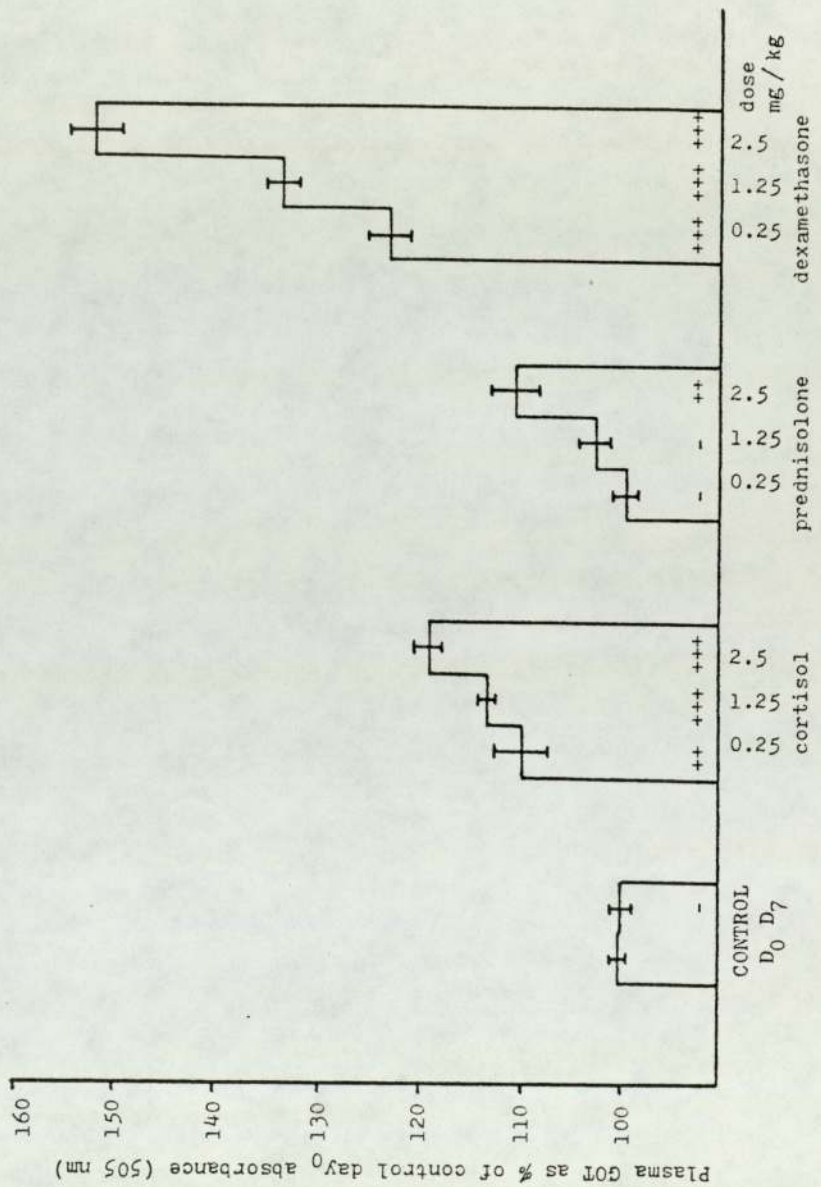
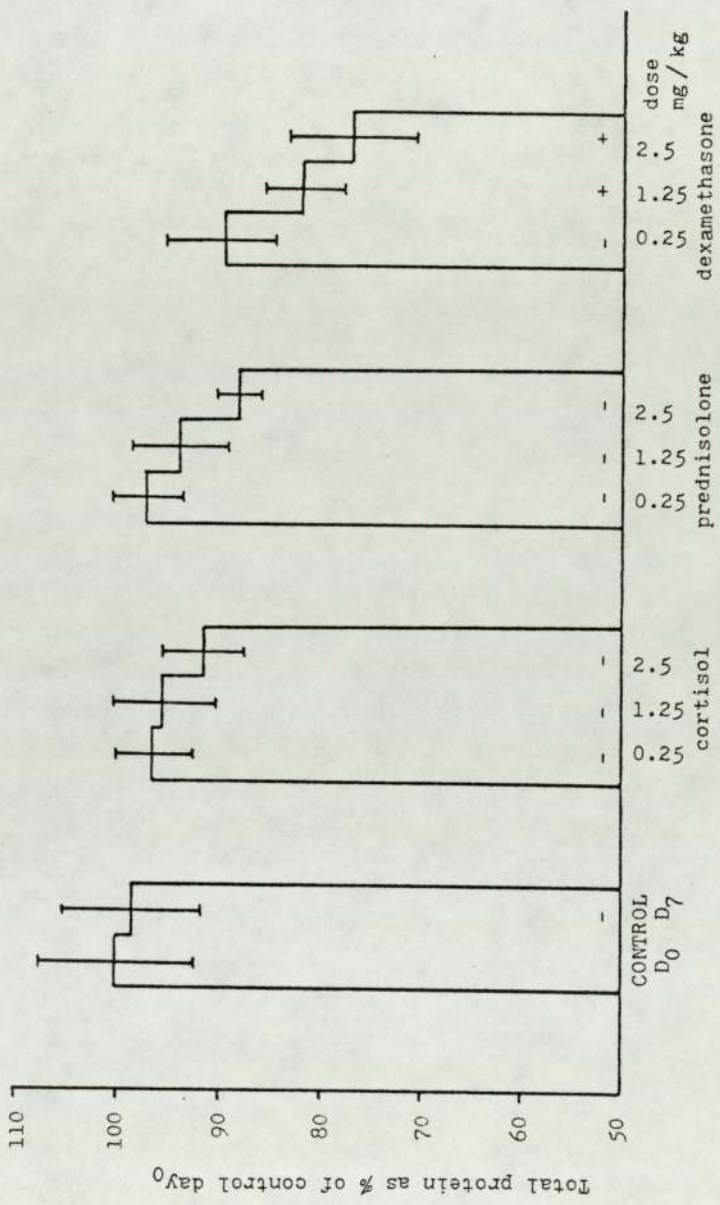


Fig 3.10 Levels of total protein in the plasma of rats after 7 daily injections of steroids at 3 different doses



3.5.3 Histology of the livers

Examination of the livers of the dexamethasone treated group showed an elevation of hepatic lipid. The liver cells were vacuolated and considerable quantities of fat were demonstrated by Sudan Black stain. The effect appeared dose reponsive.

The typical appearance of sections from livers in each dose group are shown in Figs. 3.11, 3.12, 3.13.

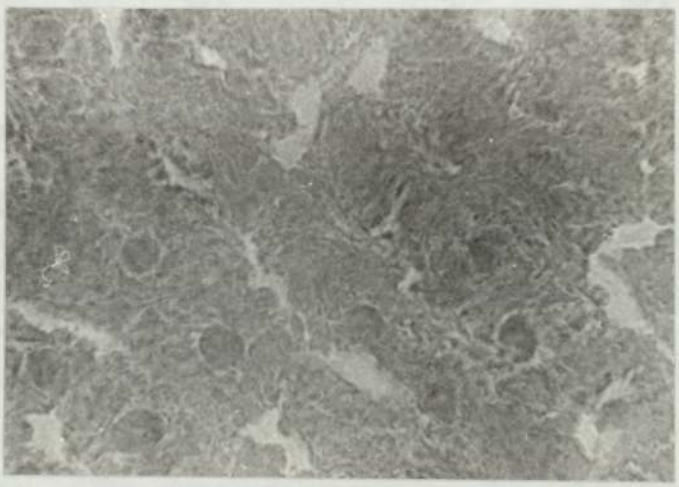
Fig. 3.11 shows (A) liver from control group, i.e. normal and (B) liver from group treated with 2.5 mg dexamethasone per kg body weight. The magnification is 400 x and the stain is Haematoxylin and Eosin. Vacuolation of the cells is visible, as are droplets (unstained) of fat within the cells.

Fig. 3.12 shows (A) normal liver and (B) liver from group treated with DMSO/arachis oil vehicle only. The magnification is 400 x and the stain is Sudan Black. A few Sudan-positive particles are visible in the normal liver. There are slightly more in the treated one.

Fig. 3.13 shows livers after dexamethasone treatment at (A) 0.25 (B) 1.25 (C) 2.5 mg per kg body weight. Many Sudan-positive particles are present, their numbers being greater for the higher drug doses.

Fig 3.11 Histology of rat liver
(Haematoxylin + Eosin stain, 400 x)

(A) Normal liver



(B) After 2.5 mg/kg dexamethasone

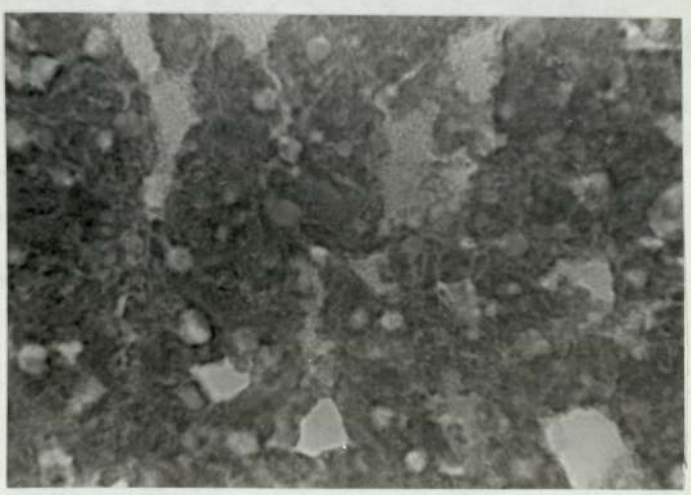
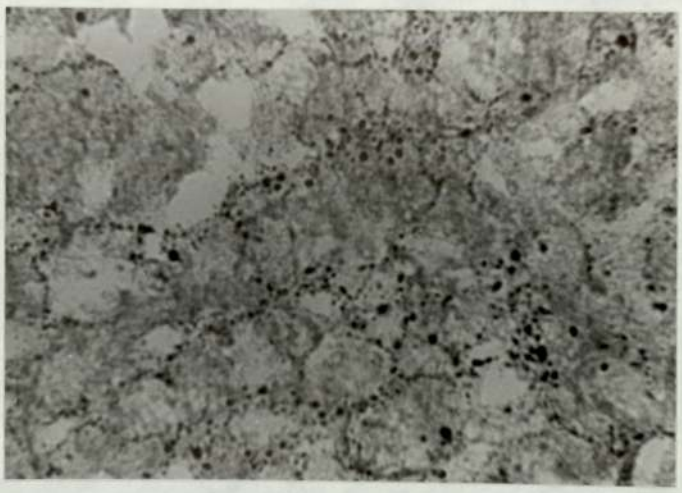


Fig 3.12 Histology of rat liver
(Sudan black stain, 400 x)

(A) Normal liver



(B) After dosing with DMSO/arachis oil vehicle only

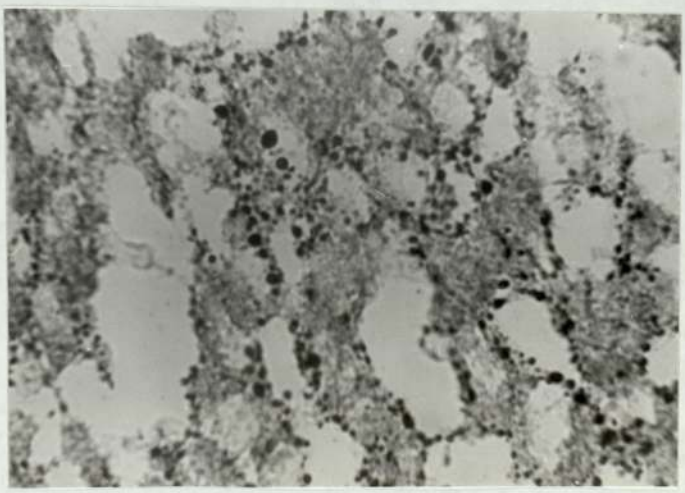
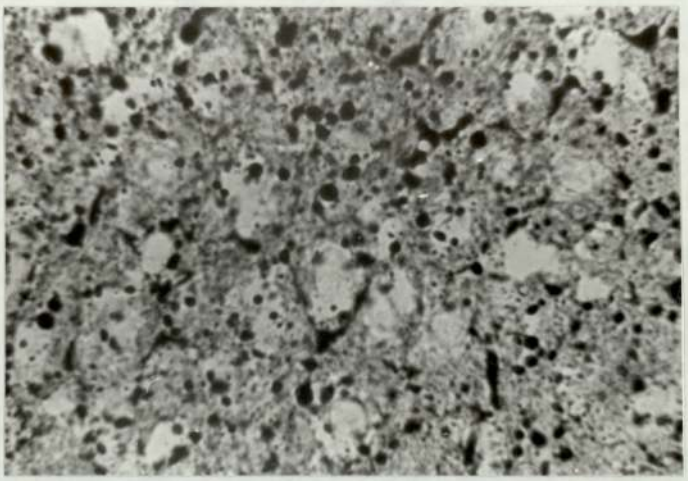
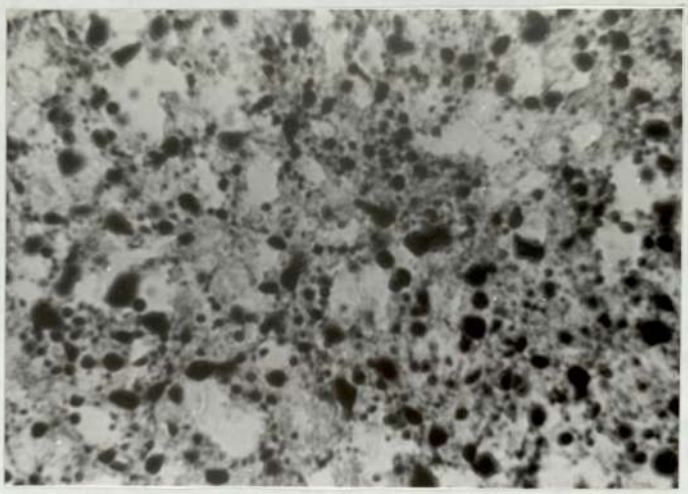


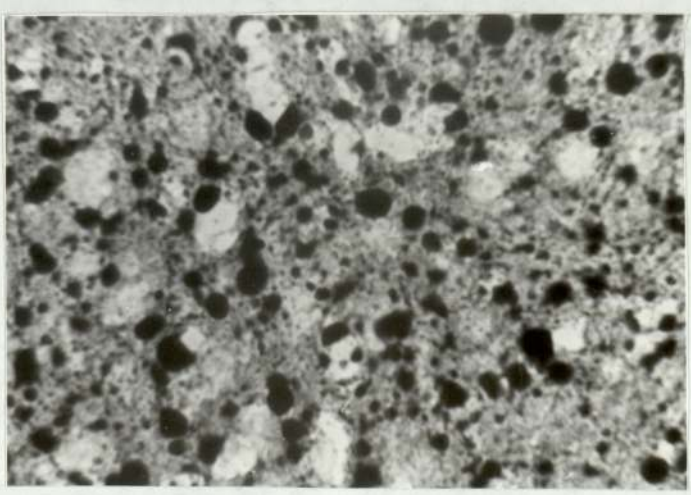
Fig 3.13 Histology of rat liver
(Sudan black stain, 400 x)
following dexamethasone treatment



(A) 0.25 mg / kg



(B) 1.25 mg / kg



(C) 2.5 mg / kg

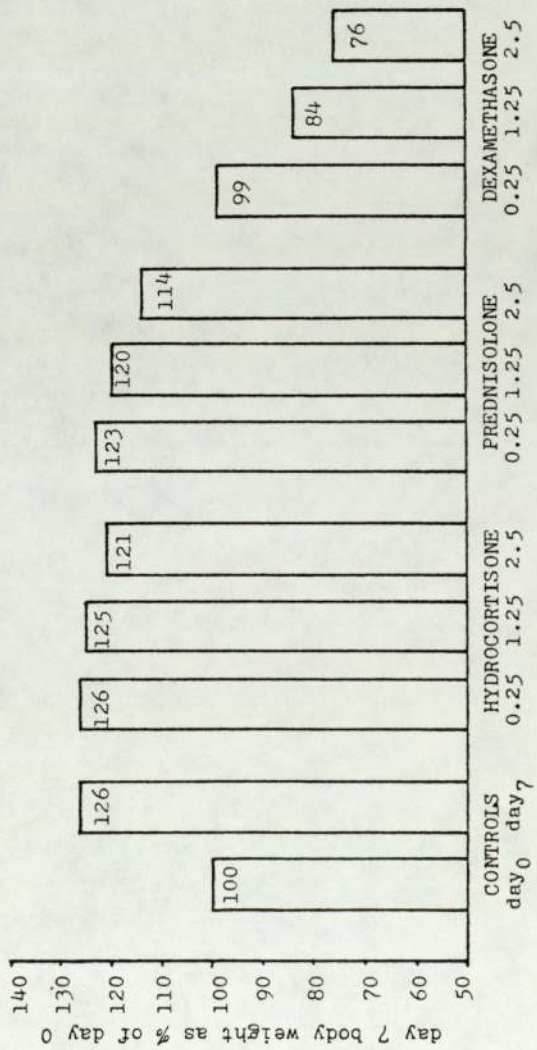
3.5.4 The effect of administered steroids on rat weight

The body weights at day 7 are presented as percentages of the weights at day 0 in Fig. 3.14.

The weight of the control group increased by 26% (due to normal growth). The weights of rats treated with hydrocortisone and prednisolone increased by a smaller amount, but actual and considerable weight losses occurred in the dexamethasone group.

These effects were dose responsive.

Fig 3.14 The effect of administered steroids on rat body weight (control day 0 = 100 %)



3.6 The effect of intra-articular administration of α_1AT and cortisol phosphate (free and encapsulated) on rabbit monoarticular arthritis

3.6.1 Joint diameter measurements

All the groups received the same treatment until day 12, when administration of preparations began. The graphs of joint diameter up to this point therefore represent only the progress of the untreated disease.

For convenience, the day 12 diameter has been used as the common reference point, and the graphs adjusted up or down accordingly so that all groups match with control at this point. The progress of the control group (untreated disease) is shown on all graphs for reference. The standard error of the mean for the control group was typically 3%. This has been omitted from the graphs for the sake of clarity.

Fig. 3.15 shows the sham treatments (encapsulated media only, EOO own blood, EOM mixed blood) which were always within 1 - 2% of the control group, i.e. indistiguishable. It also shows the treatments which were most successful: a combined administration of cortisol phosphate and α_1AT. There was no significant difference between the encapsulated and the free forms of this preparation.

Fig. 3.16 shows the effect of the cortisol phosphate (not combined with α_1AT) treatments. The effect of the low dose, high dose and encapsulated low dose are seen to be a very similar improvement over the control.

Fig. 3.17 shows the effect of α_1AT (not combined) treatments. Again, the effects of high, low and encapsulated low doses are seen to be very similar, and in fact little different from the control.

Overall, it seems that the encapsulated preparations do not show any advantage for this particular method of treatment of this model. α_1AT itself does not appear to be an effective treatment, but has a synergistic effect with cortisol phosphate giving a noticeable improvement.

The measurements of joint diameter and flexibility of the control group and the experimental groups were compared by A. Student's t-test for days 14, 36 and 60. Significant differences in joint diameter from the control group were found as follows:

Day 14 - EATCPL; ATCPL CPL. Day 36 - CPH; EATCPL; CPL; ATCPL.

Day 60 - EATCPL, ATCPL.

Fig 3.15 The effect of intra-articular administration of treatments on joint diameter measurements of rabbits with monoarticular arthritis

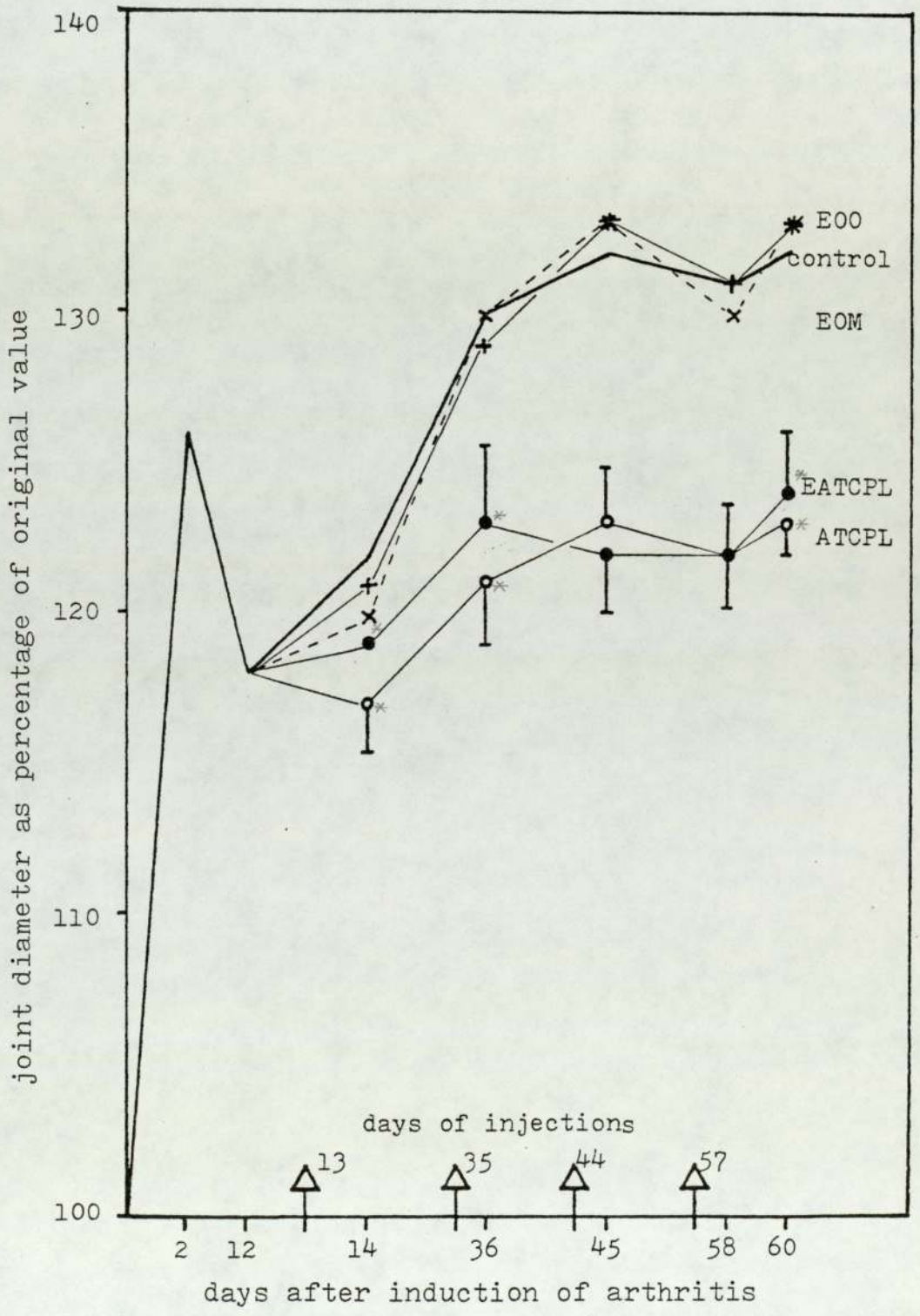


Fig 3.16 The effect of intra-articular administration of treatments on joint diameter measurements of rabbits with monoarticular arthritis

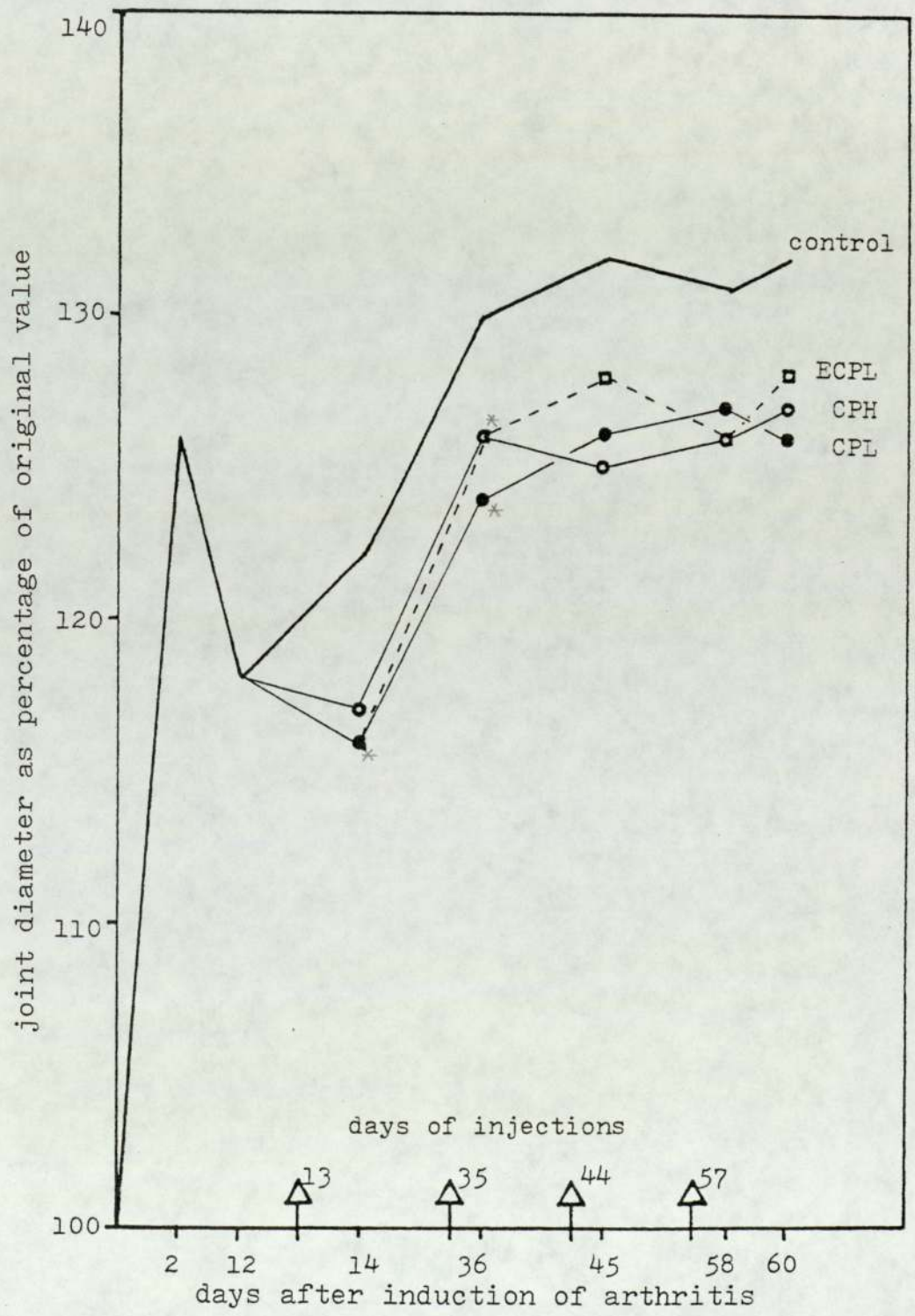
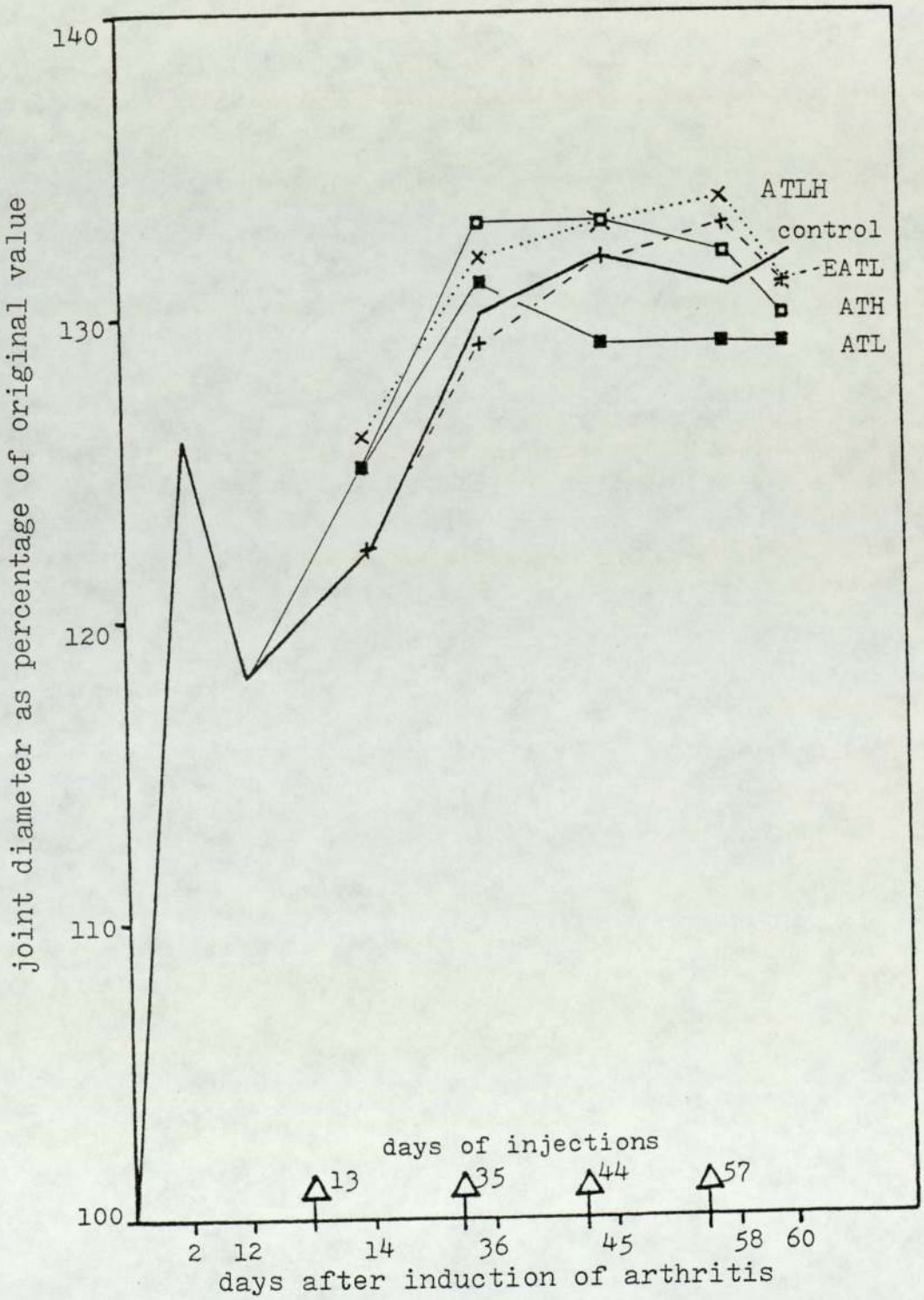


Fig 3.17 The effect of intra-articular administration of treatments on joint diameter measurements of rabbits with monoarticular arthritis



3.6.2 Joint angle measurements

These measurements were made on the same animals along with the joint diameters in 3.6.1, and the results are presented in much the same way in Figs 3.18, 3.19 and 3.20. The joint flexibility on day 12 has been taken as the reference point (this was in fact typically a loss of 12° of movement compared with normal). Joint angles are presented thereafter as degrees compared with the maximum angle on day 12, and show a clear and progressive loss of flexibility. The control group is shown on each figure. The standard error of the mean was typically 12° (which is large, and due to the considerable variation between animals in response to the disease - see discussion).

Fig. 3.18 shows the sham treatments (indistinguishable from control) and the combined α_1AT + CP treatments (best of all treatments). Fig, 3.19 shows a lesser improvement due to cortisol phosphate treatments. Fig 3.20 shows the α_1AT treatments to be scattered about the control.

Once again, encapsulation shows no advantage, α_1AT cannot be considered a treatment but is synergistic with cortisol phosphate. Significant differences from control were found on Day 36 for CPL and ATCPL; on Day 60 for EATCPL and ATCPL. (Student's t-test as for joint diameter.)

Fig 3.18 The effect of intra-articular administration of treatments on joint angle measurements of rabbits with monoarticular arthritis

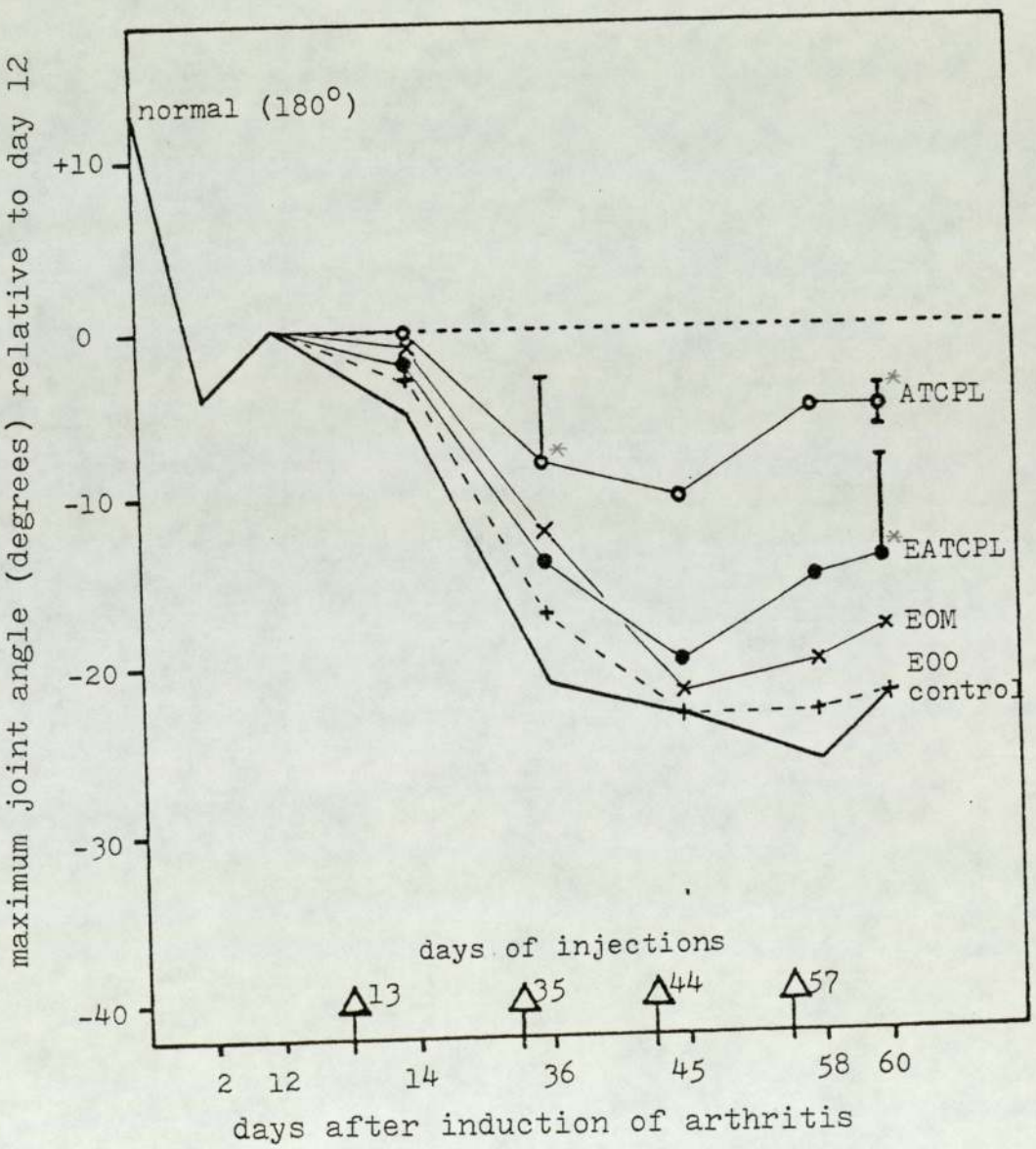


Fig 3.19 The effect of intra-articular administration of treatments on joint angle measurements of rabbits with monoarticular arthritis

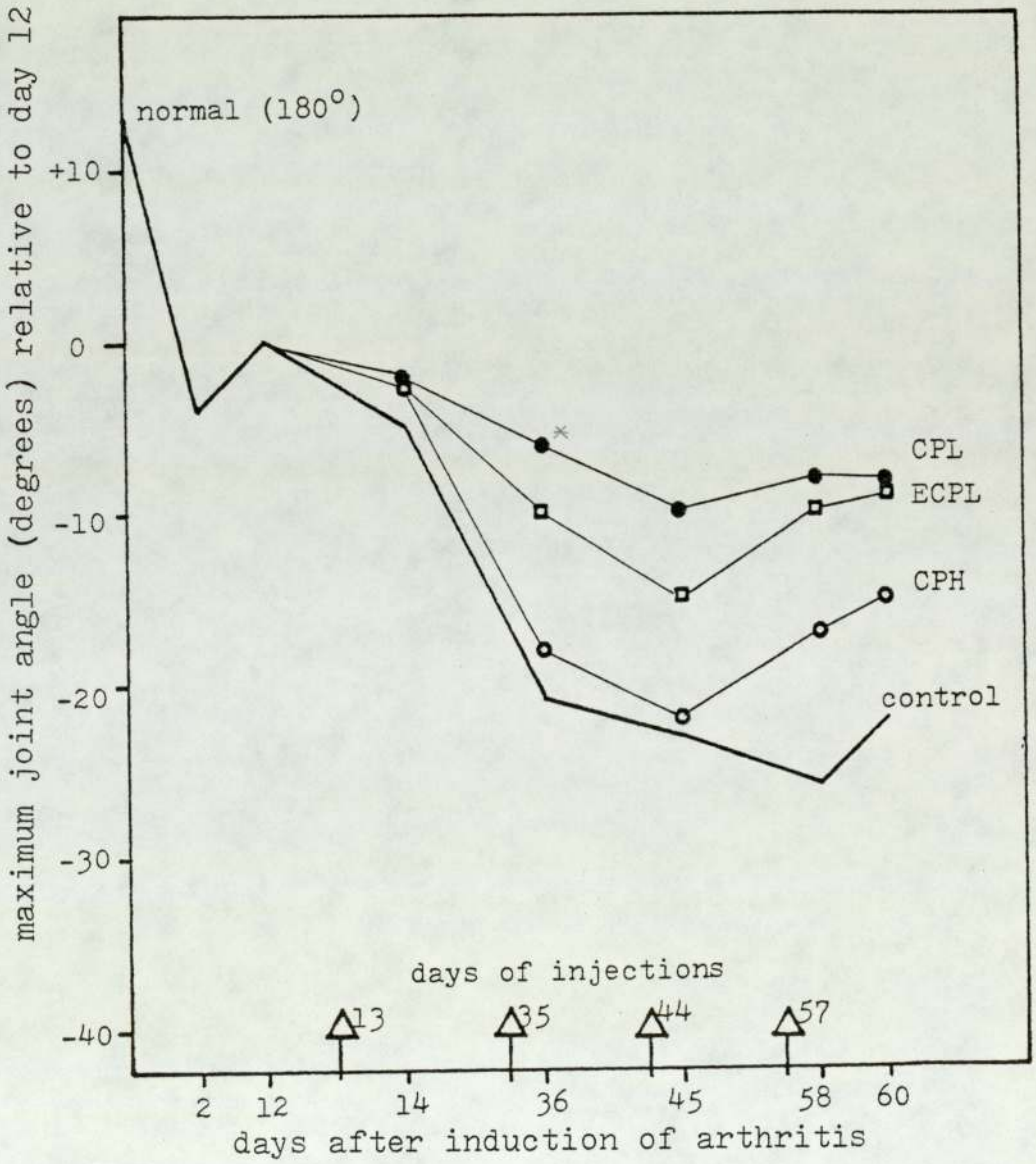
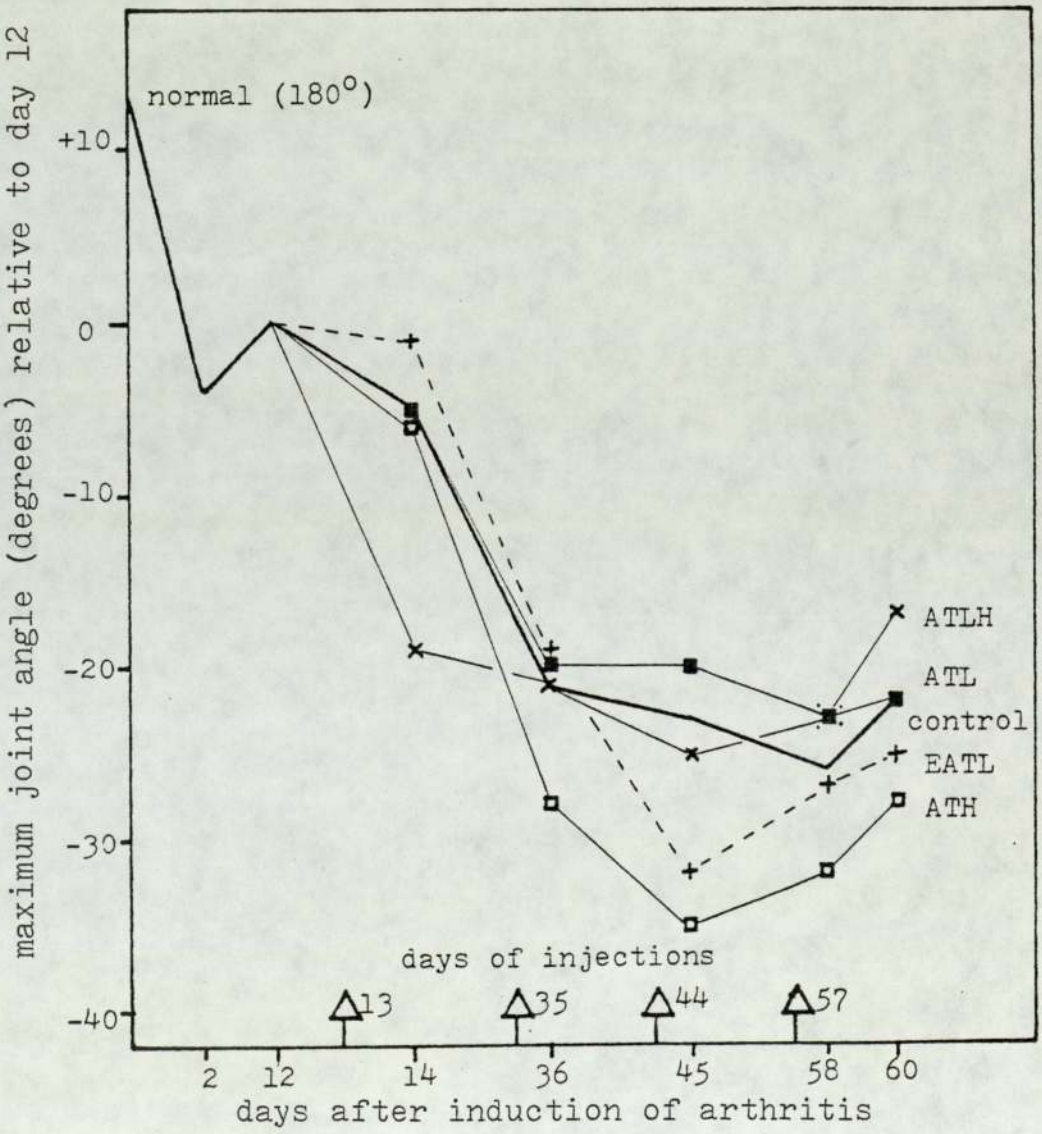


Fig 3.20 The effect of intra-articular administration of treatments on joint angle measurements of rabbits with monoarticular arthritis



3.6.3 Non-arthritic joint diameter and angle

Fig. 3.21 shows the percentage changes in joint diameter caused by treatment of non-arthritic joints. (A) refers to the unchallenged (i.e. left) knee of the arthritic rabbits receiving the treatments previously discussed. These joints received an injection of an equal amount of normal saline on each occasion the arthritic joint received drug injections. The diameter increased by a maximum of 5% more typically 3%. No progressive increase was observed (by day 60 the increase was of the order of 2%.)

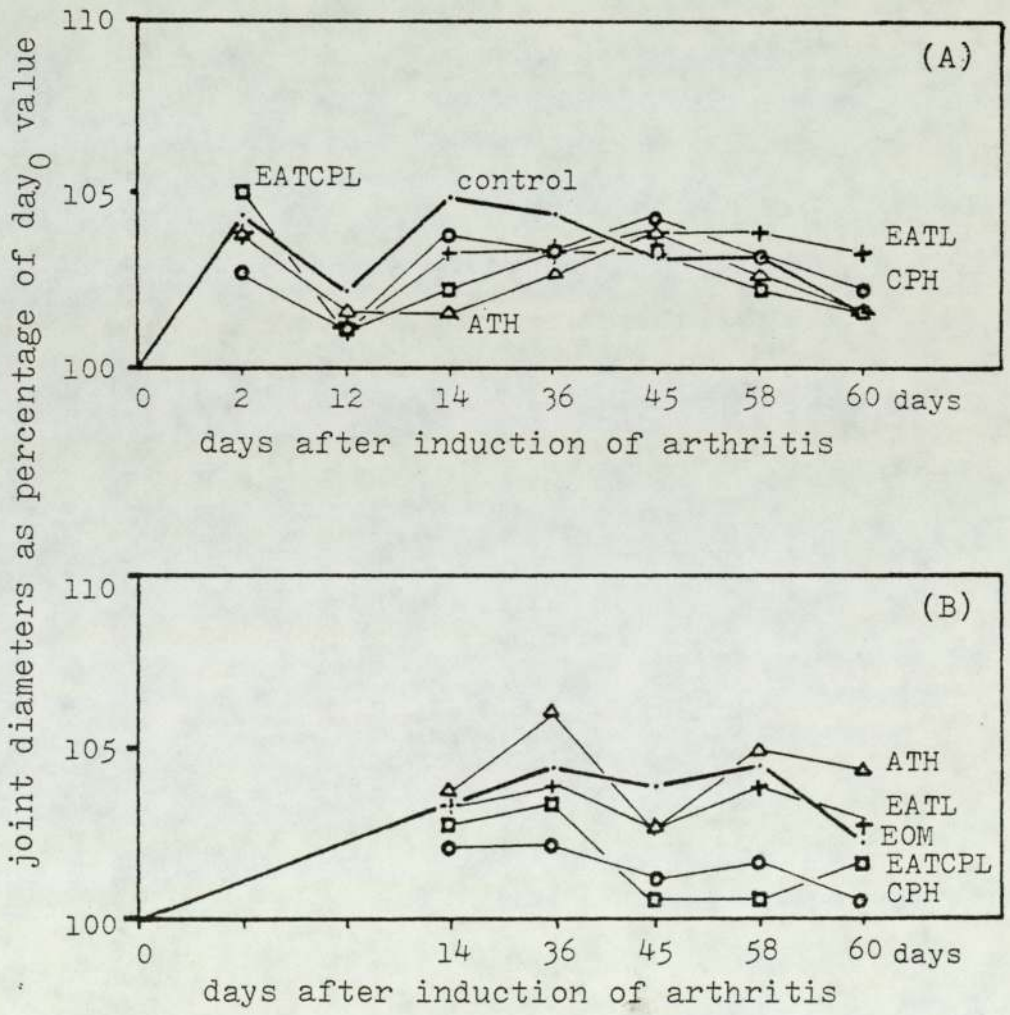
Fig 3.21 (B) shows the percentage change in joint diameters in normal non-arthritic rabbits receiving the same drug treatment as the arthritic groups (only 5 preparations were tested). The maximum increase observed was 6% for ATH, day 36. The overall average was 3% and no progressive increase was observed.

Joint angles for both experiments were almost always 180°, i.e. no loss of flexibility. The only exceptions were a few individuals (non-arthritic) which suffered a 5° loss of flexibility for two days after injections but always regained the normal state.

Fig 3.21 Joint diameter changes in non-arthritic rabbit knees under various treatments

(A) Unchallenged knee of monoarticular arthritic rabbits(saline injections only)

(B) Knee of non-arthritic rabbits (drug treatment as arthritic group)



3.6.4 Total protein in synovial fluid

Inflamed membranes can permit the entry of protein into the synovial fluid, thus an increase in total protein is a measure of the inflammatory process.

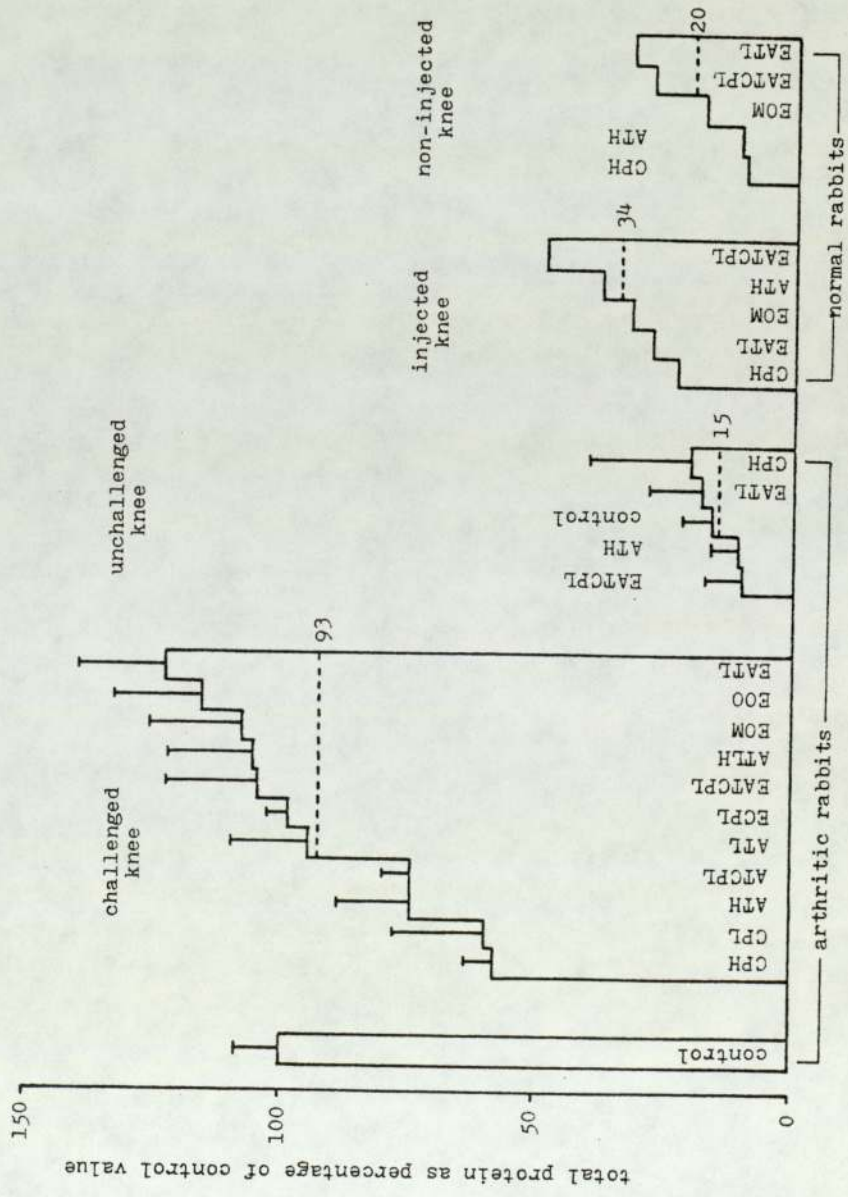
Fig. 3.22 shows total protein in the synovial fluid of groups of arthritic and non-arthritic rabbits receiving various treatments. The total protein found in the arthritic joints of an untreated control group was taken as 100 % and others expressed accordingly.

The elevation of protein in arthritis was clearly shown. The control group's arthritic knees had levels of total protein very much higher (highly significant, $P < 0.01$) than in the non-arthritic knees of any group (highest was CPH arthritic group).

Although differences were observed, only the cortisol phosphate treatments produced a significant reduction ($P < 0.05$). Group means are shown as dotted lines.

No standard errors are indicated for the non-arthritic rabbit groups, because these were in fact only pairs of animals. Taken as a whole, however, they confirm that the increased protein levels in the arthritic group were not due to the drug treatments by repeated intra-articular injections.

Fig 3.22 Levels of total protein in the synovial fluid of groups of arthritic and non-arthritic rabbits receiving various intra-articular treatments



3.6.5 Acid phosphatase in synovial fluid

Acid phosphatase is a lysosomal 'marker' enzyme, and its presence is indicative of cellular destruction.

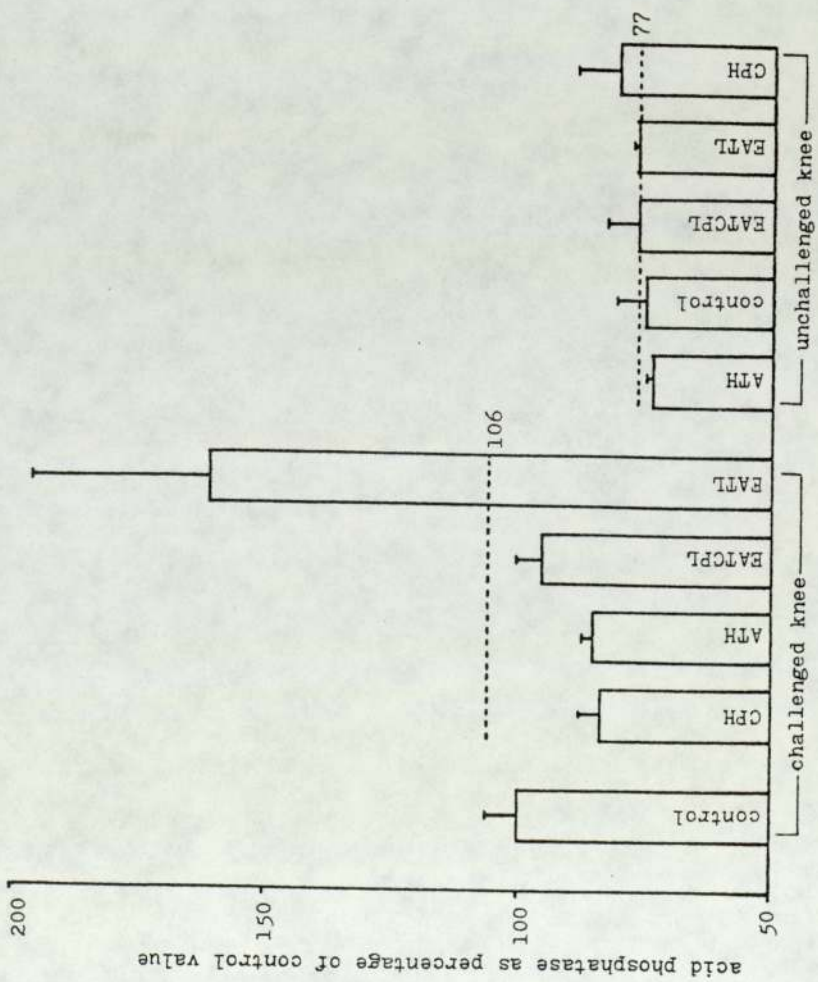
Fig. 3.23 represents measurements on the same groups and in the same form as for the total protein measurements (Section 3.6.4, Fig. 3.22). Unfortunately acid phosphatase is particularly labile in storage, and it was not practicable to perform the necessary tests on all the samples immediately after, so the results are less complete than for other parameters.

The arthritic joint in an untreated control group was again taken as 100%. (Dotted lines are group means.)

The elevation of acid phosphatase levels in arthritis was clearly shown. The control group's arthritic knees had levels very much higher (highly significant $P<0.01$) than in the non-arthritic knees of any group (highest was the CPH arthritic group).

Both CPH and ATH produced a significant lowering ($P<0.05$) of the enzyme level. EATCPL also lowered the enzyme level, but not significantly. There was a 60% increase of acid phosphatase level in EATL group. However, this had a high standard error and was not validly significant by t-test.

Fig 3.23 Levels of acid phosphatase in the synovial fluid of groups of arthritic [redacted] rabbits receiving various intra-articular treatments



3.6.6 Neutral protease in synovial fluid

The level of protease activity measured by neutral protease assay essentially represents the erosive potential of the synovial fluid.

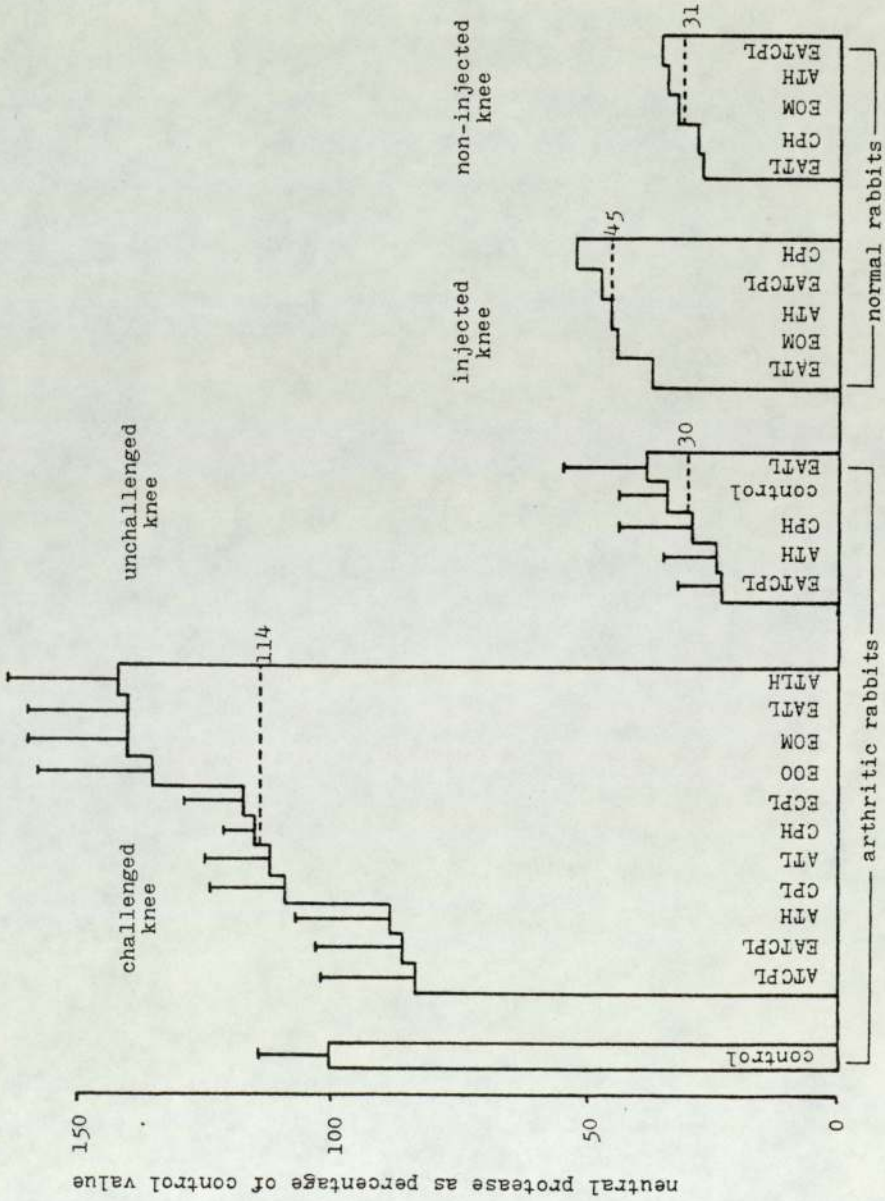
Fig. 3.24 shows the results for neutral protease in the same groups and in the same way as for total protein. The arthritic joint in the control group has again been taken as 100 %, and dotted lines are group means.

The elevation of neutral protease in arthritis was clearly shown. The control group's arthritic knees had levels of neutral protease very much higher ($P < 0.001$) than in the non-arthritic knees of any group.

The treatments ATCPL, EATCPL and ATH gave levels lower than control, although this was statistically insignificant. The treatments EOM, EATL and ATLH gave levels significantly higher than control ($P < 0.05$), in the first 3 instances presumably due to increased phagocytosis of extra cellular material resulting in increased lysosomal enzyme leakage. Injections of a high level of foreign protein (ATLH) had the same effect.

For assay, the samples from non-arthritic rabbit pairs were pooled. A slight increase in neutral protease was observed with all treatments compared with saline, but much less than the effect of arthritis.

Fig 3.24 Levels of neutral protease in the synovial fluid of groups of arthritic and non-arthritic rabbits receiving various intra-articular treatments



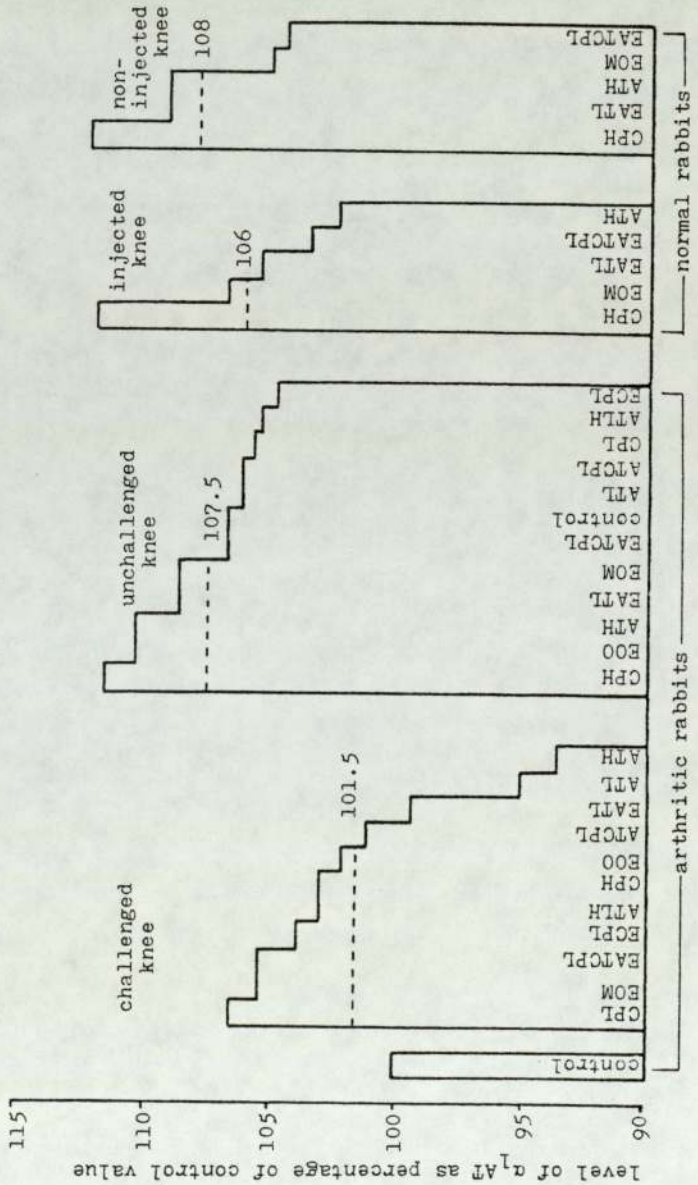
3.6.7 α_1AT in synovial fluid by TIC

Fig. 3.25 shows the results for α_1AT in the same groups and in the same way as for total protein. The arthritic joint in the control group has again been taken as 100%. The mean value of the four sets of results is shown by the dotted lines.

It should be noted that the assay method responds to active α_1AT, not to inactive complexes with proteases. The amount of active α_1AT was in fact found to be least for arthritis treated with a high dose of α_1AT. This result is in agreement with the lack of anti-inflammatory activity of α_1AT measured by other parameters.

Overall, it was clearly shown that lower levels of active α_1AT were a feature of arthritis. There was very little difference between the joints of the non-arthritic rabbits (i.e. the drugs were similar to saline in their effect). The non-diseased joint of the rabbits with mono-articular arthritis was generally similar to the non-arthritic rabbits. The main exception was the treatment with a high dose of cortisol phosphate. This produced higher levels of free α_1AT in both joints for both arthritic and non-arthritic animals. (Samples were pooled for assay, so standard deviations are not given.)

Fig 3.25 Levels of α_1-antitrypsin in the synovial fluid of groups of arthritic and non-arthritic rabbits receiving various intra-articular treatments



3.6.8 Macroscopic examination of dissected rabbit joints for visible destruction (clinical score)

The overall score (maximum possible = 99) for the different treatments was as follows:

untreated normal rabbits	0
treated normal non-arthritic rabbits	3
ATCPL treated arthritic rabbits	35
CPH " " "	43
CPL " " "	43
EATCPL " " "	45
ECPL " " "	55
untreated arthritic rabbits (control-1)	63
EOM treated arthritic rabbits	63
ATH " " "	65
untreated arthritic rabbits (control-2)	67
EATL treated arthritic rabbits	67
EOO " " "	68
ATLH " " "	68
ATL " " "	71

The non-zero score for treated normal rabbits was due to a little discoloration due to haemoglobin released from the encapsulated preparation.

The clinical score is not an exact measure, but the figures may be generally interpreted as follows:

Unsuccessful treatment (not significantly different from untreated arthritis) - EOM, ATH, EATL, EOO, ATLH, ATL.

Slight improvement - ECPL.

Definite reduction in severity of disease - CPH, CPL, EATCPL.

Greatest effect (from severe to mild disease) - ATCPL.

A selection of photographs is given as Figs 3.26 - 3.39 These were taken from the 80 used for the clinical score, chosen to illustrate the effects which were observed in each group. These are explained thus:

3.26 : ATH treated normal non-arthritic animal. The joint appears perfectly normal.

3.27 : EATCPL treated normal non-arthritic animal. The joint appears normal, except for a slight discoloration of the synovial membrane (due to haemoglobin products from the encapsulation) which is not actually pathological.

3.28 : ATCPL treated arthritis. The mild arthritis is shown only in a slight thickening of the synovial attachment, and a little hypertrophy of the synovial membrane.

3.29 : CPH treated arthritis. As 3.28.

3.30 : CPL treated arthritis. As 3.28 plus mild pannus on one side of the femoral condyle.

3.31 : EATCPL treated arthritis. The joint is discoloured by haemoglobin from the encapsulated preparation. More extensive pannus, and synovial space obliterated.

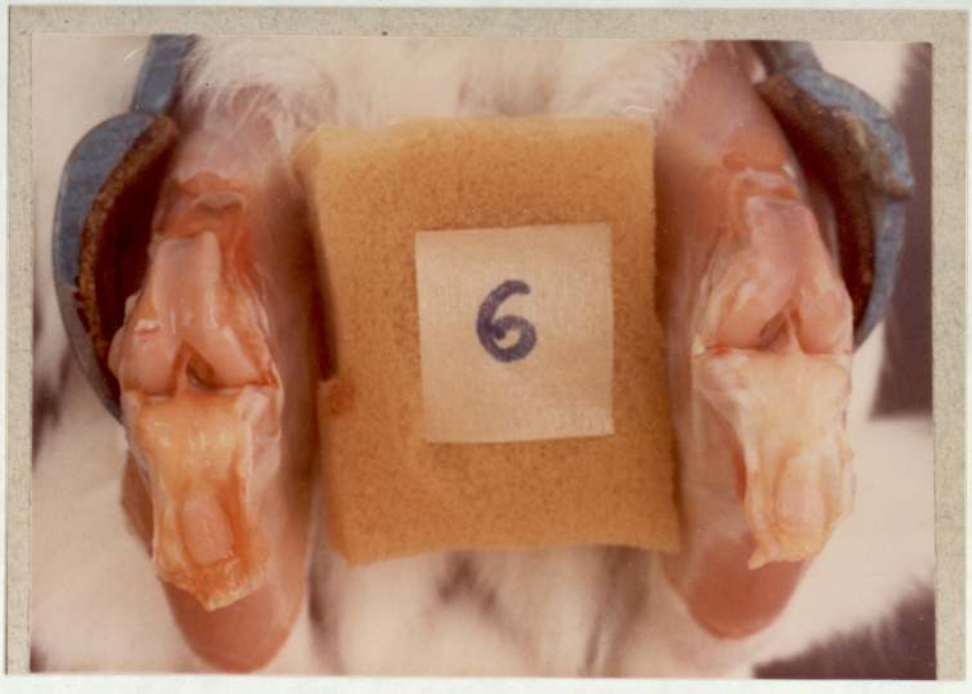
3.32: ECPL treated arthritis. As 3.31 plus slight erosions on femoral condyles.

3.33 : Untreated arthritis. The structure of the joint is almost completely obliterated by extending fibro-cellular pannus. The joint articular surface cartilage, the side tendon cartilage, the patellar insertion surface cartilage and the femoral cartilage are all eroded and replaced by pannus. The joint capsule is thickened. The synovial membrane is hypertrophied, discoloured and has a rough villous appearance.

3.34: EOM,3.35:ATH,3.36:EATL,3.37:E00,3.38:ATLH, and 3.39ATL treated arthritis all show no evidence of amelioration of the disease, only increasingly severe examples of joint destruction, with well marked erosion of the bone.

Figs 3.26 & 3.27 Macroscopic examination of dissected rabbit knees

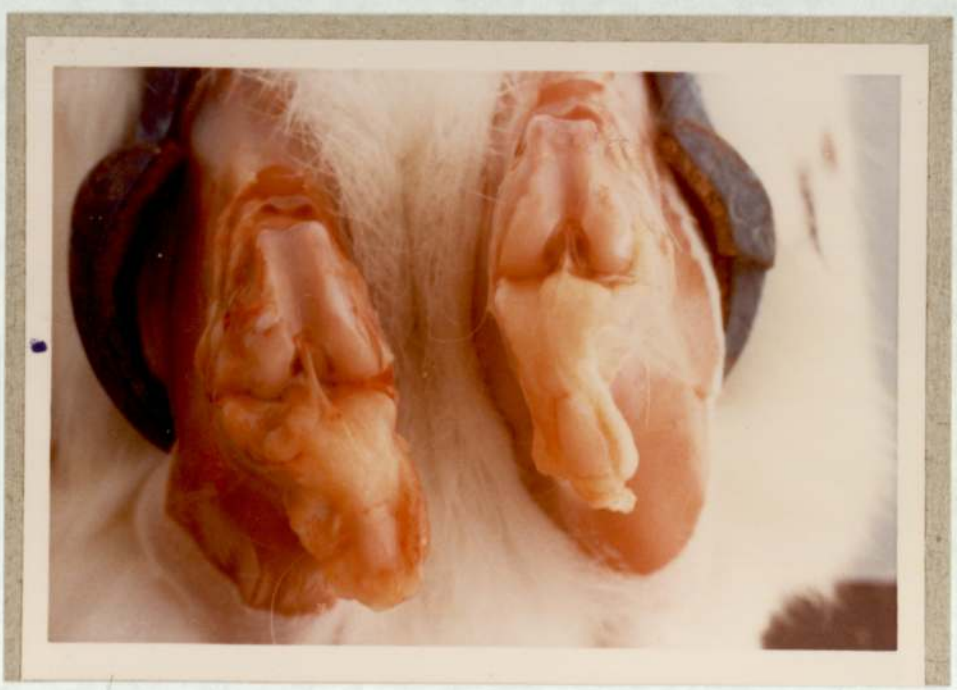
3.26
ATH normal



3.27
EATCPL normal

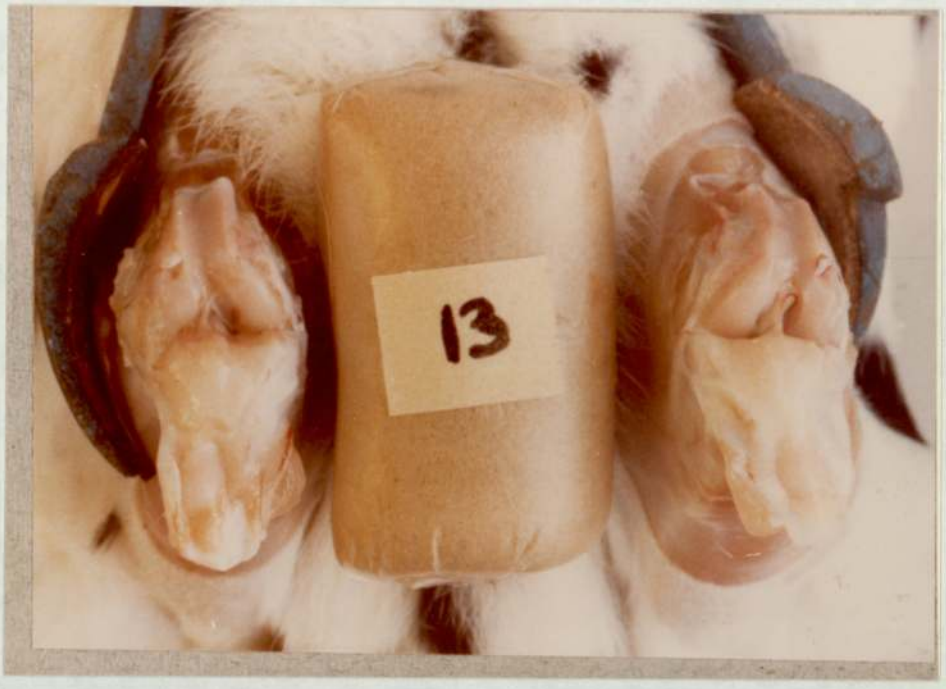
Figs 3.28 & 3.29 Macroscopic examination of dissected rabbit knees

3.28
ATCPL arthritic

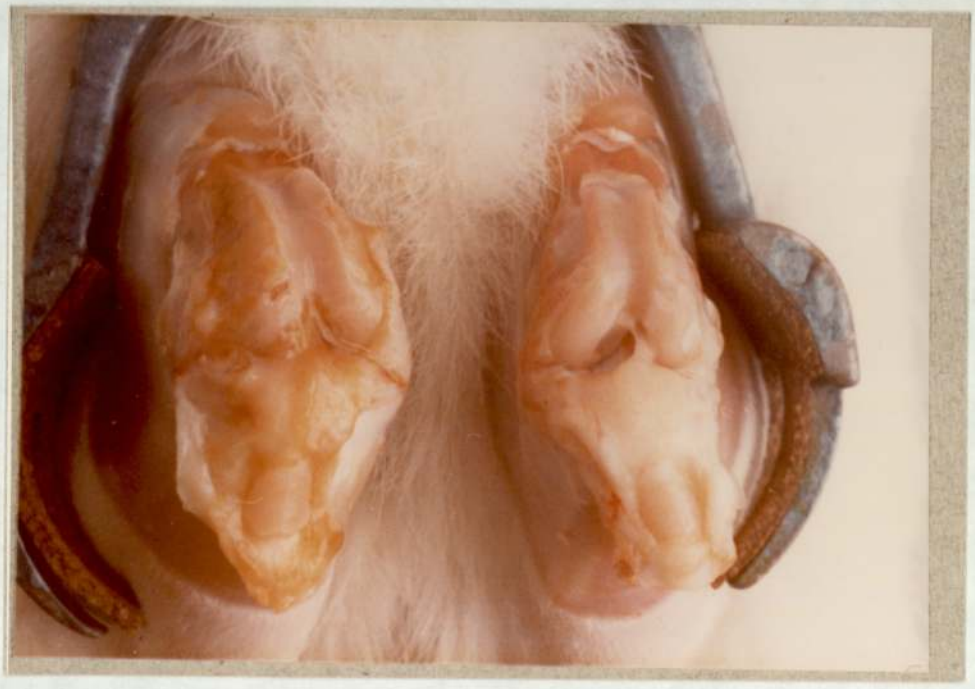


3.29
CPH arthritic

Figs 3.30 & 3.31 Macroscopic examination of dissected rabbit knees

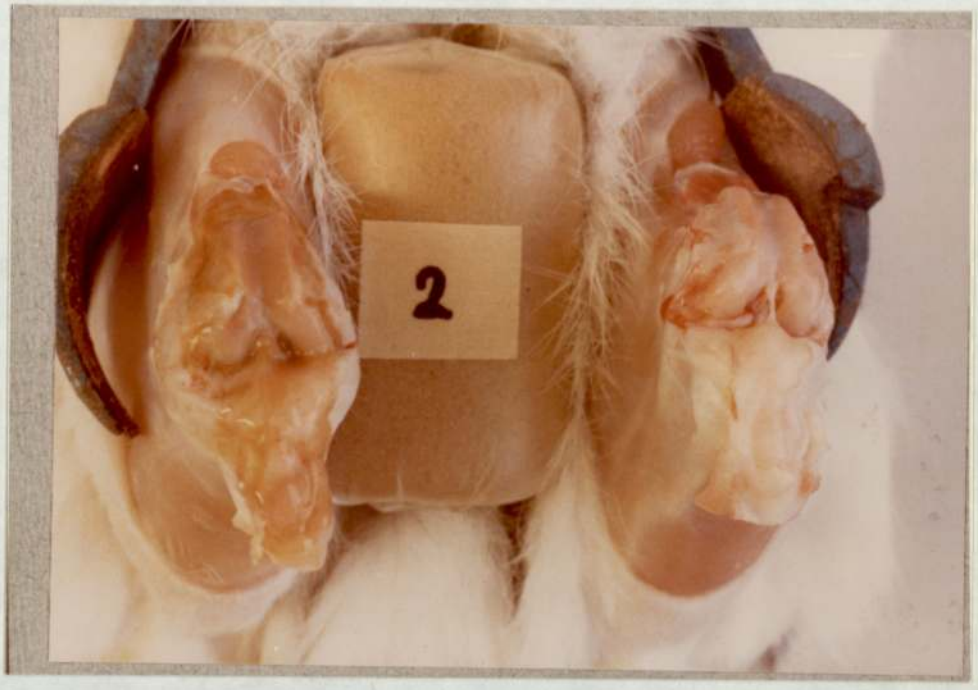


3.30
CPL arthritic



3.31
EATCPL arthritic

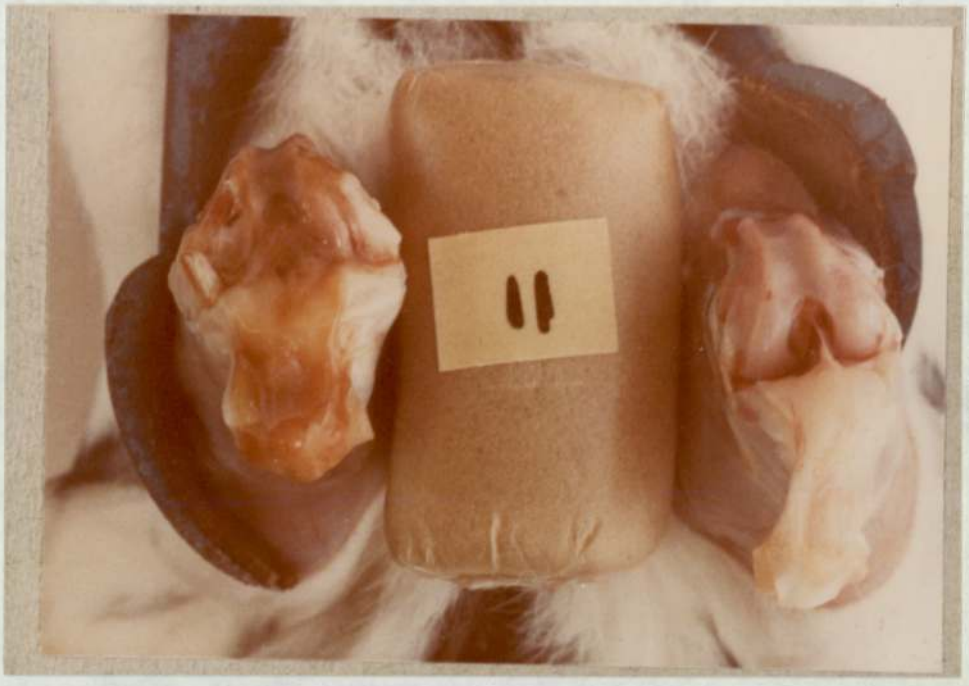
Figs 3.32 & 3.33 Macroscopic examination of dissected rabbit knees



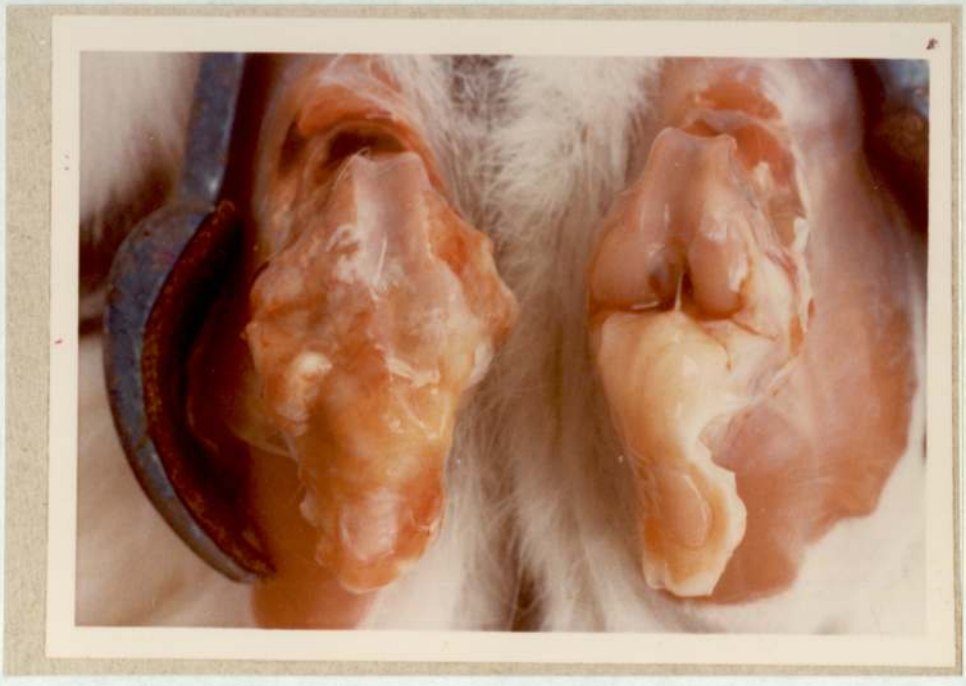
3.32
ECPL arthritic

3.33
untreated arthritic

Figs 3.34 & 3.35 Macroscopic examination of dissected rabbit knees

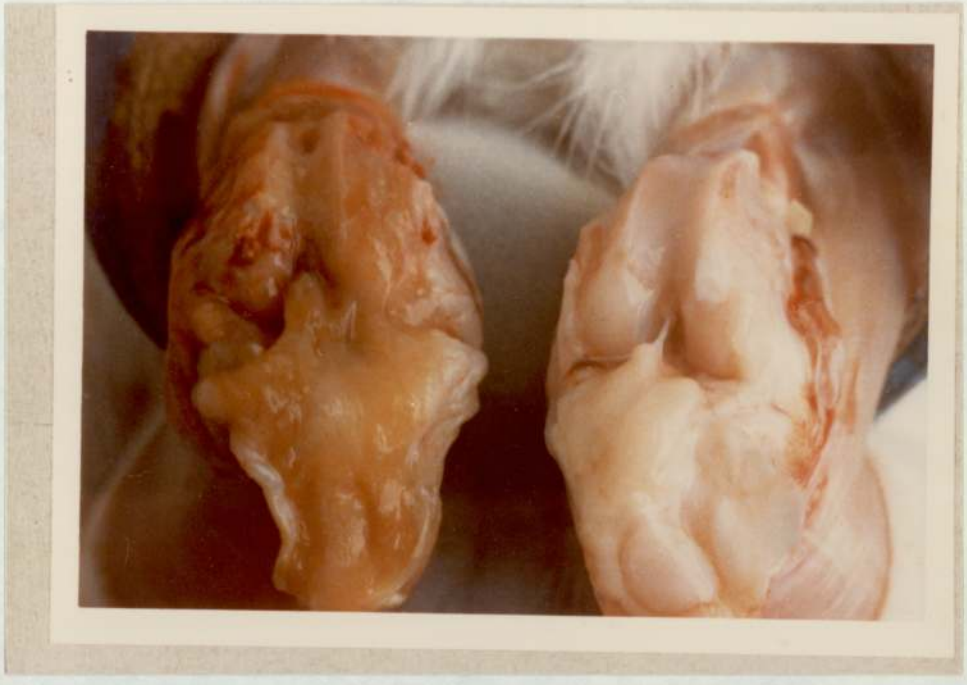


3.34
EOM arthritic

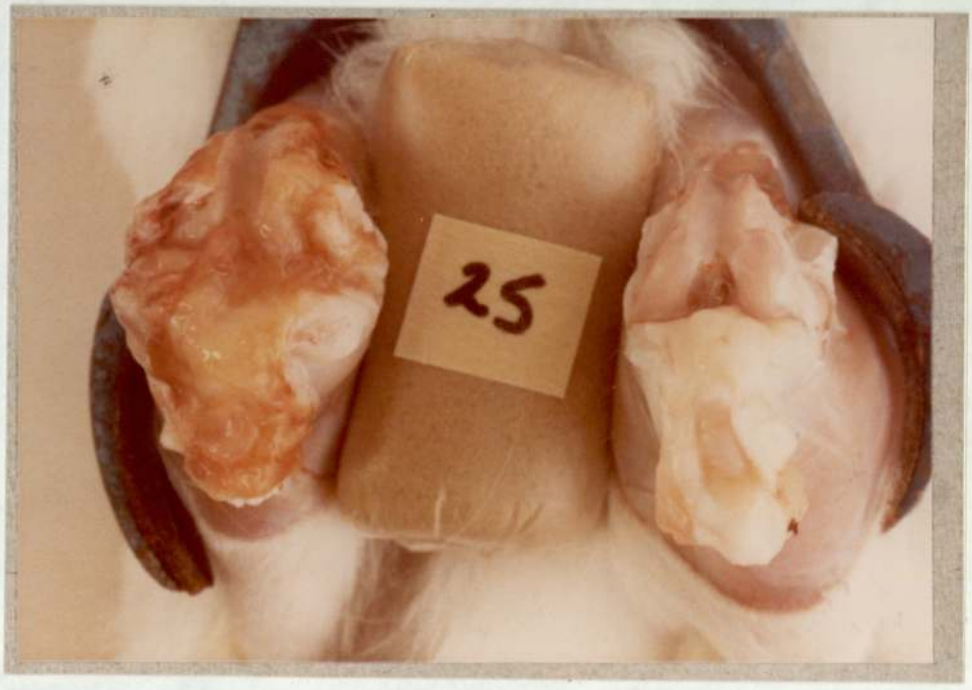


3.35
ATH arthritic

Figs 3.36 & 3.37 Macroscopic examination of dissected rabbit knees

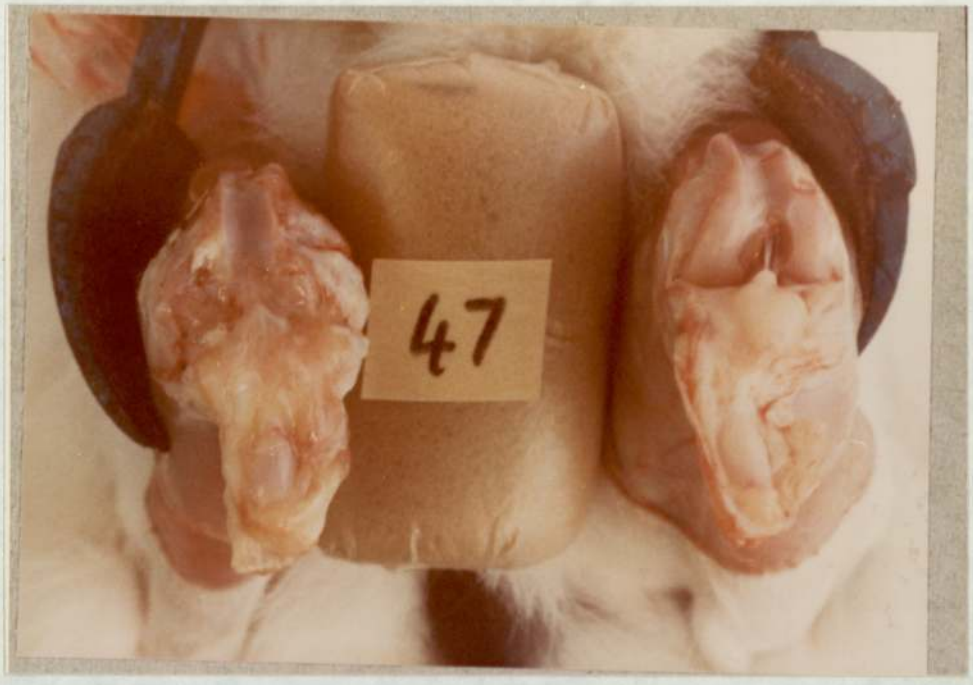


3.36
EATL arthritic

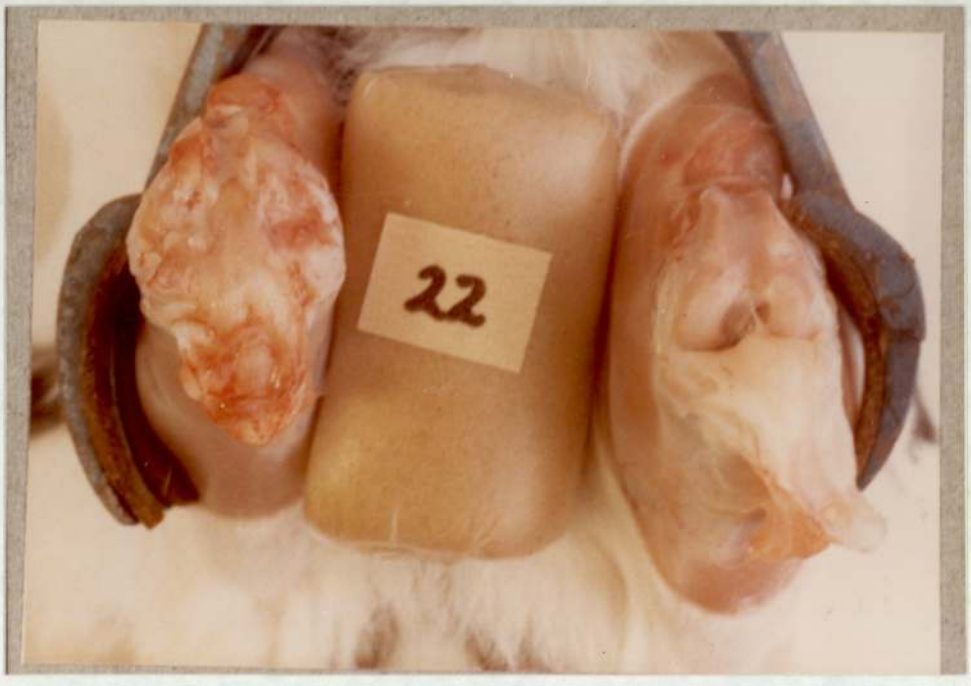


3.37
EOO arthritic

Figs 3.38 & 3.39 Macroscopic examination of dissected rabbit knees



3.38
ATLH arthritic



3.39
ATL arthritic

3.6.9 Histological examination of rabbit synovial tissue

With the assistance of an independent expert on rheumatological histopathology, slides of prepared tissue sections were examined and put into the following groups according to the apparent severity of the disease.

(a) Nil :	normal, and treated normal non-arthritic rabbits.
(b) Trace:	CPH, EATCPL, ATCPL treated.
(c) Mild:	CPL, ECPL, ATH treated.
(d) Moderate:	ATL, EATL treated.
(e) Severe:	ATLH, EOO, EOM treated, plus untreated.

For illustration, some micrographs of synovial sections are given as Figures 3-40 to 3-47.

Three types of tissue occur, and the appearance varies from place to place within each synovium.

Fig 3-40 shows typical adipose synovial tissue, which consists mainly of a single layer of synovial cells and underlying adipose. (The term synovial membrane is a misnomer, since it is not a true membrane but a layer of specialized connective tissue.

Fig 3-41 shows the next most common, loose connective tissue, i.e. areolar type, which is more nuclear. There are 2 or 3 layers of synovial cells and underlying loose connective tissues containing arterioles.

Fig 3-42 shows fibrous tissue. Usually synovial cells form a single layer with underlying fibrous tissue, which comes close to the synovial membrane. Normal synovial membrane contains no collagen, but there may be underlying collagen very close to it.

The results of examination of the tissue samples from the various groups are as follows. Note that the photographs are only illustrative and do not cover all the features found by visual inspection of all parts of the set of slides.

Control - untreated arthritis (Fig 3-43): The synovial membrane was hypertrophied and possessed many villous processes. The synovial tissue was very cellular, infiltrated mainly with PMNs. A lesser number of plasma cells were present, as were a few lymphocytes (collectively called "round cells"). The synovial membrane surface had a layer of fibrinoid material, some of which was necrotic (the non-necrotic was probably due to blood fibrin clotting the fluid).

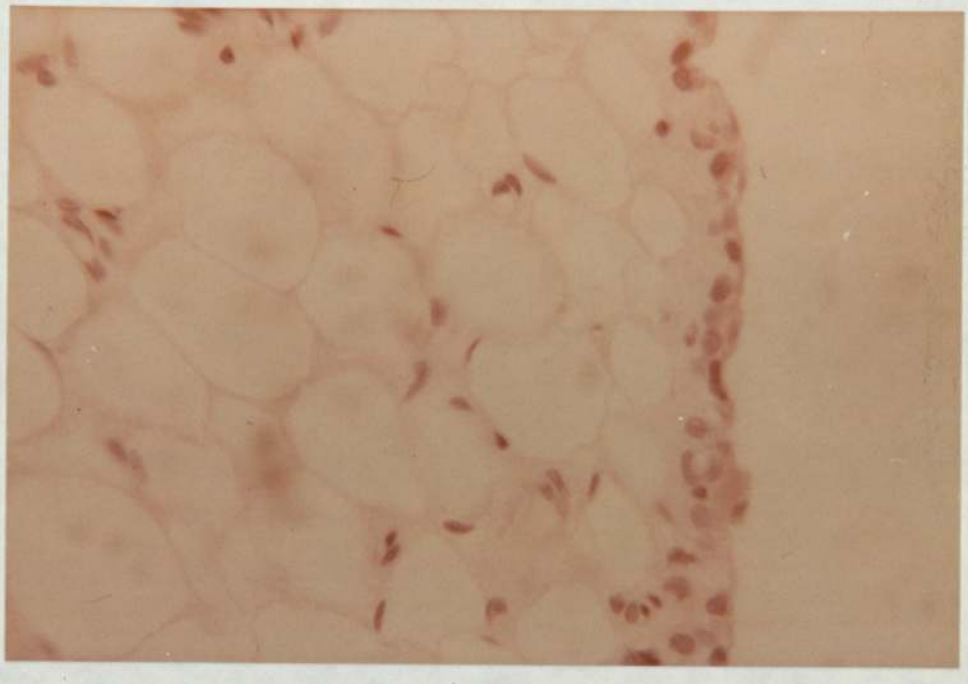
EATL (Fig 3-44). These showed a condition similar to the control, with infiltration by PMNs, accumulated round cells, fibrinoid necrosis patches. The membrane was hypertrophied and villous.

ATH (Fig 3-45). Membrane hypertrophied and villous. Infiltration by PMNs (mainly) and round cells. Fibrinoid material in places on and near the surface.

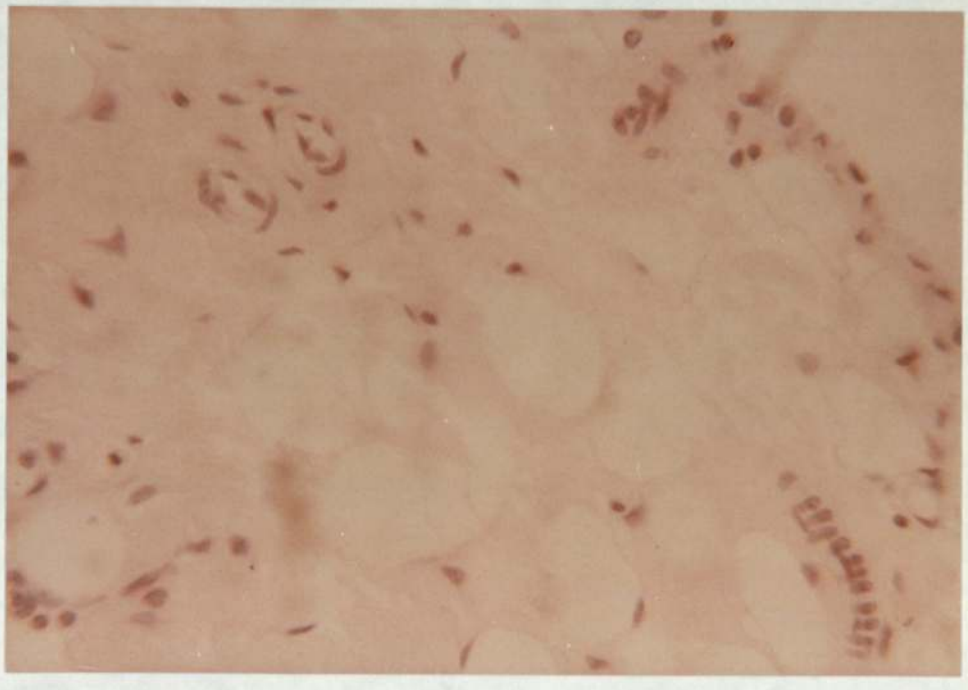
CPH (Fig 3-46). The synovial tissue was much less cellular. Though some PMNs and plasma cells were present, there was no marked infiltration. There was a fair amount of fibrinoid material.

EATCPL (Fig 3-47). The synovial membrane was not hypertrophied, but was patchy and variable in appearance. Some areas seemed normal, while others had some cellular infiltration by PMNs and round cells. It appears as if there was inflammation present at some stage, but this later started to clear up.

Figs 3.40 & 3.41 Histology of rabbit synovial tissue

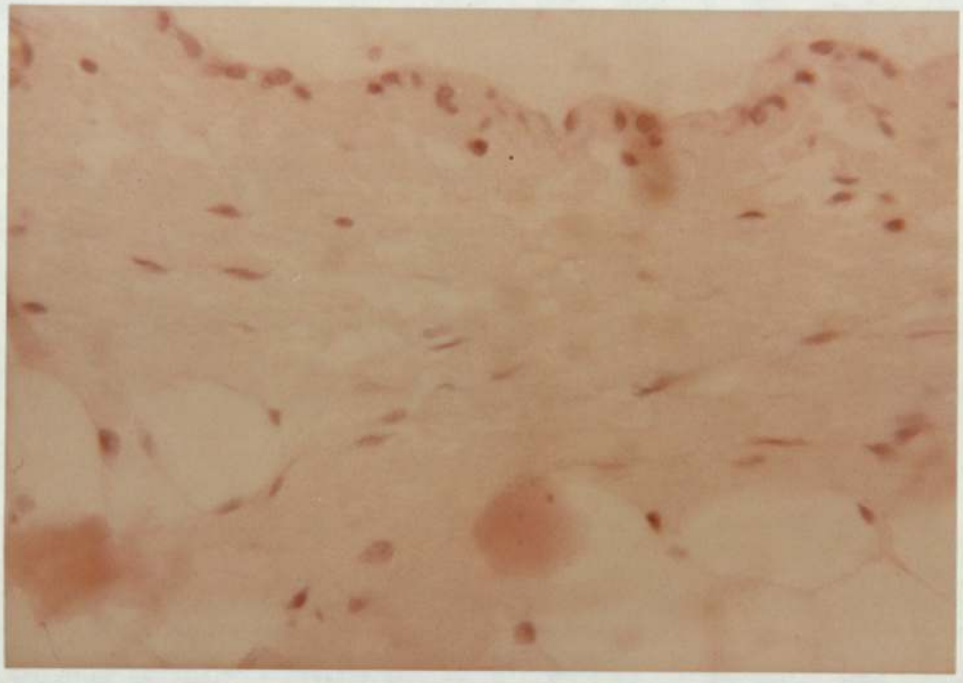


3.40 normal adipose tissue

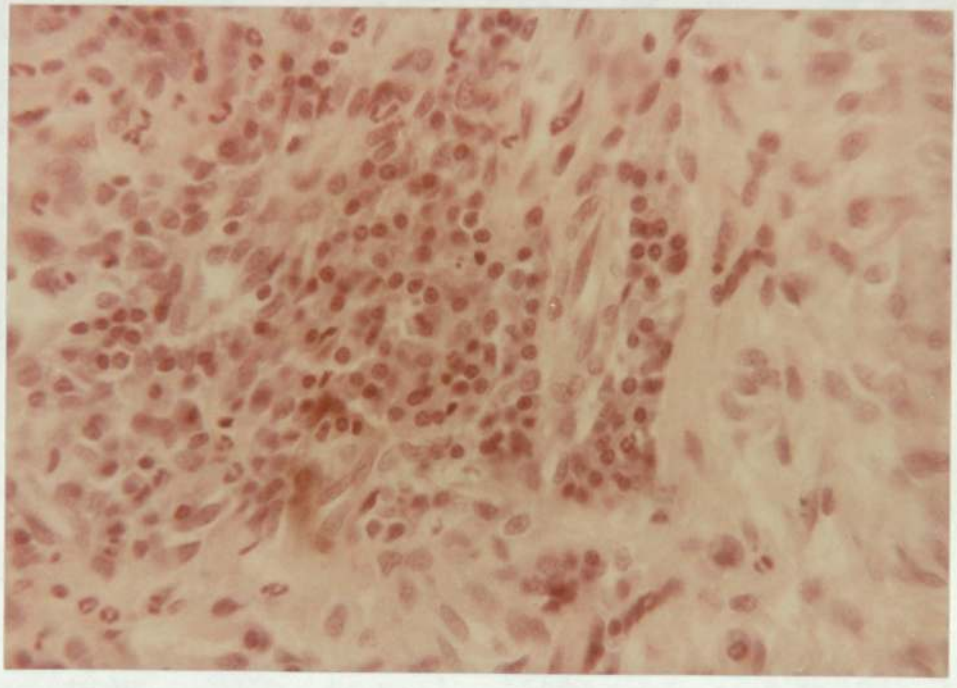


3.41 normal connective tissue

Figs 3.42 & 3.43 Histology of rabbit synovial tissue

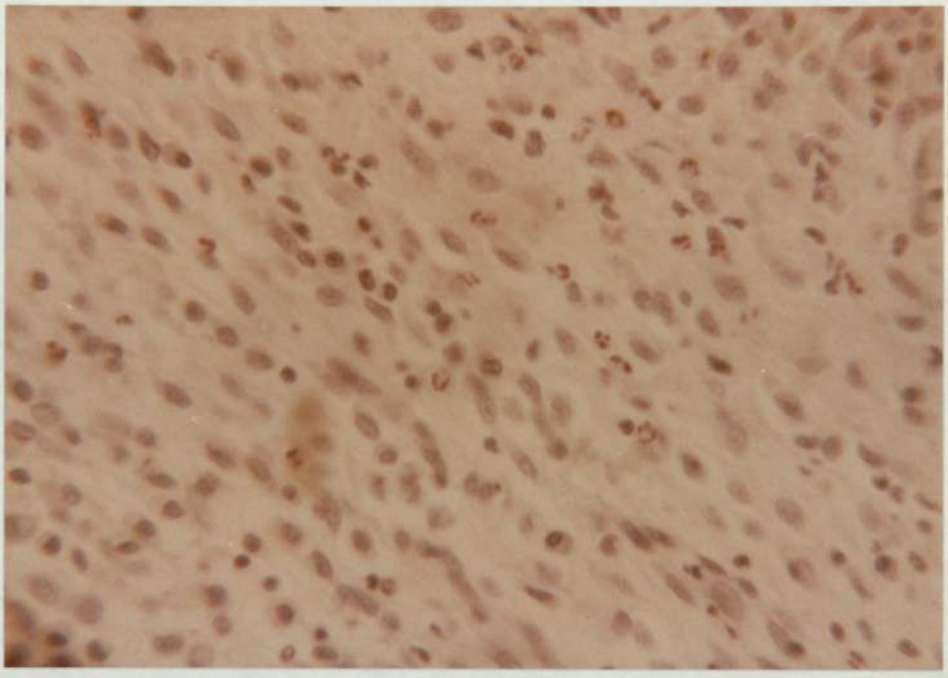


3.42 normal fibrous tissue

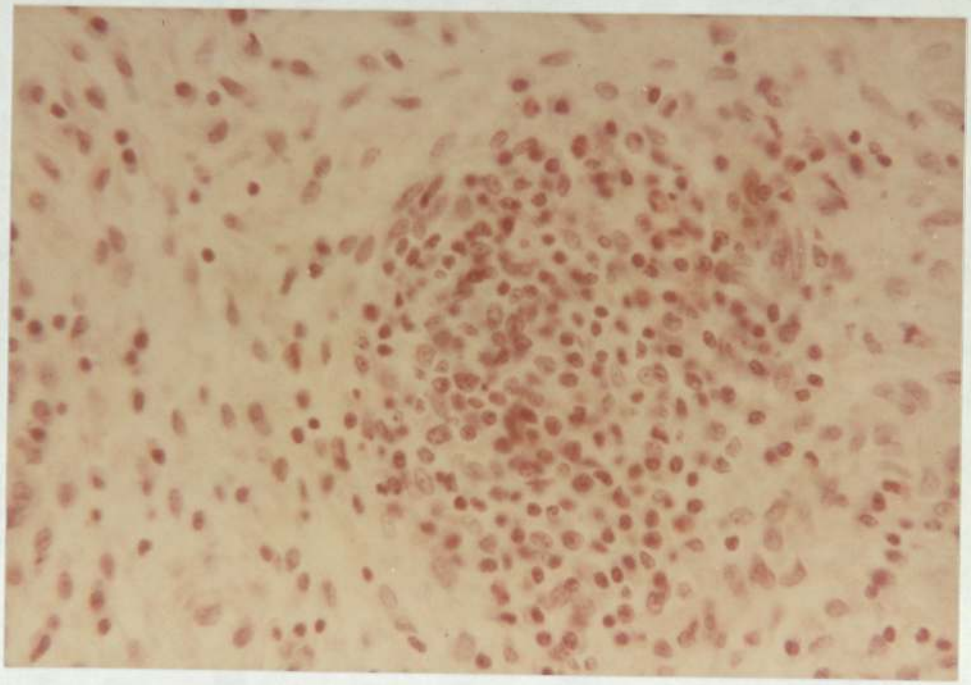


3.43 untreated arthritis

Figs 3.44 & 3.45 Histology of rabbit synovial tissue

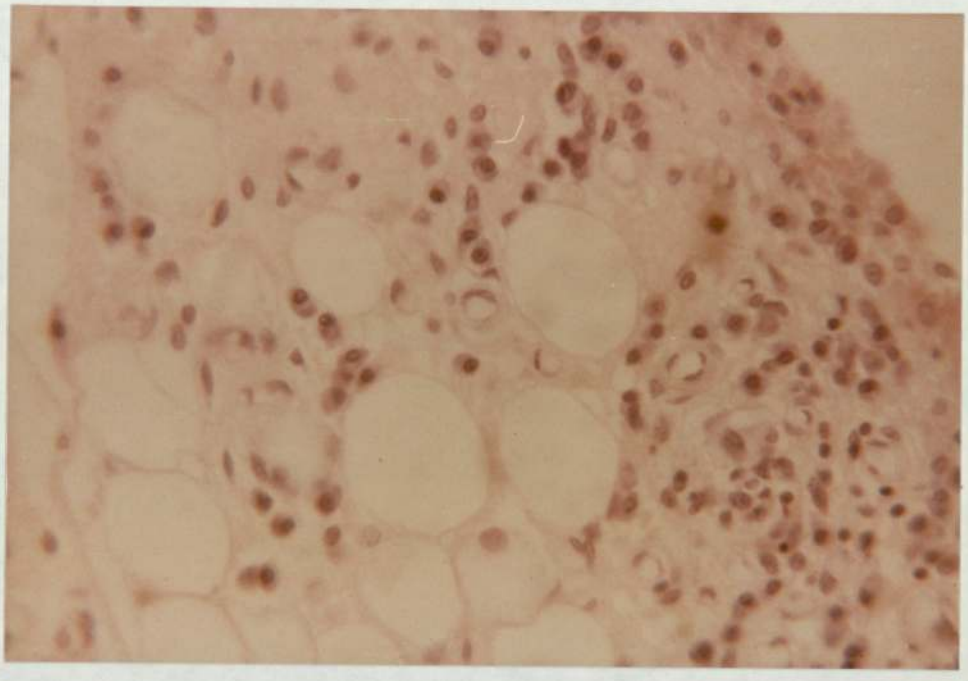


3.44
EATL arthritic

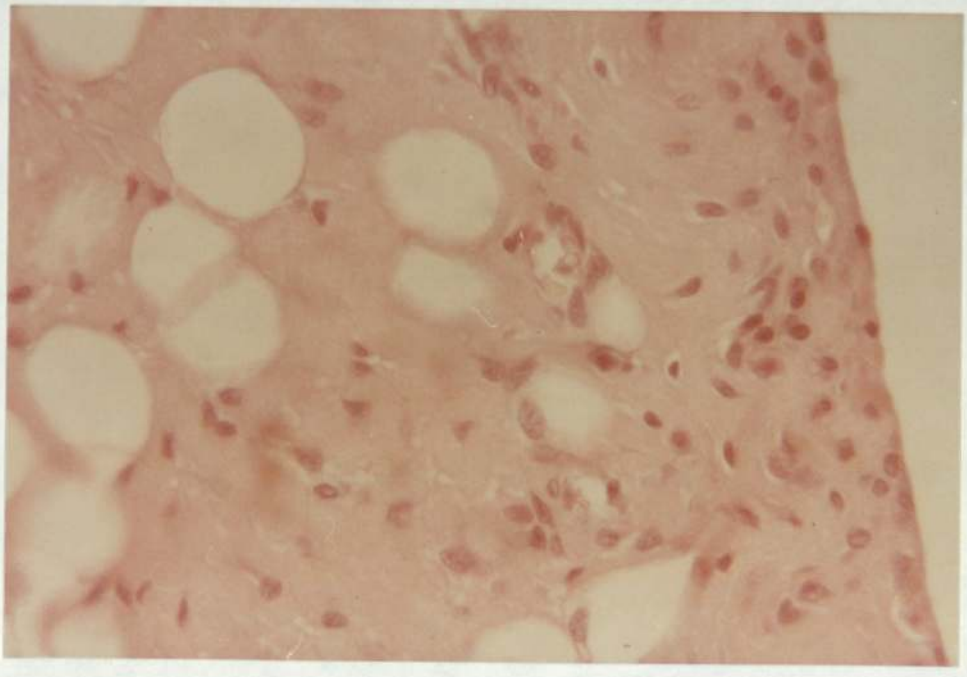


3.45
ATH arthritic

Figs 3.46 & 3.47 Histology of rabbit synovial tissue



3.46
CPH arthritic



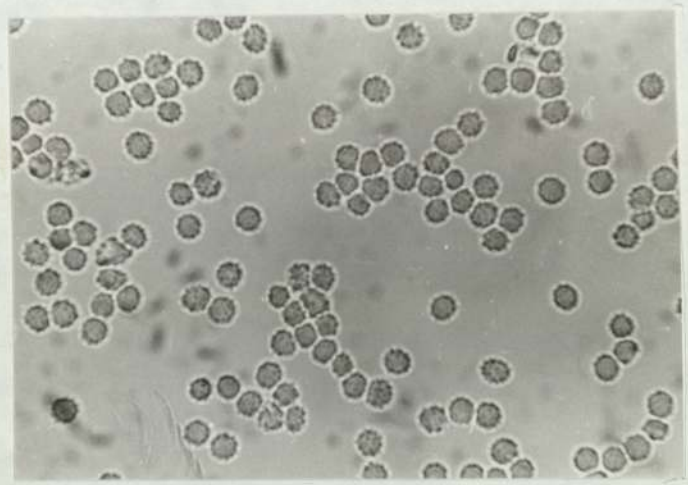
3.47
EATCPL arthritic

3.7 In vivo survival of erythrocytes after encapsulation

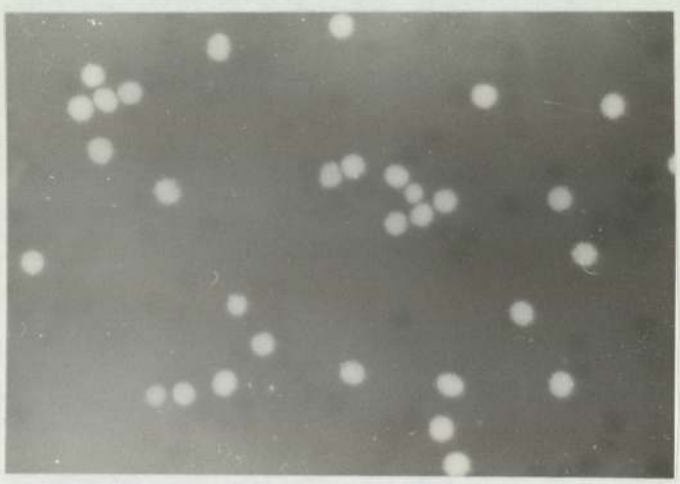
The appearance of erythrocytes labelled with FITC is shown in photomicrographs Fig. 3.48. (A) shows a mixture of labelled and unlabelled RBCs under normal tungsten lighting, where they appear identical. (B) shows the identical microscope field photographed under UV (deuterium) lighting. The FITC labelled cells alone appear as bright (green) spots. (C) shows a different field with combined tungsten and UV lighting. Both sorts of cells are visible and clearly different.

The photographs show cells which have been treated with FITC after encapsulation. This was found to give a stronger label than encapsulation of FITC solution, and made cell counts easier.

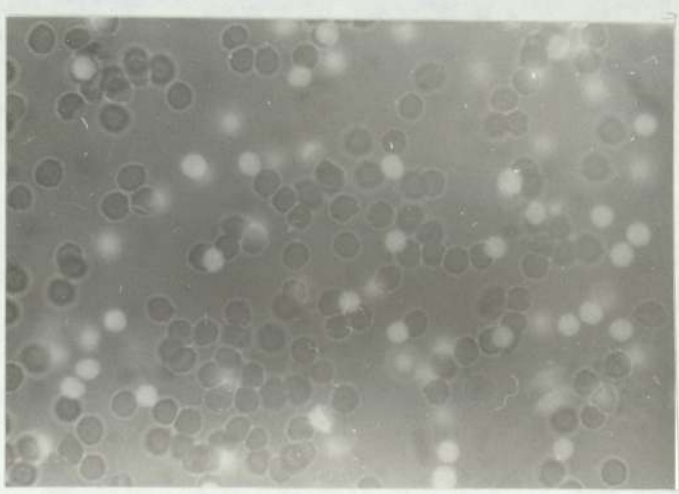
Fig 3.48 Photomicrographs of FITC labelled RBCs



(A) tungsten lamp



(B) UV lamp



(C) tungsten + UV

3.7.1 Survival of FITC encapsulating RBCs (rat's own)

A group of 4 rats received preparations of their own erythrocytes, encapsulating FITC. Results of relative counts of blood samples are shown in Fig. 3.49.

3.7.2 Survival of RBCs labelled with FITC after encapsulation (rat's own RBCs)

A further group of 4 rats received RBCs which had been labelled after encapsulation. Results are also shown in Fig. 3.49

3.7.3 Survival of RBCs labelled with FITC after encapsulation (mixed rat RBCs)

A group of 5 rats had RBCs from pooled blood labelled after encapsulation and the mixed RBCs administered as usual. Results are also shown in Fig. 3.49.

To see if the decline in labelled cells followed an exponential decay, the combined results from 3.7.1 - 3 were plotted on a semi-log graph given as Fig. 3.50. Resultant figures give a reasonably linear plot, except for a persistent irregularity around 15 - 20 days.

Fig 3.49 Survival in the circulation of erythrocytes labelled by encapsulating FITC, or by FITC treatment after sham encapsulation

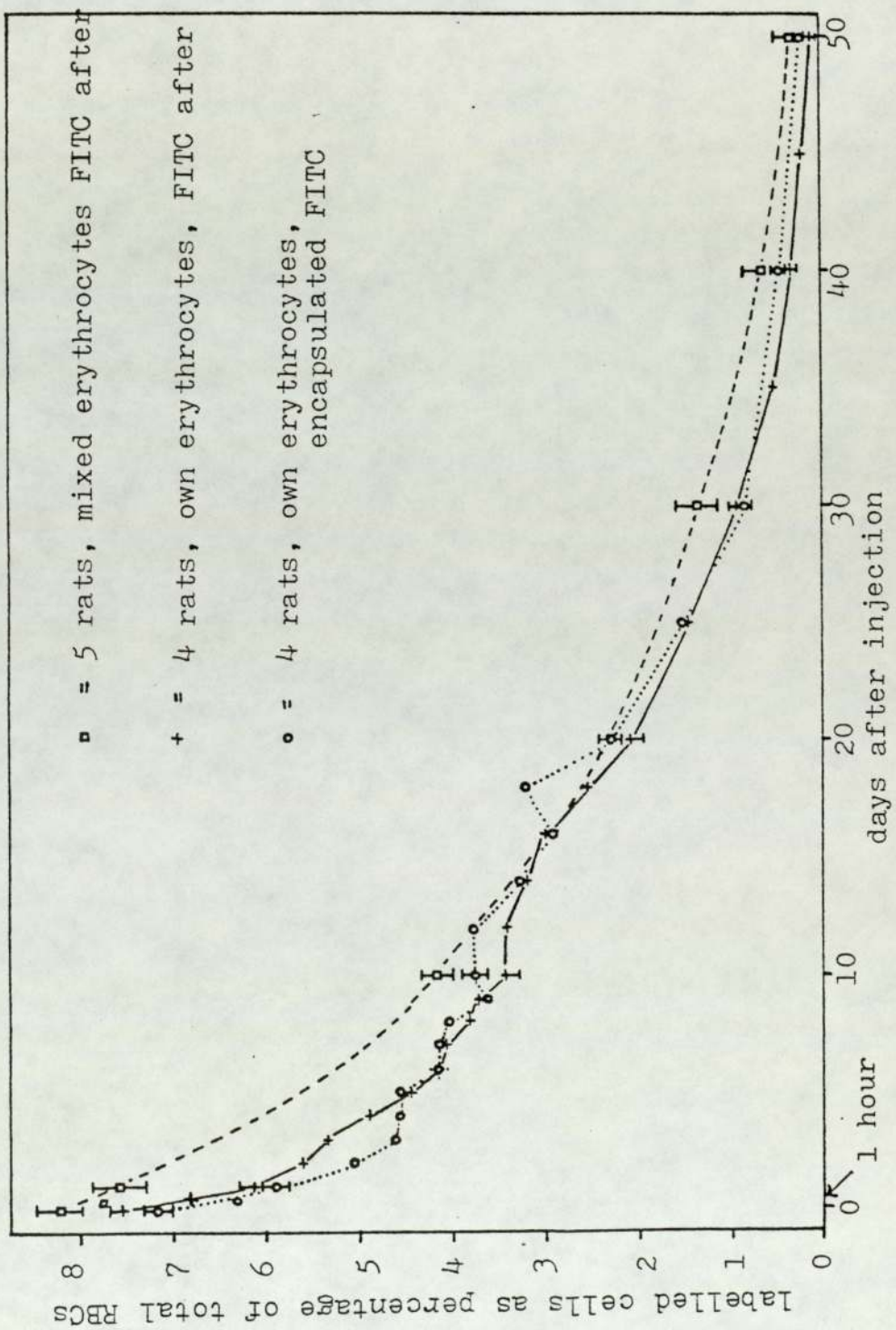
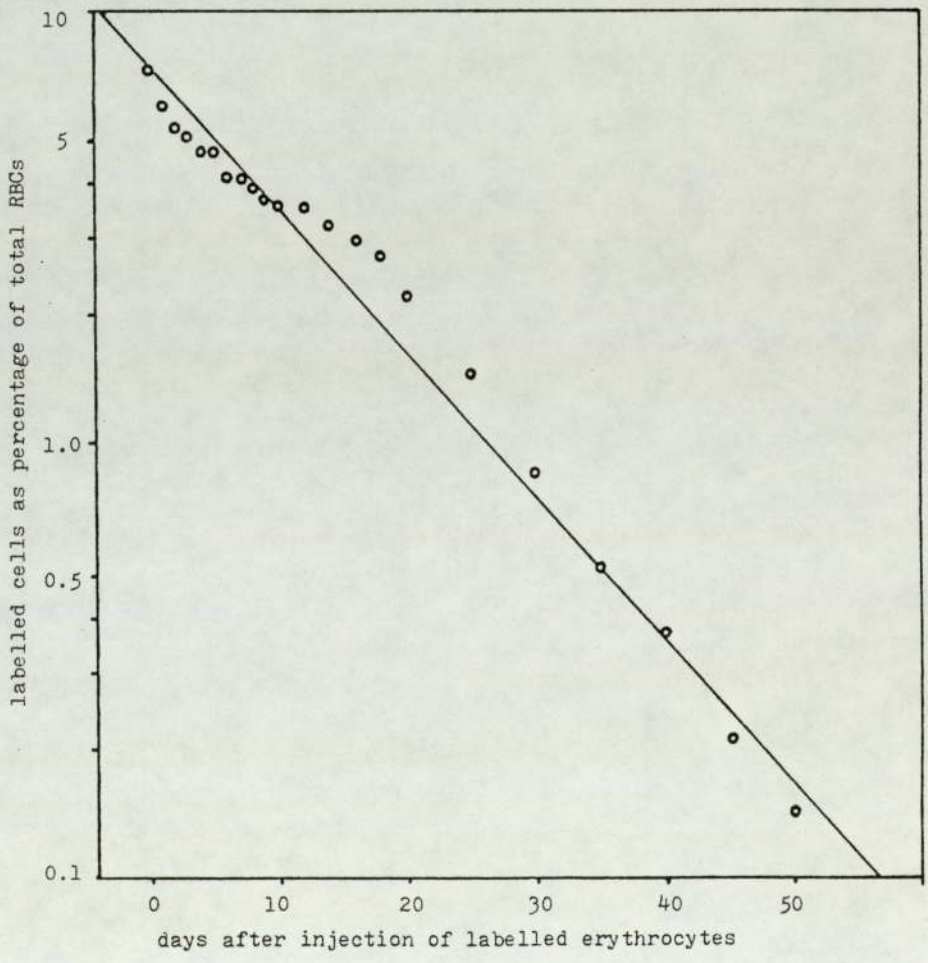


Fig 3.50 Survival in the circulation of erythrocytes labelled with FITC: semi-log plot of pooled results as in Fig 3.49

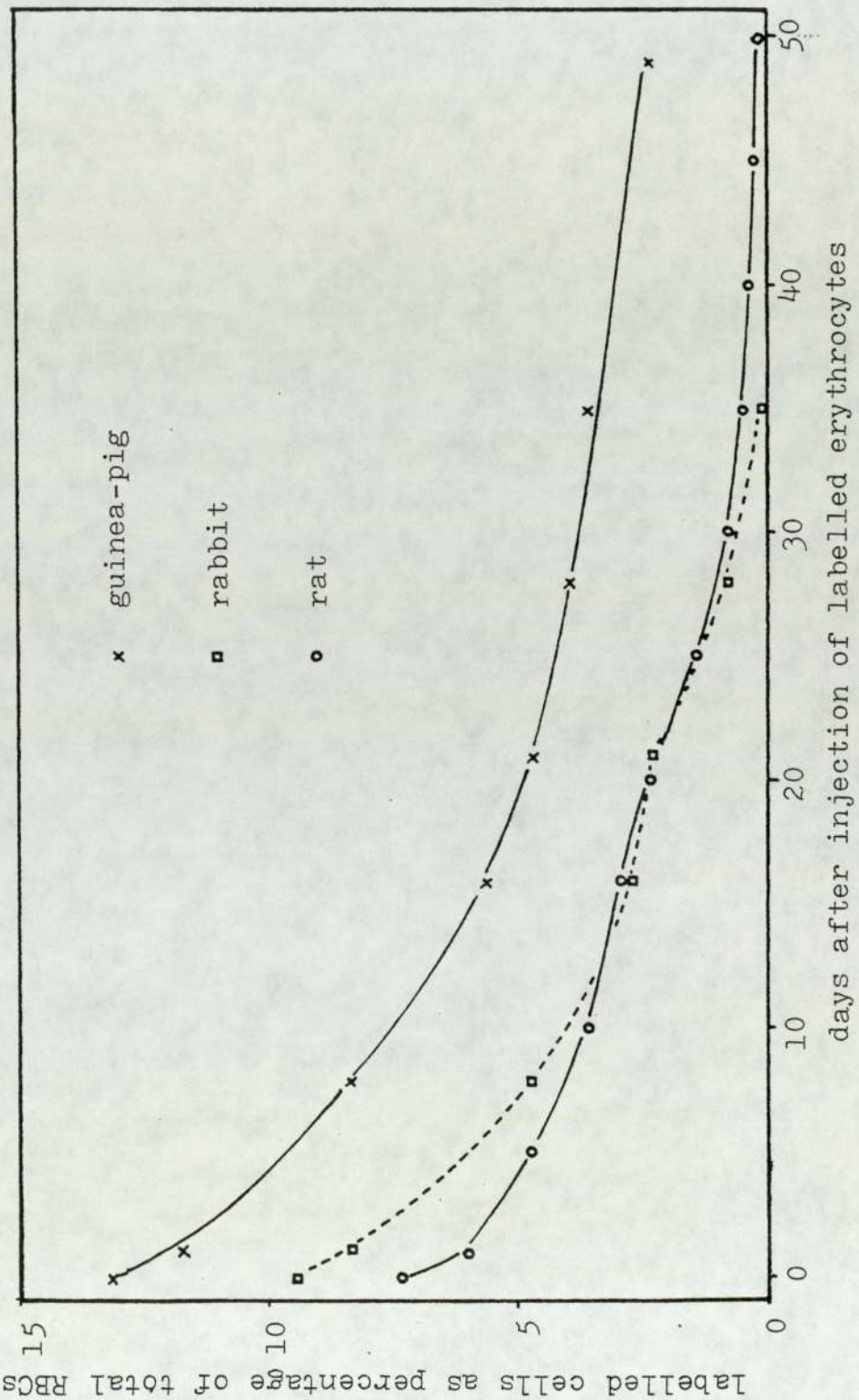


3.7.4 Survival of RBCs labelled with FITC after encapsulation (rabbit & guinea-pig own RBCs)

The experiment on rats (Section 3.7.2) was repeated with guinea-pigs and with rabbits. The results of survival of labelled RBCs in circulation for these two species are given in Fig. 3.51. The equivalent curve for the rat (from Fig. 3.49) is given for comparison.

The same sort of decay curve was observed for all three species. Some labelled RBCs were still present in the guinea-pig circulation at the termination of the experiment after 50 days.

Fig 3.51 Survival in the circulation of FITC labelled erythrocytes in the guinea-pig, rabbit & rat



3.7.5 In vivo survival of rat erythrocyte ghosts labelled by encapsulation of FITC

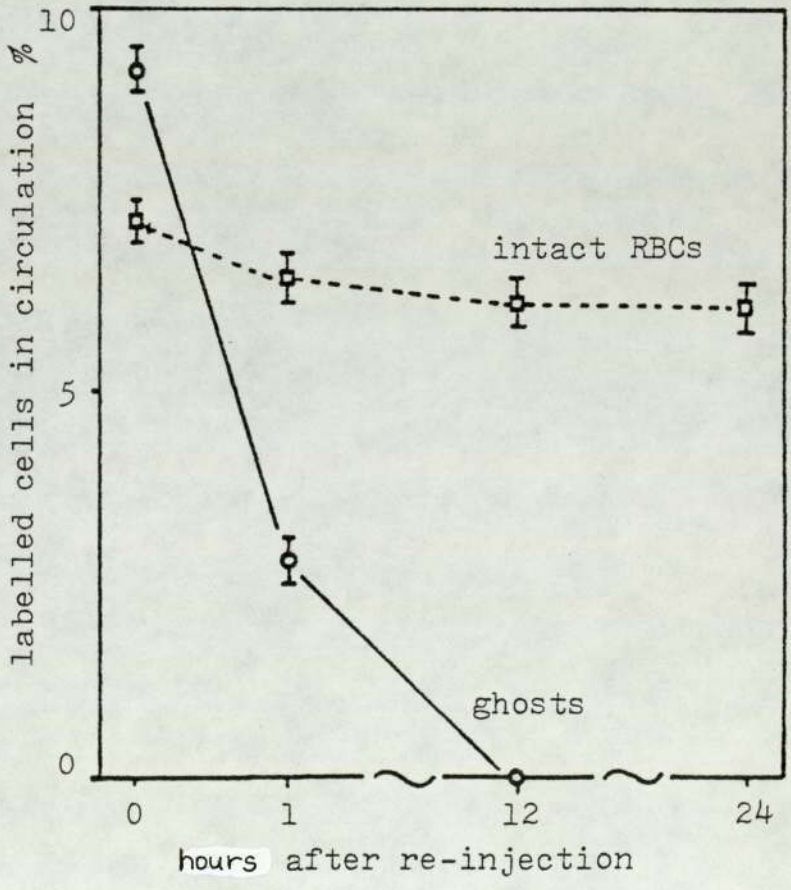
Fluorescence measurements showed these ghosts to be rapidly eliminated from circulation.

Blood was removed from each of ten rats, the RBC treated and cells returned to the original rat so that on average 9.2% (s.e.m. 0.26%) of the circulating RBCs were labelled ghosts (initial count immediately after injection).

After 1 hour this value fell to 2.8% (s.e.m. 0.29%) and after 12 hours no labelled cells could be detected at all. At this time it was noted that some of the macrophages exhibited fluorescent staining, presumably due to engulfing labelled ghost cells.

A comparison of the survival of FITC-labelled ghosts and of FITC-labelled RBCs (which had been treated by the encapsulation procedure) is given in Fig. 3.52.

Fig 3.52 Survival in the circulation of the rat by ghosts and intact erythrocytes, both labelled by encapsulation of FITC



3.8 The effect of intravenous administration of corticosteroids encapsulated in RBCs on adjuvant arthritis in the rat

Foot volumes were used as a measure of the disease, and the effect of cortisol phosphate administered in various ways was noted for the acute and chronic phases. Prednisolone sodium succinate was also tested.

3.8.1 Comparison of cortisol phosphate, encapsulated and in solution, intravenously and sub-cutaneously

Results are shown in Fig. 3.53 for the effect of different methods of administration on arthritic foot volume. It is clear that the encapsulated steroid was more effective than the free steroid in suppressing inflammation in both the acute and chronic phases. This effect was not due to the RBCs themselves (i.e. by a counter-irritant mechanism) since encapsulated media had no anti-inflammatory effect. Intravenous cortisol phosphate had virtually no effect, probably due to very rapid metabolism.

Fig. 3.54 shows the changes on the non-injected foot. This suffered chronic inflammation, which was however suppressed most effectively by the encapsulated drug and whose onset was delayed by at least two days.

Fig 3.53 The effect of administration of cortisol phosphate (encapsulated and in solution) on foot volume of rats with adjuvant arthritis (injected foot)

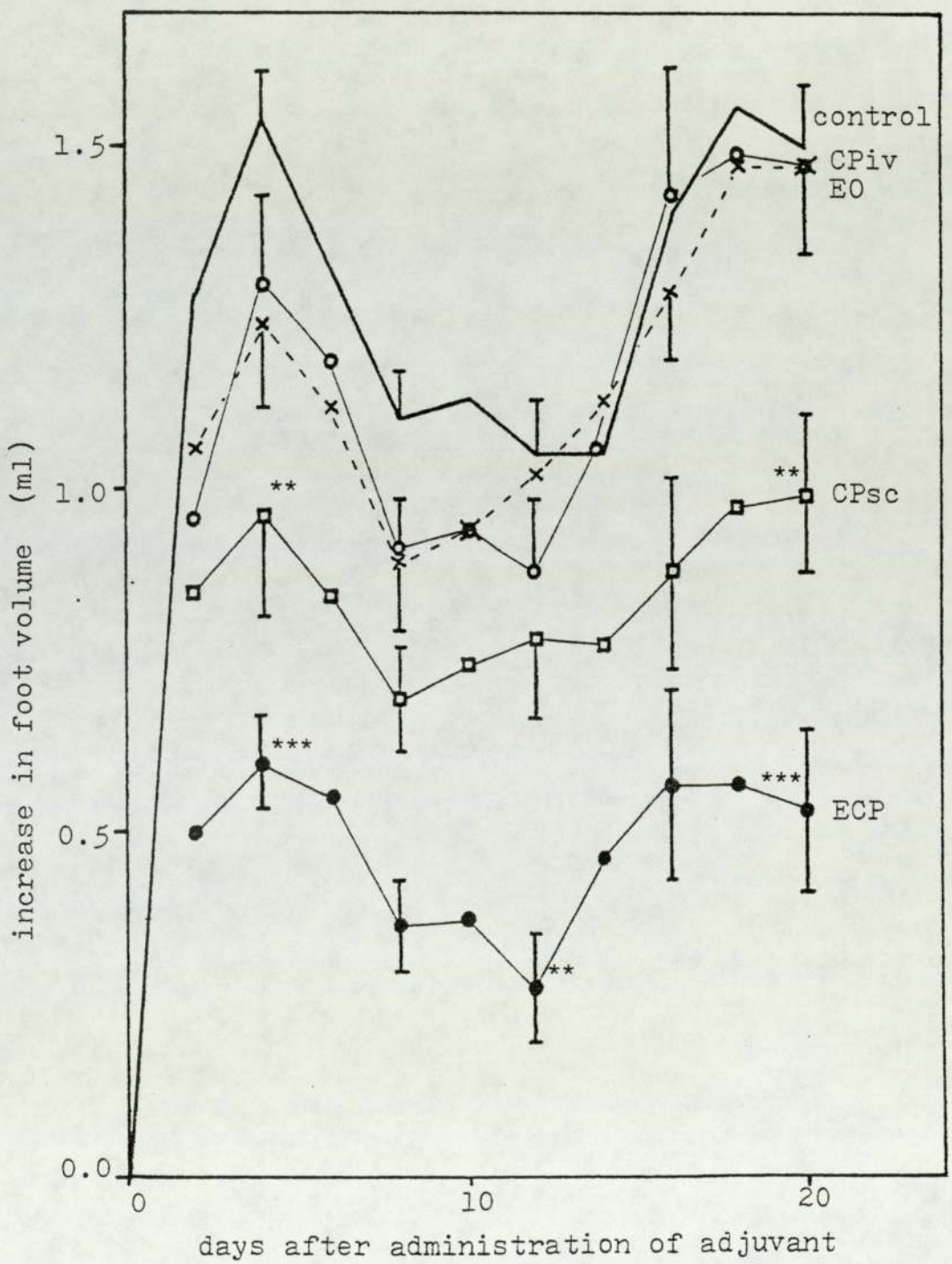
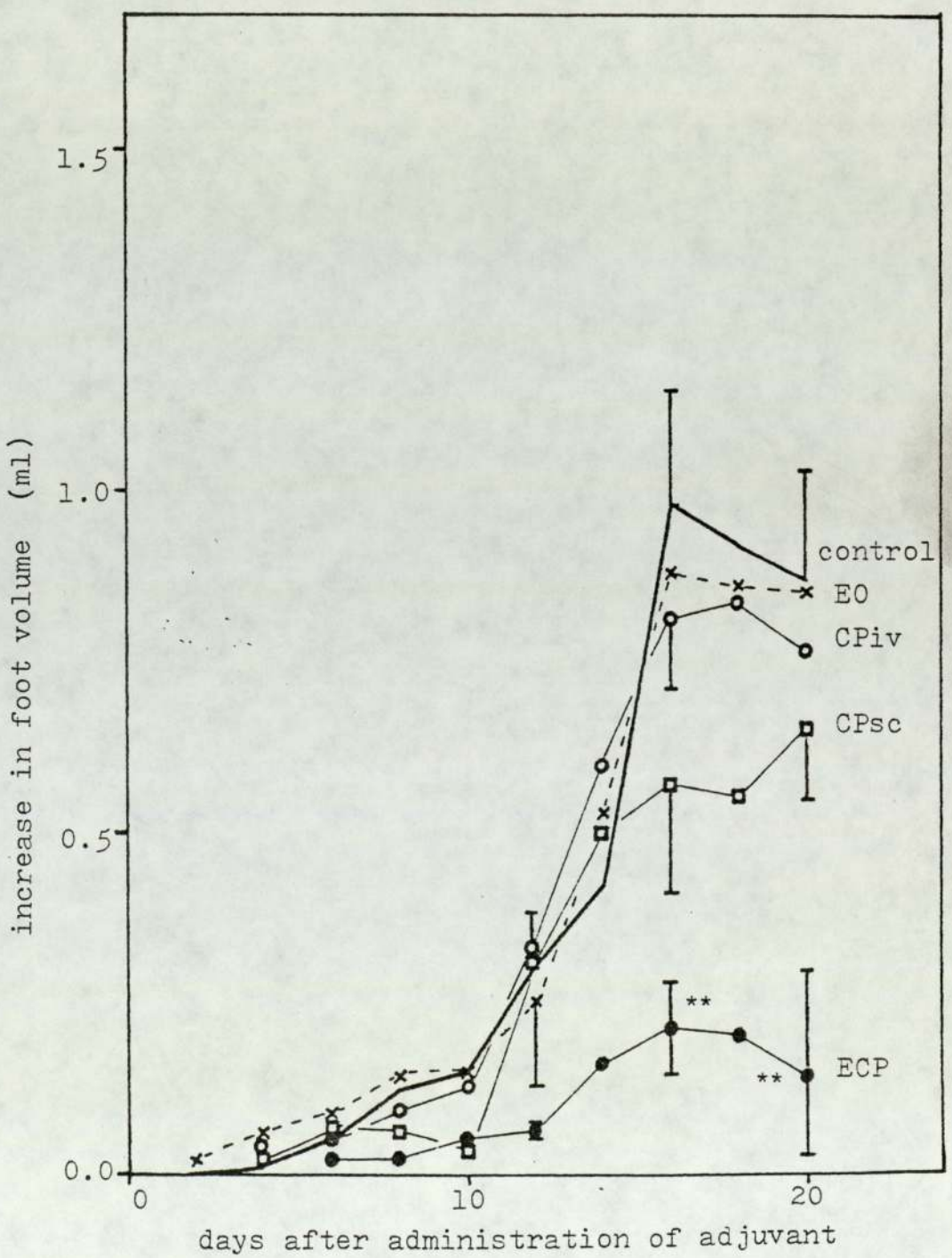


Fig 3.54 The effect of administration of cortisol phosphate (encapsulated and in solution) on foot volume of rats with adjuvant arthritis (non-injected foot)



3.8.2 The effect of encapsulated cortisol phosphate at 3 different dose levels

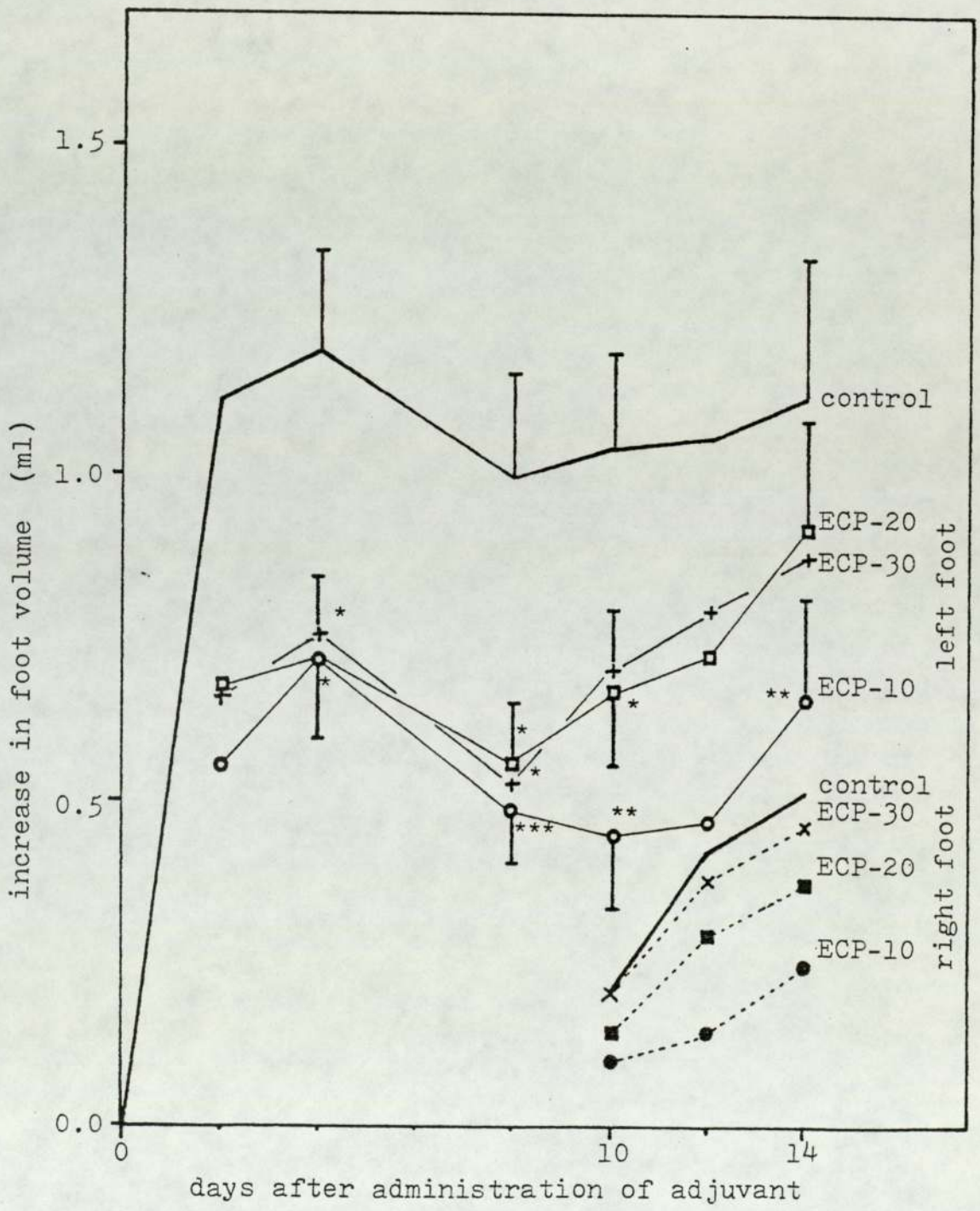
Results are shown in Fig. 3.55 for both the arthritic and non-arthritic foot.

All three dosages reduced the inflammation in the acute phase by a significant amount. The higher doses were, however, no different at all from the lowest dose. In the chronic phase, only the lowest dose produced a significant reduction in inflammation.

Similar effects were observed for the non-injected foot, in that the lowest dosage had the greatest effect.

It should be noted that the doses were nominal, assuming the efficiency of encapsulation was the same for different concentrations of cortisol phosphate.

Fig 3.55 The effect of administration of encapsulated cortisol phosphate at 3 different dose levels on foot volume of rats with adjuvant arthritis (left = injected foot)



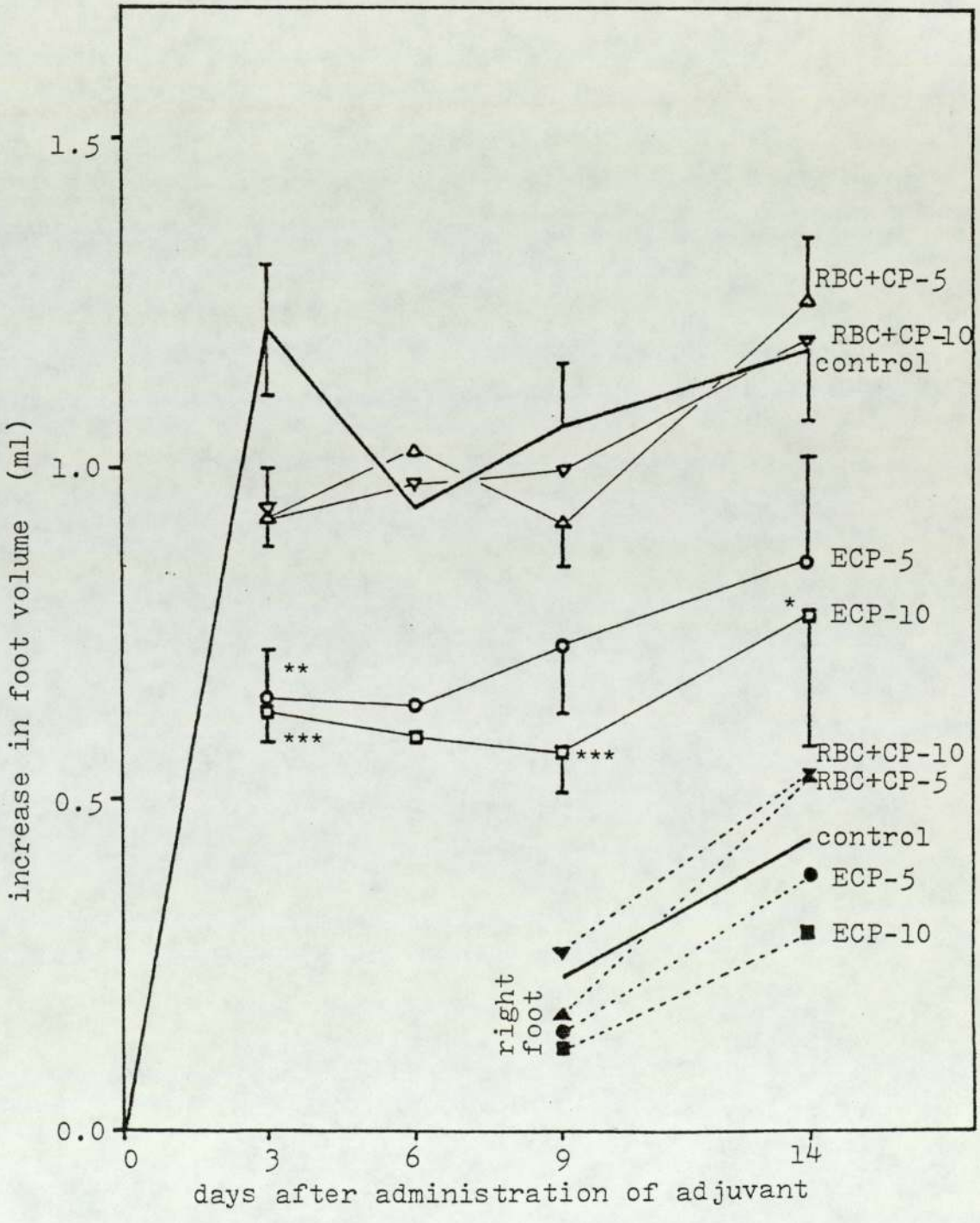
3.8.3 Comparison of encapsulated cortisol phosphate with the same drug incubated with RBCs

Cortisol phosphate was encapsulated at nominal 5 and 10 days' dosage, and equivalent preparations made up containing RBCs with which the drug had been incubated for the same time, but not encapsulated. The effects on foot volume are shown in Fig. 3.56.

There was no statistically significant difference between the two dose levels in either case, though the higher dose gave least inflammation. The encapsulated preparations were effective in reducing inflammation throughout the experiment. The non-encapsulated treatments gave some relief at day 3 but were otherwise indistinguishable from the controls.

The encapsulated treatment also reduced inflammation in the untreated foot.

Fig 3.56 The effect of administration of cortisol phosphate (encapsulated or incubated with RBCs) on foot volume of rats with adjuvant arthritis (left = injected foot)



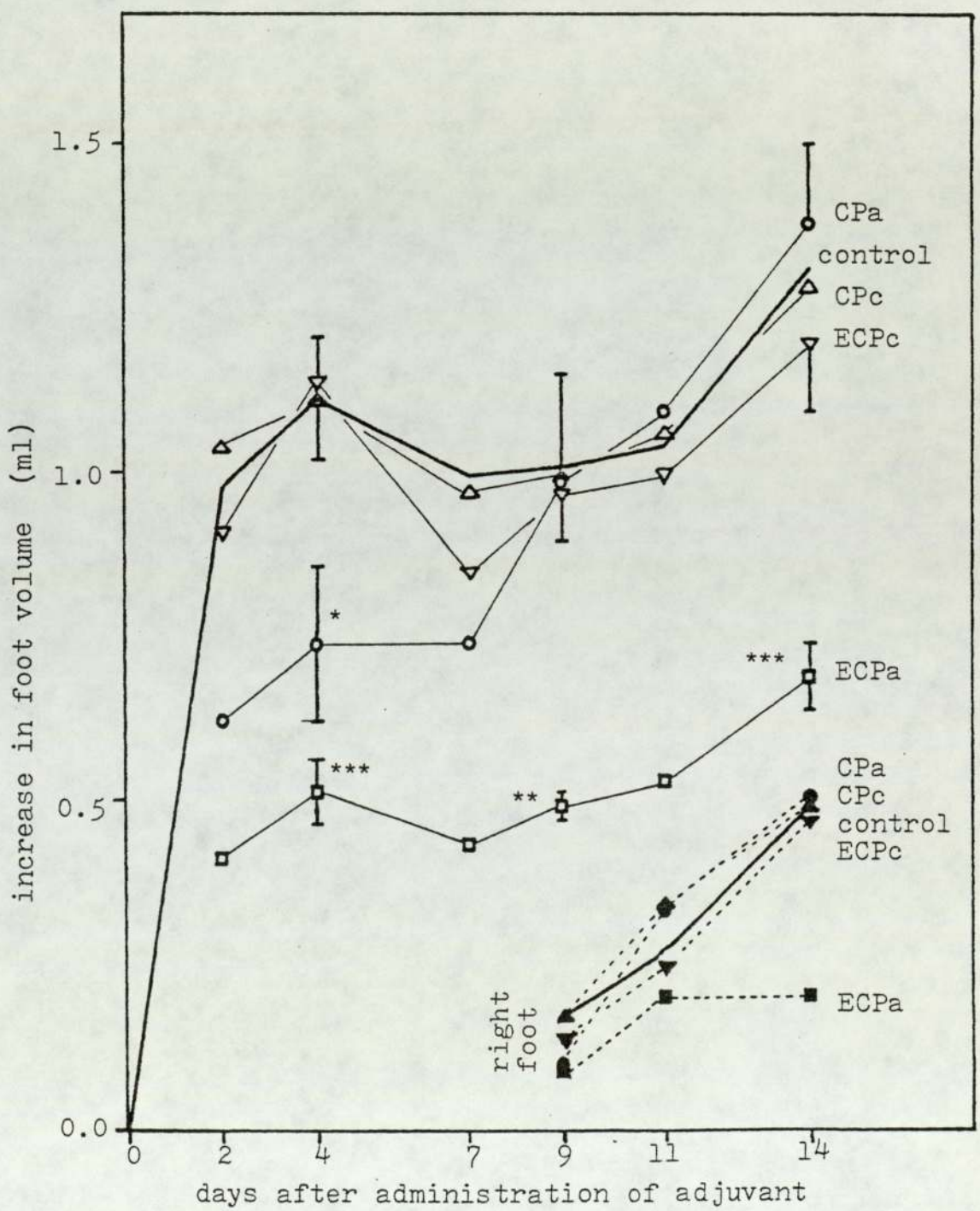
3.8.4 Comparison of free and encapsulated cortisol phosphate, injected in either the early (acute) or late (chronic) phase of adjuvant arthritis

A 7-day dose of encapsulated steroid administered on day 0 was compared with daily doses of free steroid for 7 days. Two other groups of animals received no treatment for the first 7 days, then either an encapsulated 7-day dose or 7 daily doses of the free drug. Results are shown in Fig. 3.57.

It is clear that the encapsulated preparation on day 0 achieved the greatest suppression of inflammation in both the chronic and acute phases. Daily doses of free cortisol phosphate held the disease in check during the acute phase (though less effectively than the encapsulated preparation). However, the inflammation rapidly progressed to match the controls upon ceasing the treatment. Other treatments had no significant effect.

The early treatment with encapsulated cortisol phosphate also greatly reduced the inflammation in the untreated foot.

Fig 3.57 The effect of administration of free and encapsulated cortisol phosphate in either the early or later phase of the disease on foot volume of rats with adjuvant arthritis (left = injected foot)



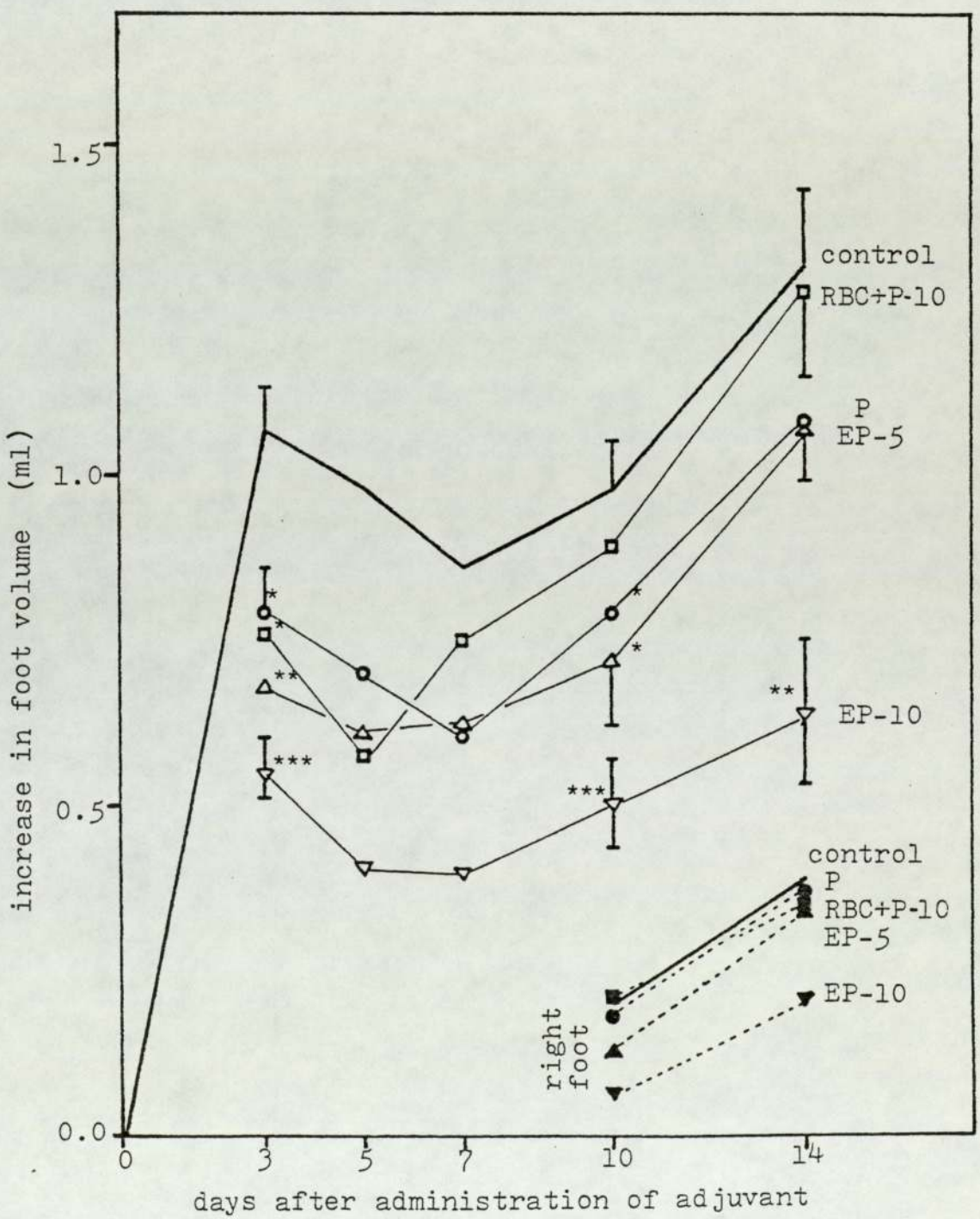
3.8.5 Comparison of encapsulated prednisolone sodium succinate with the same drug in solution or incubated with RBCs

5-day and 10-day doses were made up as for cortisol phosphate, and the results of their administration are shown in Fig. 3.58.

The greatest reduction in inflammation was achieved by the encapsulated 10-day dose. The 5-day dose had a lesser but significant effect, which was virtually identical with daily injections of the free drug. A 10-day dose incubated with RBCs produced an effect in the early stages similar to the encapsulated 5-day dose, but regressed to the control by the end of the experiment.

The encapsulated 10-day dose was likewise the most effective in keeping down inflammation of the untreated foot.

Fig 3.58 The effect of administration of prednisolone Na succinate (solution, encapsulated or incubated with RBCs) on foot volume of rats with adjuvant arthritis (left = injected foot)



3.9 In vitro tests of encapsulated preparations

3.9.1 Tests of the slow release of encapsulated drugs using dialysis sacs as model membranes (rabbit RBC)

Fig. 3.59 shows the release rate of methylene blue through a dialysis sac (modified Hanks' medium on inside and outside). The dye represents an encapsulated drug, and the sac represents the cell membrane in this simple model. The release rate curve is of the form which suggests the rate to be proportional to the concentration difference between inside and outside the membrane.

Fig. 3.60 shows the results of similar tests with cortisol phosphate inside the sac. A similar shape of curve is observed, which is reasonable since the two molecules are of comparable size (methylene blue, MW = 374; cortisol phosphate, MW = 486.5). The presence of (non-encapsulating) erythrocytes had overall no significant effect, nor did the presence of α_1AT.

For comparison, sacs were also tested containing encapsulated preparations. As is also shown on Fig. 3.60, these gave a much lower release rate, due to the lower amount of drug present, converted to cortisol within the cell, and possibly due to the slower release mechanisms from erythrocytes.

Fig 3.59 The release rate of methylene blue through a dialysis membrane (sac)

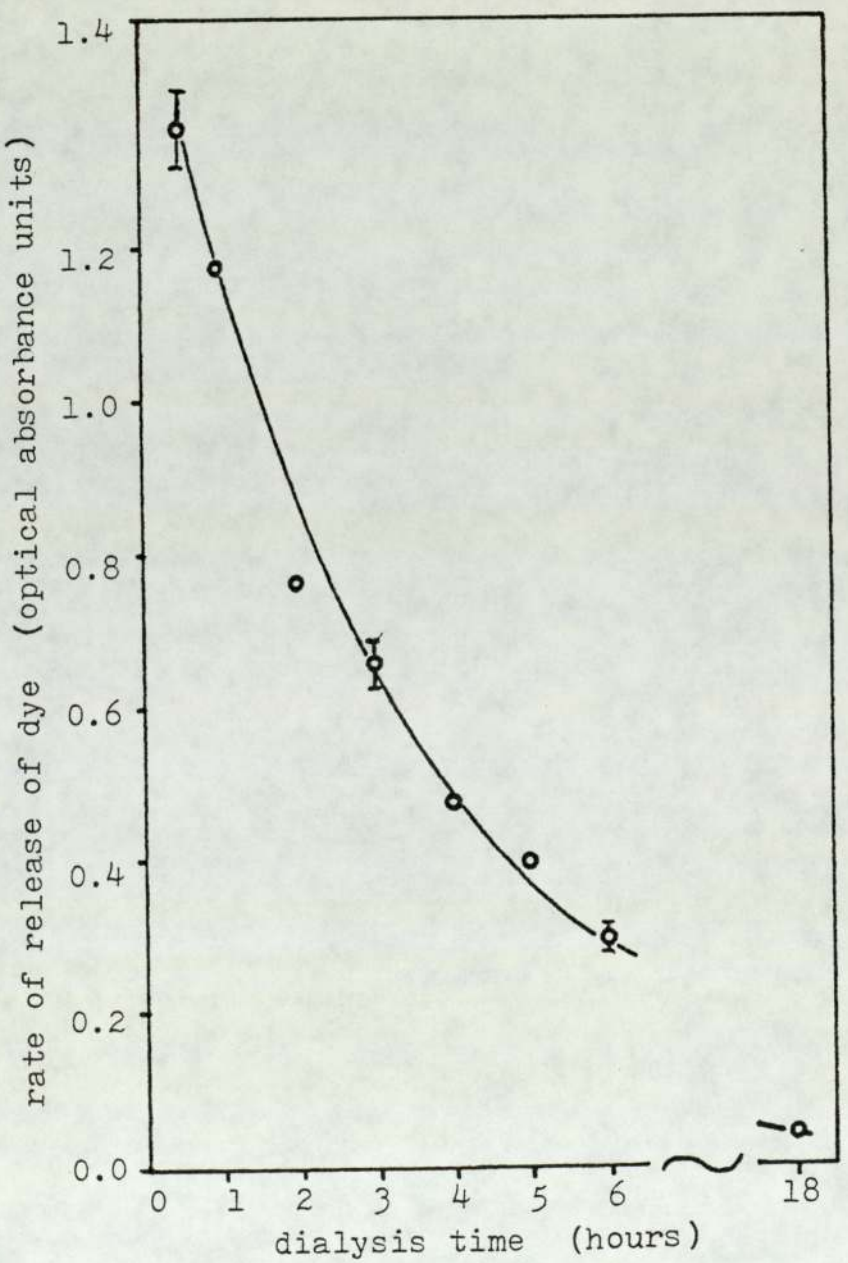
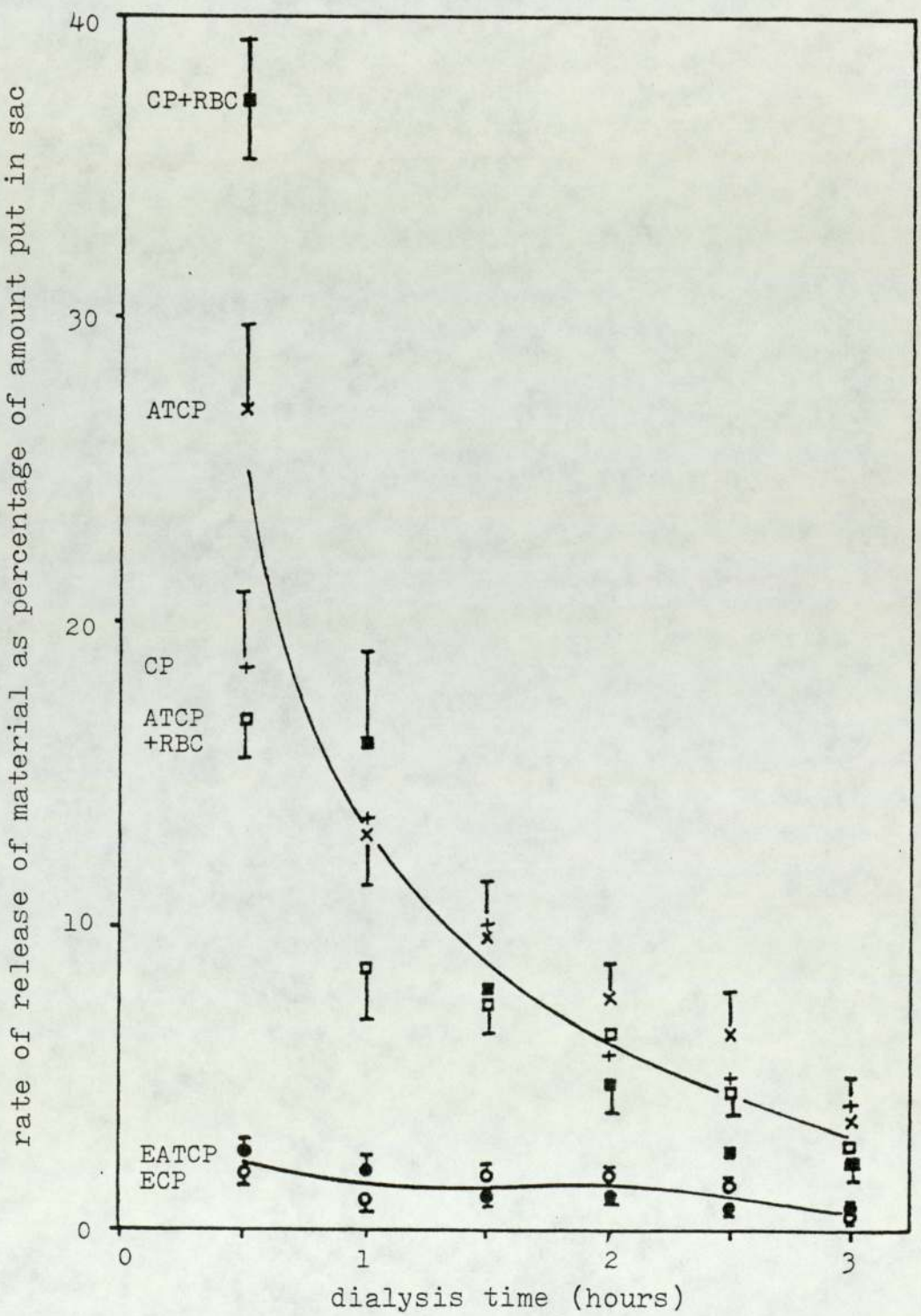


Fig 3.60 The release rate of cortisol in free and encapsulated preparations through a dialysis membrane (sac)



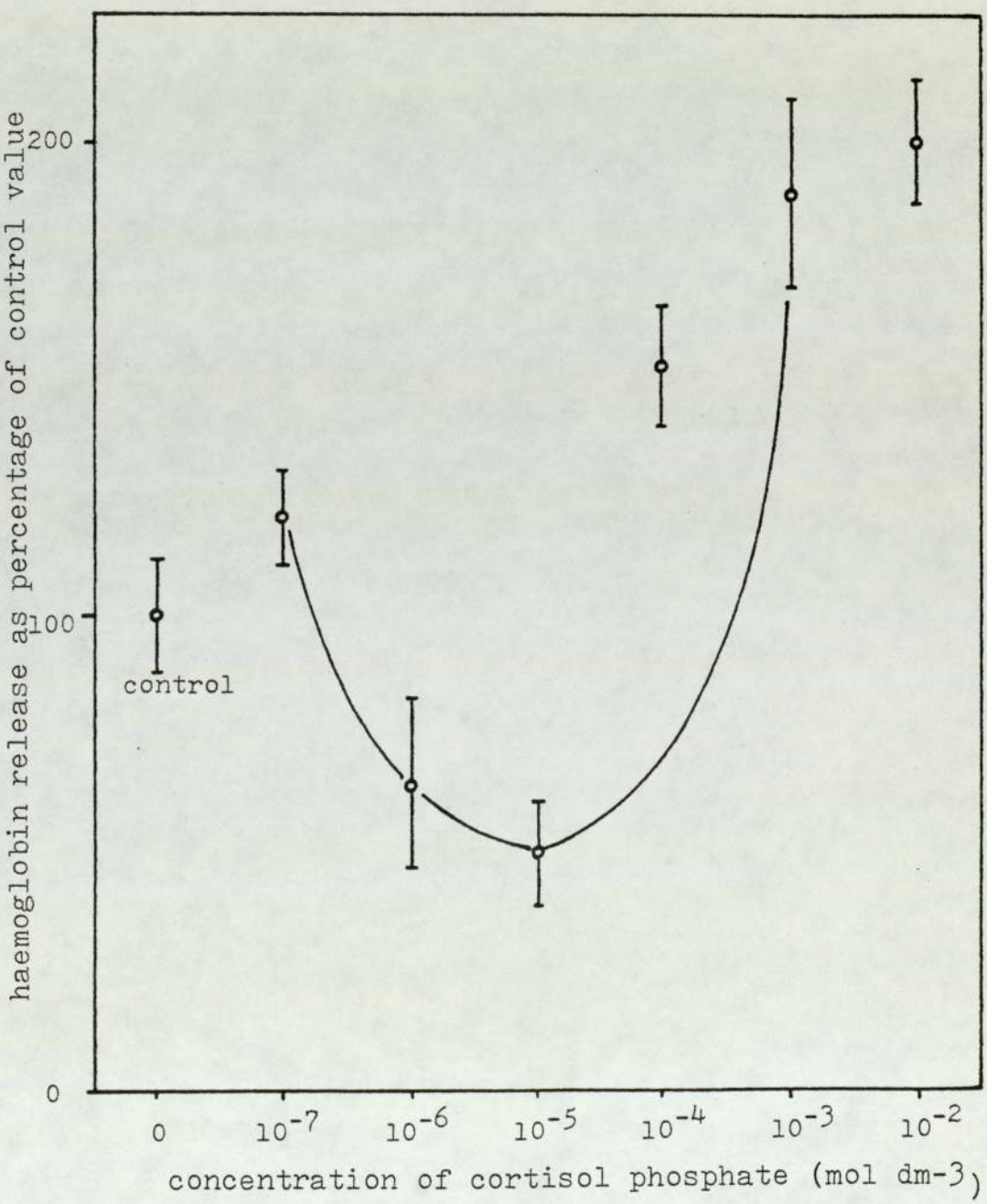
3.9.2 Stabilizing action of free and encapsulated cortisol phosphate on the rabbit RBC membrane

Incubation of RBCs at 37° C was used to give a slight lysis allowing release of haemoglobin. The amount of haemoglobin released (measured by its light absorption at 543 nm) indicated the degree of lysis.

The release by untreated normal erythrocytes was taken as 100 % . The lysis for encapsulated media only was therefore 217 % (standard deviation 1 %) suggesting that the encapsulation process itself destabilizes the membrane to some extent. However, a standard encapsulation of cortisol phosphate gave a thermal lysis of only 41 % (s.d. 2 %) i.e. more stable than a normal cell.

The degree of lysis for a range of dilutions of cortisol phosphate is shown in Fig. 3.61. It will be seen that at the lowest level measured the RBCs show similar stability to normal cells (i.e. the incubation destabilization is balanced by the drug stabilization). At the highest levels the drug is definitely de-stabilizing, but there is an intermediate optimum in which the treatment is stabilizing to this particular test.

Fig 3.61 The effect of cortisol phosphate on the stability of the rabbit RBC membrane at different concentrations



3.9.3 Investigation of erythrocyte membrane charge by microelectrophoresis after various treatments (rat and human RBC)

The electrophoretic mobility, U, is a measure of the membrane charge of a cell. In this experiment, the voltage was the same for all samples, hence U is directly proportional to the reciprocal of the time. Statistical tests of significance were therefore applied directly to the experimental results, i.e. times.

Table 3.2 Electrophoretic mobility of erythrocytes

RBC	U	time s	S.D.	s.e.m	t test	P 0.05
rat fresh	1.813	9.024	0.424	0.095	-	-
rat aged	1.815	9.012	0.356	0.080	0.096	>
rat sham encap	1.769	9.243	0.383	0.086	1.560	>
rat CP encap	1.815	9.007	0.334	0.075	0.148	>
rat ghost	1.757	9.304	0.464	0.104	2.016	<
other rat aged	1.800	9.091	0.404	0.090	0.480	>

From the results, only the grey ghosts showed any significant difference in charge.

A similar comparison was carried out between fresh and aged human RBC. No significant difference was found. Results were as follows.

Table 3.3 Electrophoretic mobility of human erythrocytes

RBC	U	time s	S.D.	s.e.m.	t test	P 0.05
human fresh	1.506	10.860	0.346	0.077	-	-
human aged	1.561	10.473	0.394	0.088	1.57	>

N.B. The term 'aged' is used for the erythrocytes which were kept in a test tube at room temperature for a time equivalent to the encapsulation procedure (as opposed to freshly withdrawn cells) to see the effect of the environment alone on the cells.

3.9.4 TLC examination of the contents of rabbit RBCs following encapsulation of cortisol phosphate

Spots of encapsulation supernatant liquor and of lysed erythrocytes (following encapsulation) were co-eluted with standard solutions of cortisol phosphate (5 mg ml^{-1} in water, R_f values 0.00 and 0.00) and cortisol (5 mg ml^{-1} in acetone, R_f values 0.723 and 0.729)

The supernatant liquor produced faint but detectable spots with R_f values of 0.723 and 0.736, which was interpreted as a trace of cortisol. The lysed erythrocytes produced definite spots with R_f values 0.750 and 0.764, which were interpreted as a significant amount of cortisol.

There is thus some action by the cells during encapsulation which results in some of the cortisol phosphate being converted to free cortisol. This was probably due to hydrolysis by erythrocyte acid phosphatase.

3.9.5 Investigation of the site of acid phosphatase activity in rabbit RBC by TLC

Sample mixtures of (a) rabbit erythrocyte membranes with cortisol phosphate and (b) rabbit erythrocyte cytoplasm with cortisol phosphate were co-eluted with standard solutions as in 3.9.4. (Cortisol R_f values 0.736 and 0.723.)

The membrane mixtures (a) gave very strong spots with R_f values 0.716 and 0.723, indicating a considerable amount of cortisol. By contrast, the cytoplasm mixtures (b) only gave very faint spots with R_f values 0.696 and 0.709 , indicating only a trace of cortisol.

Hence the main site of hydrolysis of cortisol phosphate appears to be the cell membrane (where acid phosphatase is predominantly situated). The cytoplasm has some enzyme activity, but contributes much less to production of free cortisol from the phosphate.

3.9.6 In vitro phagocytosis of encapsulated preparations (rabbit & guinea pig RBC)

Phagocytosis of erythrocytes following encapsulation was observed by optical microscopy. Figs 3.62 & 3.63 illustrate processes which were observed, and show the visible differences between viable, lysed and engulfed erythrocytes.

The following combinations were tested:

(a) guinea pig PMN + rabbit RBC, encapsulated α_1AT
(b) guinea pig PMN + rabbit RBC, encapsulated CP
(c) rabbit PMN + guinea pig RBC
(d) rabbit PMN + rabbit's own RBC
(e) rabbit PMN + other rabbits' RBC
(f) rabbit PMN + rabbit RBC, encapsulated α_1AT
(g) rabbit PMN + rabbit RBC, encapsulated CP

It was observed that RBCs from different species (groups (a), (b), (c)) produced immediate and rapid phagocytosis, both with and without encapsulated material. Conversely, PMNs remained inert to RBCs of the same species (groups (d) - (g)) at the time of mixing, and when observed an hour later. Complete phagocytosis was observed in all cases when the slide was sealed to its cover-slip and left overnight. This

is most probably attributable to lack of oxygen causing premature aging of the RBC, which would then be recognized as 'foreign' by the PMN and thus engulfed.

The process of phagocytosis occurred as follows. Within a few seconds of mixing with alien RBCs, the PMNs became active: pseudopodia appeared, and the cells were very motile. Movement of the inner cell structure of the PMN was also observed.

On contact with the RBC, the pseudopodia of a PMN would elongate until the RBC was completely surrounded by PMN cytoplasm. In the early part of the engulfment, the RBC was lysed, changing its appearance from that of a viable cell to a (slightly smaller) ghost. An individual PMN would engulf up to three RBCs (visible as vacuoles within the PMN cytoplasm). It could then denature further RBCs without engulfing, by attachment and lysing, leaving the RBC ghosts outside the PMN.

Each engulfment took 3 to 5 minutes: after 15 minutes the slide preparation mainly showed lysed RBCs and PMNs with 1 to 3 engulfed RBCs inside.

Fig 3.62 Legend

(1) PMNs and RBCs, immediately after mixing

(2) & (3) various stages of phagocytosis

(4) PMNs containing 1 and 2 phagocytosed RBCs

a = live RBC

b = lysed RBC

Fig 3.63 Legend

(1) & (2) various stages of phagocytosis by PMN already containing an engulfed RBC

(3) PMNs containing up to 3 engulfed RBCs

(4) phase contrast view: differentiates between live (white halo) and lysed (dark) RBCs

a = live RBC

b = lysed RBC

Fig 3.62 Phagocytosis of drug-loaded rabbit erythrocytes (artificially 'aged') by rabbit macrophages

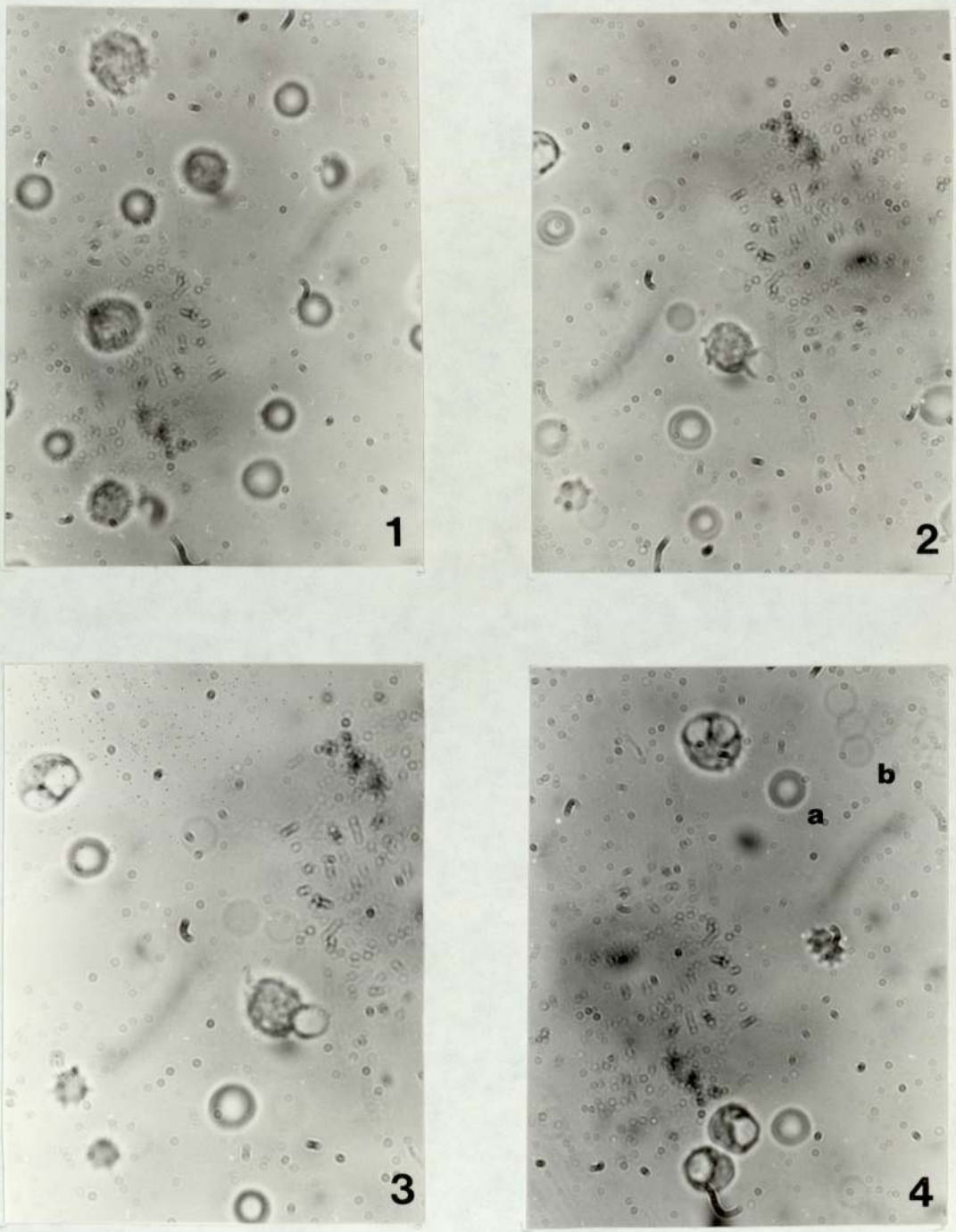
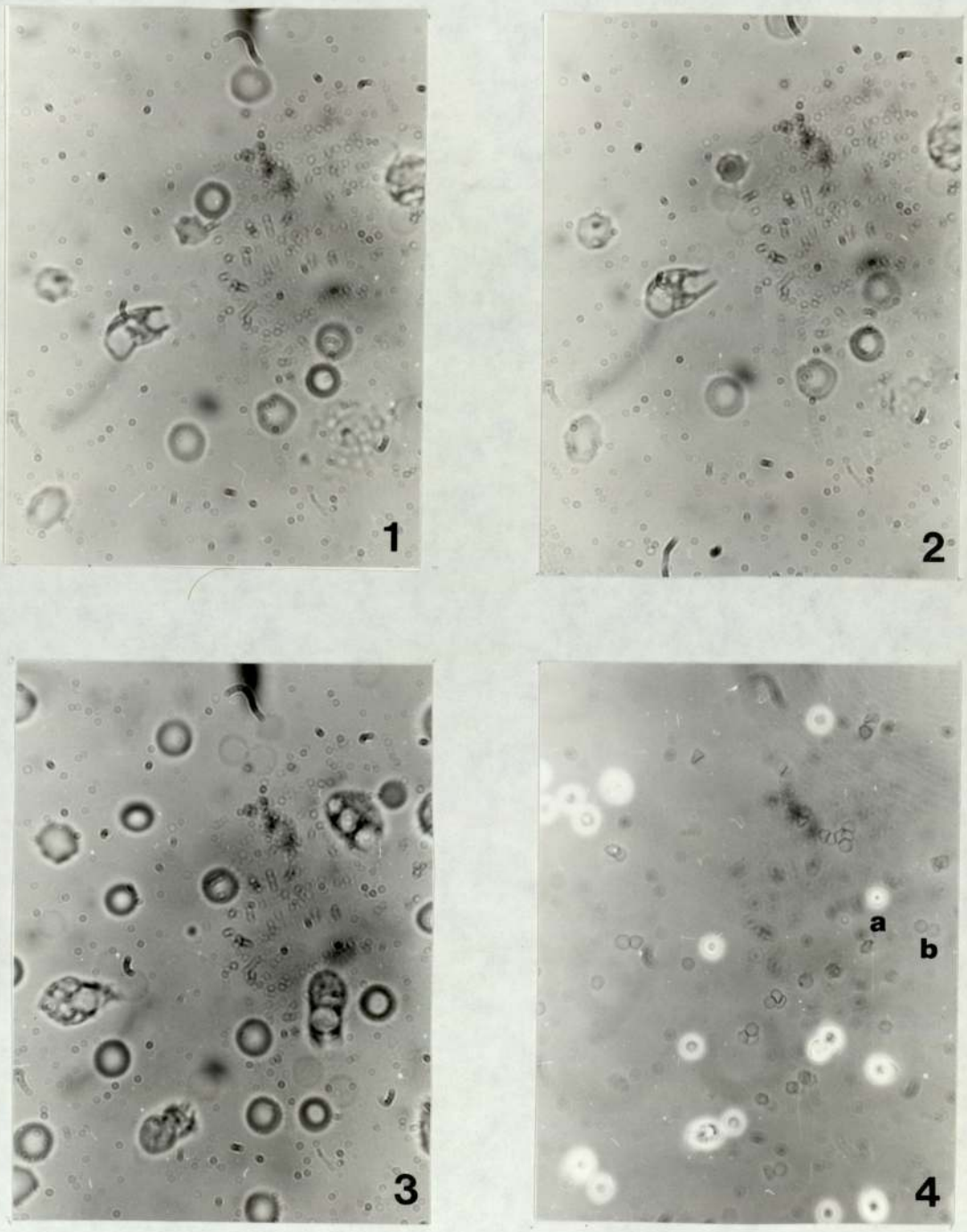


Fig 3.63 Phagocytosis of drug-loaded rabbit erythrocytes (artificially 'aged') by rabbit macrophages



3.9.7 Estimation of encapsulated α_1-antitrypsin by a trypsin inhibitory capacity assay (rabbit RBC)

Frozen samples were thawed and diluted 3 x prior to assay, and this allowed for in calculations.

For standards of 10 mg ml^{-1} of solid α_1AT in plasma, the average of the TIC assay was 5.0 mg ml^{-1}. Hence the α_1AT used was about 50 % active. For standards of the same concentration but measured in the presence of haemolysed red blood cells, the assay was 2.5 mg ml^{-1}. Hence the presence of RBC haemolysate interferes with the assay to reduce the reading by 50 %. Thus the TIC assay values were multiplied by 4 to measure the α_1AT in the samples for comparison with the amounts used in the treatment.

In the experiment, the mean TIC assays were 0.839 mg ml^{-1} encapsulated α_1AT (x 4 = 3.356) and 1.308 mg ml^{-1} α_1AT in the supernatant (x 4 = 5.232). The volume at haemolysis was made up of 5 ml packed RBC, 4 ml of α_1AT solution of concentration 20 mg ml^{-1} and 1 ml of water. Thus a total of 80 mg α_1AT was present.

The assays account for 3.356 x 5 = 16.78 mg (21 %) in the RBC, plus 5.232 x 5 = 26.16 mg (33 %) in the supernatant, with 37.06 mg (46 %) unaccounted for.

3.9.8 Estimation of encapsulated α_1-antitrypsin by "rocket" immunoelectrophoresis (rabbit RBC)

The original solution of α_1AT (made up as 20 mg ml^{-1}) used for encapsulation was used as a standard. Hence the α_1AT in the supernatant was measured as 14.8 mg ml^{-1} and that in the lysed erythrocytes was 0.8 mg ml^{-1}.

A total of 80 mg of α_1AT was added as 4 ml of solution. The volume of packed RBC was 5 ml, and the total volume at haemolysis was 10 ml.

Thus $14.8 \times 5 = 74$ mg α_1AT (92.5%) remained in the supernatant, $0.8 \times 5 = 4$ mg α_1AT (5%) was encapsulated, and 2 mg (2.5%) is unaccounted for.

3.9.9 Estimation of encapsulated cortisol phosphate by a fluorimetric assay (rabbit RBC)

Readings from a spectrofluorimeter were converted to amounts of cortisol using a standard curve.

Table 3.4 Cortisol assay

	cortisol (mg)		
	total	free	as phosphate
in erythrocytes	0.263	0.163	0.100
in supernatant	1.128	0.312	0.816
in washings	0.635	0.512	0.123
Σ	2.026	0.987	1.039

During the encapsulation 3.2 mg of cortisol phosphate was added, equivalent to 2.4 mg of cortisol. The volume of packed RBC was 3 ml, and the total volume 4.44 ml at point of haemolysis. From the assay, 15.5% of added cortisol is unaccounted for, 47% remains in the supernatant, 26.5% is washed off, and 11% is associated with the erythrocytes.

In the process, roughly half the cortisol salt is converted to free cortisol (62% in the erythrocytes).

3.9.10 Estimation of encapsulated cortisol phosphate by a fluorimetric assay after chloroform extraction (rat RBC)

Table 3.5 Cortisol assay: cell uptake

cortisol phosphate added (mg)	cell uptake of cortisol phosphate			
	total (mg)	total (%)	free (%)	as phosphate (%)
0.0	0.007	0.3	0.1	0.2
6.7	0.41	6.1	2.7	3.4
14.3	2.09	14.6	7.1	7.5
30.6	0.61	2.0	1.6	0.4

The values for zero cortisol added indicate the blank error of the method. Cortisol was added entirely as the phosphate.

It will be seen that the efficiency of the method is reduced if the concentration of CP is too high. The addition of 14.3 mg of cortisol$_{\text{phosphate}}$ (≡10 mg CP of 96 % purity - mainly due to moisture) was the optimum in this series, corresponding to a 10 day dose for treating adjuvant arthritis.

3.9.11 Estimation of encapsulated cortisol phosphate by measurement of inorganic phosphate after enzymatic hydrolysis (rat RBC)

The phosphorus concentration (as free phosphate) was measured in the cells and the supernatant liquid of a standard encapsulation in which 16 mg of the steroid salt was used. The measurements were made with and without enzymatic hydrolysis, to allow for phosphate other than that added as cortisol phosphate. Results were as follows.

Table 3.6 ppm P in cells and supernatant

phase	ECP	EOM	difference
cell + enzyme	380	196	184
supernatant + enzyme	380	200	180
cell	70	44	26
supernatant	38	42	-4

For a mass balance, the molecular weight of CP is 486.5, that of P = 31; there was 1 ml of packed cells and 5 ml of supernatant. Thus 2.9 mg was encapsulated (18%) and 14.1 mg left (88%) in the supernatant (total 17 mg or 106%).

4 DISCUSSION

4.1 The anti-inflammatory effect of α_1AT on the rat carrageenan induced oedema

In this acute model of inflammation α_1AT did not prove to be anti-inflammatory, whether injected into the footpad together with the carrageenan or when injected systemically (sub-cutaneous) for 4 days.

It is known that the initial phase of the oedema is due to the release of histamine and serotonin. The oedema is maintained in a plateau by kinin-like substances (released from the plasma substrates by the carrageenan) until the second phase of development, which is due to the release of prostaglandin-like substances (Di Rosa & Willoughby 1971; Di Rosa, Girond & Willoughby 1971).

Di Rosa & Willoughby (1971) have linked the prostaglandin activation to leucocytic exudation and migration, which has been recognized as important for the full development of carrageenan oedema (Vinegar et al 1971; Van Arman, Risley & Kling 1971).

Since α_1AT is not known to inhibit histamine, prostaglandin or leucocytic exudation, this result is to be expected. However, Van Arman et al (1965) and Di Rosa & Sorrentino (1968) have achieved substantial inhibition of carrageenan oedema with antiproteases (soybean trypsin inhibitor in the paw, trasylol intraperitoneally). This was probably due to the inhibition of the kinin-like substances in the plateau phase of the oedema.

It may therefore be possible that although no inhibition of carrageenan oedema by α_1AT was observed in this experiment, higher doses of α_1AT might prove effective. However, it seems more likely that α_1AT has little effect on this type of inflammatory oedema.

4.2 The anti-inflammatory effect of α_1AT on the rat adjuvant arthritis model

The result of this experiment suggests that α_1AT had anti-inflammatory action very similar to therapeutic dosage with cortisol phosphate: nearly 50 % inhibition of oedema was achieved throughout the course of the disease and in fact α_1AT proved better at inhibiting the spread of the disease as measured in the foot which was not injected with adjuvant.

The severity of the disease may be modified by steroidal (and non-steroidal) anti-inflammatory drugs (Pearson & ward 1959; Newbould 1963), although no drug has been found that will cure the established disease in this model. The model does not appear to select drugs that are any different from those detected by other anti-inflammatory models.

It has been suggested (Persillin 1972) that there is liver damage in the acute phase resulting in a release of lysosomal enzymes into the circulation from the liver. Invading macrophages in the inflamed joint release further lysosomal enzymes, as does the proliferating synovial tissue, giving rise to high serum protease levels.

During both the acute and chronic inflammation of rat adjuvant arthritis there is a fall in the serum trypsin inhibitory capacity (TIC) (Parrott 1976), and it may be noted that α_1AT is a major contributor to TIC activity in the rat (Huttunen & Korhonen 1973).

The high serum protease levels present in adjuvant arthritis may lead to the build-up of a pool of α_1AT - protease complexes in the circulation, which might inhibit further synthesis of active antiprotease by a feedback mechanism. Even if the complexes are rapidly removed, a high equilibrium level may be maintained if the rate of synthesis of active α_1AT is sufficiently high.

It is estimated that the daily injections of 10 mg of α_1AT during the course of the disease increased the plasma level of α_1AT by 25%, allowing for the purified α_1AT having 50 to 75% activity. In fact measurements of TIC on pooled plasma samples at the end of the experiment (day 20) showed an increase of some 20% of TIC in the rats which had been treated with α_1AT (TIC = 2.67 μmol cm^{-3} min^{-1}) compared to control untreated rats (TIC = 2.23 μmol cm^{-3} min^{-1}). (Normal non-arthritic rats had TIC = 3.13 μmol cm^{-3} min^{-1}.)

Thus the additional supply of α_1AT by injections may have corrected the lack of active α_1AT in the circulation (as complexed with proteases) and so allowed it to act as an anti-inflammatory substance in rat adjuvant arthritis.

Other anti-proteases, such as Trasylol (bovine serum inhibitor = aprotinin) have been found anti-inflammatory in the rat adjuvant arthritis model (Foerster 1969).

4.3 The stimulatory action of various steroidal anti-inflammatory drugs on plasma α_1AT and α_2M levels in the rat and a dose response investigation with liver damage assessment

The initial result confirms the findings of Parrott & Lewis (1977) in that cortisol treatment increases the α_1AT levels in the rat and also depresses the α_2M levels. (The assay does not strictly measure α_2M but a similar globulin which appears to fulfil the same function in the rat as does α_2M in humans, and whose concentration directly correlates with TPE activity in the serum (Ganrot 1973).)

In fact a similar result was found with a number of anti-inflammatory steroids (hydrocortisone, betamethasone, prednisolone, methylprednisolone, triamcinolone and dexamethasone) of which the synthetic steroids were the most active. The elevation of α_1AT corresponded to the anti-inflammatory potency of the steroid. Penicillamine did not alter the α_1AT nor α_2M levels and nor did non-anti-inflammatory steroids.

The subsequent dose response experiment showed that the elevations of plasma α_1AT followed the dose levels. It is of interest that significant elevations were obtained by doses regarded as therapeutic. There was evidence that steroids affected other liver functions (total protein, bilirubin & GOT) and that these too were

dose responsive.

Plasma total protein levels were depressed, but significantly so only by the highest doses of dexamethasone. Kim & Kim (1975) presented evidence which showed that glucocorticoids inhibit the synthesis of liver proteins in vivo. It is also possible that the total protein was depressed due to liver damage, as the liver toxin dimethylnitrosamine is known to depress plasma protein levels (Lewis et al. 1979).

Bilirubin assay is a test of normal liver function, i.e. excretion. Elevations in bilirubin are found in drug toxicity. Except for the lowest dose of hydrocortisone, all the other doses and the other steroids increased the bilirubin levels dramatically. This result points to a strong degree of liver damage, especially in the highest doses of steroids.

GOT tests for normal liver enzymatic activity. Serum levels of the enzyme increase whenever tissues containing it (e.g. liver, heart, kidney) are acutely destroyed, due to the release of the enzyme from the damaged cells. Thus GOT is an indicator of hepatocellular damage, e.g. necrosis. Each steroid gave a significant increase of GOT, except for the 2 lowest doses of prednisolone. Such elevations probably indicate hepatocellular necrosis.

However, such necrosis could not easily be seen in the liver, since frozen sections do not provide sufficient resolution.

Thus it cannot be concluded with certainty whether α_1AT increased in plasma due to the direct action of the steroid on the liver or indirectly due to liver damage (Lewis et al. 1979; Pinals 1973; Billingham, Gordon & Robinson 1971; Neuhaus, Balegno & Chandler 1966). Although hydrocortisone and prednisolone at the lower dose elevated α_1AT without altering the other parameters, which suggests direct action by the steroid on synthesis of α_1AT, the literature evidence points to hepatic synthesis of plasma proteins in response to injury. (Ibid.) Investigations with actinomycin show this to be de novo synthesis after injury (Billingham, Gordon & Robinson 1971; Neuhaus, Balegno & Chandler 1966).

The histological evidence of liver damage after steroid treatment varies in the literature. Gotjamanos (1970) reported the development of fatty changes in livers of mice resulting from sub-cutaneous adminstration of cortisone. Hepatocytes were mildly affected when a dose of 10 mg kg^{-1} was used, while at 50 mg kg^{-1} or higher severe fatty changes resulted. Similarly the development of hyperlipemia and lipid deposition in the

livers of rats given cortisone acetate (6.25 mg s.c. daily) was found by Hill & Droke (1963).

On the other hand, Alterman & Ahmad (1953) concluded that negligible changes occurred in livers of rats given 50 mg kg^{-1} of cortisone daily for 10 days. This suggests that there is a species difference in the susceptibility of liver to cortisone induced changes. Other workers report an increase in hepatic glycogen rather than lipid after cortisone treatment.

The fatty changes were clearly demonstrated in the livers of rats treated by dexamethasone (s.c.) in the work reported in this thesis. At all three doses (0.25, 1.25, 2.5 mg kg^{-1}) there was an increase of fat droplets as demonstrated by Sudan Black stain, and this was obviously dose responsive. The doses used in this experiment seem considerably lower than those mentioned by other workers, but the anti-inflammatory index of dexamethasone is roughly 25 times higher than cortisol or cortisone in man, and 200 times higher in cortisol sensitive animals (such as rodents) which would mean that the lowest dose of dexamethasone used is roughly equivalent to 50 mg kg^{-1} cortisol in the anti-inflammatory index. Such a dose produced severe fatty changes in the livers of mice and rats (Gotjamanos 1970; Hill & Droke 1963) and it suggests that the anti-inflammatory potency

of steroids and their damaging effect on the liver are interrelated.

The livers of rats treated with hydrocortisone and prednisolone were not examined histologically since no noticeable effect was expected according to the literature findings, and the biochemical tests were consdiered more sensitive and quantitative in detecting liver damage.

The route of administration of corticosteroids is certainly important in the effect on the liver (Frenkel & Havenhill 1963). Intraperitoneally injected and ingested corticoids were relatively ineffective due to their quick absorption, reduction and conjugation during their primary passage through the liver, followed by quick renal excretion. Intramuscular and sub-cutaneous injections led to a continuous and prolonged absorption of corticoid, which dissolves in the lipid, bypassing the liver and hence becoming biologically available before its passage through and inactivation in the liver.

Finally, the effect of corticosteroids on the rat weights reflects their catabolic activity (increased rate of gluconeogenesis, drain of protein reserve, and reduced mass of muscle). Hydrocortisone and prednisolone groups gained less weight than controls (normal growth) but the dexamethasone groups showed considerable loss of weight.

4.4 The effect of intra-articular administration of α_1AT and cortisol phosphate (free and encapsulated) on rabbit monoarticular arthritis

4.4.1 Measurements of joint diameter and flexibility

Measurement of joint swelling (which reflects the action of inflammatory mediators) provides a very satisfactory means of monitoring the progress of antigen induced arthritis, and correlates very well with the joint pathology.

The joint mobility measurement is a more variable parameter, but it provides another continuous assessment which indicates the course of the arthritis. The flexibility measurements are due to swelling induced by inflammation and the development of pathological lesions within the joint. The loss of joint mobility demonstrates the rapid progress of the disease in the rabbits. Some of the rabbits developed severe flexion deformities known as contractures a few weeks after challenge which restricted the movement of the affected joint by as much as 50%. In a small group size (5 animals) the occurence of a contracture in a single rabbit greatly affected the group average measurement.

As expected, cortisol was an effective agent in suppressing inflammation measured by joint diameter and mobility. Surprisingly, the high dose of steroid was less anti-inflammatory in joint mobility than the low dose and the encapsulated dose. It is possible that the synovial fluid phosphatase action lysed the more soluble cortisol phosphate to the less soluble cortisol (which thus achieved a higher concentration in the higher dose of CP) and resulting insoluble crystals may have caused irritation as in gout.

Temporary exacerbation of inflammation (flares) after intra-articular steroid injections have been known to occur in clinical practice.

The erythrocyte capsule probably protected the joint from insoluble steroid by retaining it within the cell membrane and only permitting slow release. Phosphatase activity within the erythrocyte certainly lyses the phosphate ester to the free steroid.

Alpha-1-antitrypsin showed no anti-inflammatory activity in these measurements, since proteases are probably not major mediators of inflammation demonstrated as swellings.

However, the combination of cortisol phosphate and α_1AT in both free and encapsulated forms had the best anti-inflammatory action. From injections of these preparations into non-arthritic joints it was found that these agents did not produce an irritant action in the normal joint. (An insignificant fluctuation of up to 5% was observed.) However, introduction of the treatments into already highly inflamed joints may bring about exacerbation of the condition. Indeed, a mild temporary swelling of the rabbit's joints was observed in the first 24 to 48 hours after each intra-articular injection, especially with the α_1AT and the encapsulated preparations.

The protein agents represented materials of possible antigenic origin for the rabbit, in that human α_1AT was used and no attempt was made to type the rabbit red blood cells prior to encapsulation. The presence of such antigen could mediate further inflammatory activity. It is well known that steroids act as immunosuppressive agents, and it is this action which might abolish the irritant action of both α_1AT and the erythrocyte capsule when steroid is included in the formulation. Thus the mixed preparation might be allowed to work to its full potential, i.e. as an anti-inflammatory as well as an anti-erosive preparation.

4.4.2 Biochemical assays on rabbit synovial fluids

The biochemical data show that α_1AT tended to suppress protease activity and thus the erosive potential of the synovial fluid more effectively than the steroid, since steroids are not agents which inhibit proteases directly. The combined preparation had a similar effect to α_1AT. (These results were not however significant by t-test, probably due to a high standard error of the mean.)

On the other hand the steroids were most effective at the biochemical level in controlling inflammation: total protein was depressed significantly by both doses of cortisol phosphate. A combination of α_1AT and steroid also gave a result lower than control, but not by a significant amount. [Both cortisol and α_1AT lowered n.p. significantly acid phosphatase, which represents the degree of cellular destruction (lysosome marker enzyme).

Encapsulated α_1AT and cortisol phosphate also gave a result lower than control but not statistically significant. However, in the encapsulated preparations erythrocyte acid phosphatase probably contributes to the assay result. The high level of acid phosphatase in the EATL group cannot be explained by this, since such a high elevation was not measured in the EATCPL group.

Alpha-1-antitrypsin forms inactive complexes with proteases, which can be detected by immunological assays, thus giving a higher concentration of α_1AT than when assayed by the enzymatic method, which detects only active α_1AT (Brackertz, Hagmann & Kueppers 1975).

Parrott & Lewis (1977) and also Lewis & Capstick (1977) reported that the amount of active α_1AT varies inversely with neutral protease activity.

Such a relationship was not observed in the present study. However, the assay of α_1AT was performed on pooled synovial fluid samples and so no statistical significance can be attached to the results.

4.4.3 Macroscopic examination of dissected joints

It was shown that the injection of various treatments into normal rabbit joints had no adverse effects on the joint. Similarly the erythrocyte preparations alone (groups with encapsulated media only) were well tolerated by the joint and did not appear to make the existing inflammation any worse.

The strongest anti-inflammatory action was produced by cortisol phosphate, whereas α_1AT did not reduce the inflammation as assessed in the dissected joints. However, the combination of cortisol phosphate and α_1AT produced the best anti-inflammatory effect, the free preparation rating slightly higher than the encapsulated preparation. Overall, the encapsulation of treatments proved to be of no advantage as intra-articular injections.

When the knee joints were dissected three days afer the last injection a large proportion of aged discoloured erythrocytes was present (in the encapsulated preparation groups) but not more than was delivered by the final injection. This means that between the individual injections (about 14 days) the delivered erythrocytes were cleared by phagocytosis.

4.4.4 Histological examination of rabbit synovial tissue

Injections of various treatments into normal joints does not produce any adverse reaction at the cellular level. All the non-arthritic but treated groups appeared about the same: in places there were a few PMNs and round cells and the synovial membrane had occasional villous processes. However, this variation was also observed in absolutely normal synovial samples. Saline injections likewise had no effect, although repeated intra-articular injections are reported to cause mild inflammatory responses (Cooke & Jasin 1972).

There is the same general order of treatment success as observed by the joint macroscopic examination. Cortisol phosphate, alone or combined with α_1AT (and free or encapsulated) had the strongest anti-inflammatory action and produced a marked improvement in the tissue and cellular events when compared to the controls. Unlike the macroscopic examination, where the α_1AT score was similar to that of the control, the histological examination showed that α_1AT (free and encapsulated) improved the inflammatory condition compared with controls.

Injections of high doses of α_1AT at late stages of the disease had no benefit at all, which is understandable since all the erosions had already developed by then.

Encapsulated media only (own or mixed blood) gave the same result as untreated controls, the inflammation was not aggravated (from a histological point of view) by the introduction of erythrocytes into the joint. However, the encapsulation of treatments compared to free drug therapy was not advantageous.

4.4.5 Overall conclusions on the rabbit monoarticular arthritis experiments

On the basis of this experiment, and in the light of the other experiments reported in this work, it is clear that cortisol is a relatively strong anti-inflammatory agent whilst α_1AT has weaker anti-inflammatory properties. Since inflammation is mainly due to low molecular weight inflammatory mediators such as kinins and prostaglandins, this result is not surprising. The weaker action of α_1AT is probably due to its inhibitory action on kallikrein which would depress the kinin response. However, in the later stages of the disease when proteolytic activity, including that of lysosomal origin, is involved, then α_1AT may play a role as suggested by the biochemical data.

Alpha-1-antitrypsin depressed protease, total protein and acid phosphatase levels in the synovial fluids, and this represents an anti-erosive effect. Steroid alone was not effective in depressing protease levels in the fluids to the same extent. However, the anti-erosive effect of α_1AT suffered from the disadvantage that in the absence of steroid the protein exerted an irritant action (as seen in joint diameter and flexibility measurements) which would stimulate the release of proteases. It is a paradox that although α_1AT is more effective in inhibiting proteases in the fluids, its irritant (antigenic)

reaction can result in more proteases being released. On the other hand, the steroid inhibited the inflammation and this resulted in a smaller release of protease, but once released the steroid was not effective in inhibiting the protease action.

Consequently, it is not surprising that the combination of steroid and α_1AT gave a very satisfactory performance in each test.

The overall conclusion from this experiment appears to be that it is necessary to incorporate an anti-inflammatory agent with the anti-erosive agent (α_1AT) to obtain satisfactory all-round performance on inflammatory model systems.

It had been hoped that the encapsulation would give a number of advantages for intra-articular administration of treatments. These hopes were not confirmed by this experiment. The expected advantages were as follows:

(1) the encapsulated preparation would be retained within the joint better and not allow the treatment to dissipate rapidly into the body.

(2) the encapsulated material would be more precisely targeted to the inflammatory cells by phagocytosis of the erythrocytes.

(3) a much lower amount of material would be required

to give a particular effect when supplied via the encapsulated form than by the free material.

In fact, it was found that there was no significant difference between encapsulated and free forms of treatment when administered intra-articularly. However, there was some justification of the third expected advantage, in that encapsulated material was probably more effective than the free form, since the encapsulation procedure turned out to be less efficient than was at first assumed. Nonetheless, the advantage was not as great as that reported by Dingle et al (1978) who found that entrapment in liposomes meant that steroid gave an anti-inflammatory effect in doses up to a hundred-fold less than the equivalent free drug, this being said to be due to direct incorporation of the liposomes into inflammatory cells.

No difference was found between encapsulations using the rabbit's own blood cells or pooled blood cells. This may be due to the supplied group of animals being sufficiently inbred to have the same blood group, or it may be that blood type is not important in use of the preparation for intra-articular injection.

4.4.6 Rabbit monoarticular arthritis as a practical model of rheumatic disease

It has been speculated that rabbit monoarticular arthritis may not be an appropriate model of human rheumatic disease because it is too severe to be satisfactorily treated at all: a complete cure has not been achieved by any conventional drugs, only a certain improvement or delay of the condition (Blackham 1977).

It has been shown that the best chance of ameliorating the condition is given by a dosage regime which starts as soon as possible after induction of the disease, or even doses given as pre-treatment, with conventional anti-inflammatory drugs. Even very high doses have comparatively little effect on already established disease (Ibid). Since cartilage and bone erosions are an irreversible change, effective treatment acts by the prevention of development rather than cure of these effects.

The similarity of rabbit monoarticular arthritis to human rheumatoid arthritis is described in the Introduction (Section 1.3.3). It is however worth noting here that erosions occur in animal models over a very much shorter space of time (a few weeks) than they do in human rheumatoid diseases (several years).

The main disadvantages of this model (compared with rat adjuvant arthritis) are the requirement of large amounts of therapeutic compounds (especially for oral doses) and the length of the experimental programme, both being factors which mean that the tests are comparatively expensive.

The advantages are the way in which the disease mimics the progress of human rheumatic conditions, and the larger joint size, which facilitates examination and permits intra-articular injections as a means of drug treatment, as in humans.

The most unsatisfactory feature of the experiments described in the present work was the small number (5) of animals used per group, finances being the limiting factor. This meant that the variation in response to both the induced disease and the applied treatments was even more noticeable in all the measurements performed. Even with the most careful handling of the disease induction by intra-articular injections, variations in response do exist. These were exaggerated by the small group size and although the greatest care was taken in performing the injections, there may have been some initial greater variation due to unfamiliarity with the technique.

The reliability of the results obviously decreases with group size and as an example with 4 rabbits a reduction of swelling from 5 mm to 2 mm per group is needed for a 90 % certainty of detecting as significant; with 9 rabbits a reduction from 5 mm to 3 mm would be required for the same statistical confidence (Blackham & Radziwonik 1977).

4.5 The method of encapsulation

During the research programme a few small alterations were made to the original 'minimum damage' encapsulation system (Jenner & Offord, unpublished; Jenner 1976; see section 1.7.5). One of these alterations was omitting $CaCl_2$ and $MgSO_4$ from K^+-reversed Hank's physiological medium due to problems with precipitation (see section 2.9.1). No disadvantages in the method and use of the preparation were noticed as a result.

Interestingly, Bodemann & Passow (1972) found that Mg^{++} and also Ca^{++} facilitates the resealing process in ghost preparations, and that possibly these divalent cations participate in the maintainance of cation permeability. However, the authors do not rule out the idea that in the intact red cell membrane Mn, Zn or some other metal ion plays the role which is played by Ca or Mg in the ghost preparations. On the other hand, the entry of Ca^{++} into erythrocytes is associated with the rigidity of the membrane, and the use of divalent ions for resealing ghosts should therefore be closely controlled (Ihler 1979).

The haemolysing stage was carried out at 0 °C by Jenner on the basis of the work done by Bodemann & Passow (1972) who found that resealing of erythrocytes was much slower at 0 °C than at 25 or 37 °C. The difficulty in resealing the lysed cells at low temperatures may be due to decreased fluidity of lipoprotein in the erythrocyte membrane, giving rise to increased membrane rigidity. Thus at 0 °C the haemolysed erythrocyte may retain its permeability to substances for a somewhat longer time, giving a better chance for material to be incorporated.

In the course of the present study, however, it was observed that at 0 °C the viscosity of the cell suspension was so much increased that the mixing seemed to be impaired, particularly with a small extracellular volume. A more satisfactory behaviour was achieved by carrying out the whole loading procedure at room temperature. The survival of the cells was certainly not affected by this alteration, although the effect if any on incorporation of material is not actually known.

Various loading techniques described in the literature indicate the use of both 0 °C and of ambient temperature. The evidence presented by Seeman (1967) showed that the permeable state of the cell existed only between 15 and

25 seconds after the onset of haemolysis, after which time test particles (ferritin or colloidal gold) could be found within the cells. Seeman also observed that an 'all or nothing' phenomenon controls the entry of particles such as ferritin into the cells: either there was not a single particle present or the cell was found to be richly loaded with the test particles. Although Seeman does not actually specify the temperature of his experiments, Baker (1967) also incorporated ferritin into erythrocytes in a similar experiment at 18 °C.

It was therefore concluded for the present study that if incorporation is rapid and 'all or nothing' then to maintain the pores open longer would be of no particular advantage. In fact, it might even be disadvantageous to cell viability. On a purely physical basis, there is probably some balance between having the pores open longer at lower temperatures and the lower rates of diffusion which will also occur at lower temperatures.

In general, if substances are to be encapsulated into erythrocytes the cells must be brought to the point of haemolysis to facilitate the passage of substances between the intracellular and extracellular volumes. However, it is important not to go much past this point because of the damage to the cells which will occur, affecting their

viability. It is therefore important to be able to recognize the point at which the greater part of the cells are haemolysed, and to cease dilution with water at this moment.

In the present study, several techniques were used to make the point of haemolysis as precise as possible. Firstly, it is possible to predict quite accurately the amount of water which must be added in order for the majority of cells to be haemolysed. This can be done by carrying out an osmotic fragility test on the particular cell population to be used, prior to laoding. Secondly, there is a distinct visible change in the appearance of the cell suspension as haemolysis occurs. The colour and refractive index of the cell contents normally differ from the extracellular volume so the suspension is turbid. However when haemolysis occurs there is an equilibration between internal and external solutions, resulting in the suspension becoming distinctly more clear. With practice, this could be recognized in the mixed suspension. However, I found a more sensitive test was to observe the supernatant liquid above the spun-down cells after each addition of water. The haemolysed cells are less easy to spin down at the speed used, so appear as a less sedimentable fraction on top of the pellet. At the same time, the colour of the supernatant increases (as viewed

in diffuse room light) until at the point of haemolysis the colour of the pellet and the supernatant are identical. With the help of preliminary runs for colour comparison, this was the easiest method of recognizing haemolysis, confirmed by the boundary between the pellet and the liquid becoming less distinct due to the presence of less sedimentable material. A small amount of ghosts could also be noticed, which is inevitable since the osmotic fragility of erythrocytes in any given sample will vary according to cell age and metabolic state.

Trial encapsulations were carried out whenever a different drug or concentration was to be used. Similarly the behaviour of erythrocytes from different animal species was tested in preliminary encapsulations. Rabbit erythrocytes were found to be more osmotically fragile than guinea-pig ones, which in turn were more fragile than rat erythrocytes.

The concentrations of drugs used for encapsulation were calculated on the assumption that there would be equilibration of the drug between extracellular and intracellular compartments at the point of haemolysis. Ihler, Glew & Schnure (1973) reported that haemoglobin release and enzyme (β-glucosidase) entry are parallel events, and that haemoglobin concentration after haemolysis was found equal in the intra- and extra-cellular compartments of those cells which actually haemolyse.

Since not all cells haemolyse at once, one cannot expect 100 % equilibration in a normal heterogeneous population. A 60 % incorporation of extracellular material was found under optimal conditions (Ibid). The molecular sieving property of the cells is also involved.

Similar observations were made by Jenner (1976) in encapsulation of asparaginase. The incorporated enzyme concentration was roughly the same as the pre-haemolytic concentration in the suspending medium. Since about 64 % of cells haemolysed, it was assumed that the enzyme was encapsulated in 64 % of the cells.

A certain number of cells are destroyed or lost during the encapsulation process, and so the yield of encapsulated preparation was smaller than the starting cell volume. In the present study a reduction from 1.0 to 0.7 cm^3 was generally found. Most if not all of the 'lost' cells would be ones which had probably haemolysed and perhaps transiently encapsulated some material, which would represent a discrepancy between the amount of drug incorporation immediately after encapsulation and that found in the final preparation. Taking these factors into consideration, less than 50 % encapsulation of a substance might reasonably be expected.

DeLoach and Ihler (1977) and DeLoach, Harris & Ihler (1980) have developed a dialysis procedure for loading erythrocytes with enzymes and lipids which allows for approximately 40-50 % of the added substance to be encapsulated into ghosts. In our technique, on the other hand, the maintainance of viability is an extremely important factor, and this may limit the loading capacity of our erythrocyte preparations. The characteristics of the drug to be encapsulated may be important to determine the loading success - e.g. cortisol phosphate is lysed by erythrocyte acid phosphatase to cortisol (+ inorganic phosphate) which may create a new gradient for further cortisol phosphate incorpoaraion.

It can be seen that there aremany complex factors involved in determining the amount of drug encapsulated into a sample of haemolysed cells. There certainly is a need for evaluation of the pharmacokinetics of the present encapsulation system.

Equally, the choice of drug for encapsulation is a complex matter. Experience in this research programme and appraisal of the literature suggest that the following criteria should be observed.

Sustained release therapy proves valuable when chronic dosing at frequent intervals is necessary to build up and maintain steady state plasma levels. If drugs are to be administered as encapsulated preparations, they should possess certain physical and biological properties. This assumes, of course, that once released the drug behaves the same as directly administered free drug.

(1) Sustained release dosage forms must contain more drug than a single dose of a conventional preparation. (Generally several times more.) Thus the therapeutic index of the drug should be sufficiently high to enable the safe administration of the required dose as a single entity in the sustained release preparation. Also the drug itself should have a reasonably long half-life to avoid the need for excessive single doses.

(2) The compounds should be active at low concentrations e.g. hormones.

(3) The pharmacological response should be related to plasma levels.

(4) In order to prevent continued drug accumulation, the substance should be excreted by apparent first order kinetics (linear pharmacokinetics) i.e. the excretion rate should be proportional to the amount available. (Zero order, where the drug is excreted at a constant rate regardless of increased availibilty is not suitable.)

The rate of metabolism must be greater than the rate of release of the drug, and hence the plasma levels should decrease.

(5) Compounds must be stable in vivo for the expected lifetime of the encapsulated preparation (60 - 100 days).

Some properties of drugs which would make them unsuitable for encapsulation were listed by Notari, DeYoung & Anderson (1975). For example a very short or very long half-life would make a drug unsuitable. A drug with a very short half-life will require too much in the encapsulated pool relative to a normal dose. A similar restriction applies to drugs with a large dose requirement due to the practical limits to the amount of material which can be encapsulated and administered. On the other hand, if a drug has a very long half-life it may not be necessary to administer it in a sustained form at all.

Most drugs are toxic in sufficiently high concentrations, and the presence of a multiple encapsulated dose of a very potent drug in the circulation can be potentially dangerous (and thus not an advisable therapy) because intravascular lysis might occur leading to dangerous levels of free drug. It is therefore desireable that a drug to be encapsulated should have a wide separation between the therapeutic and the toxic dose (see point 1, last page).

Drugs whose blood levels do not monitor biological activity are also unsuitable: if the therapeutic activity of a drug is independent of its concentration, it is unreasonable to attempt to maintain a constant blood level.

The physical properties of the drug should also be considered. Many drugs are lipophilic and are not water soluble. A number of drugs would be candidates for encapsulation into erythrocytes were it not for the fact that they cause serious damage to the red cell membrane, rendering the cell non-viable. Many drugs are so lipid soluble that they are able to permeate the membrane and exit from the cell at such a fast rate that their encapsulation is of little value in clinical application. (Chemical modifications to the structure of such drugs, or attaching them to a macromolecule are possible ways of limiting the rate of diffusion through the membrane.)

Certain compounds which satisfy the above requirements might be chosen from the many hormones, narcotic antagonists, β-blockers, anti-cancer drugs, anti-obesity drugs, anti-diabetic agents, anticoagulants, analgesics and narcotic analgesics, tranquillizers and antibiotics.

For clinical application to any extent, the method of encapsulation would need to be developed into a procedure more suitable for routine use on a larger scale. For example, a standard plastic blood transfusion bag might be used as the working vessel to minimize handling and contamination. Valve systems are available commercially to make the various additions and removals, and the bag itself can be centrifuged at the relatively low speeds required for the technique.

Recognition of the point of haemolysis would be crucial. At the very minimum, a standard colour card would help for matching with the appearance of the supernatant. However, current technology offers very precise spectrophotometric techniques and sophisticated control systems, so that full automation of at least this part of the technique is conceivable and might well even give a more satisfactory result than human judgment.

4.6 In vivo survival of erythrocytes after encapsulation (FITC labelling)

The survival of the encapsulated preparation in the circulation was tested by intravenous adminstration to the rat, guinea-pig and rabbit.

The fluorescent label was used in two ways - either it was encapsulated in the same way as a drug, or alternatively the cells were exposed to the label after the procedure of encapsulation had been carried out. Since FITC attaches to the exterior of the erythrocyte membrane when the cells are simply incubated with FITC, it is possible that the observed labelling of erythrocytes supposed to have encapsulated FITC was in part due to this external attachment.

In either method, the cells appeared most brilliant just after transfusion. After 24 hours in the blood circulation, some of the label had been lost, leaving cells which were slightly less brilliant, but still very easily visible and distinct from unlabelled cells.

This labelling enabled retransfused cells to be identified in the circulation for counting, and also gave the opportunity for their physical appearance to be compared with normal erythrocytes.

In fact, the encapsulated preparations appeared to be perfectly normal cells. Most importantly their size had not decreased, as is the case with some more harsh forms of encapsulation where membrane fragments are lost (leading to a smaller membrane area, hence smaller cells with reduced deformability). The crenated forms observed (which are also seen with normal cells) were most likely due to the conditions needed for microscopic examination. The spiked crenated forms can be induced when the cells are kept free of plasma between glass slide and cover slip (not so with plastic slide and slip) (Ihler 1979).

The results in all three species indicated that the survival time of the cells was not seriously shortened by the encapsulation and labelling procedures. The different life-spans for the three species were measured.

The rat is generally known to be a resilient experimental animal and is reputed to be able to harbour damaged cells in the circulation. It was therefore conceivable that the success of the technique of encapsulation might depend to some extent on the tolerance of the rat rather than the undamaged nature of the encapsulating cells. This was not borne out by these experiments showing normal survival in other species.

When cortisol phosphate was encapsulated in rat erythrocytes before labelling with FITC (at a dose used in adjuvant arthritis tests) no difference was observed in the survival of the preparation.

The decline of all labelled cell preparations seemed to follow an exponential decay curve, and a reasonably linear plot was obtained when results were plotted on semi-logarithmic graph paper. However, a certain persistent irregularity in the cell counts and therefore these plots occurred around 15 - 20 days. Mathematically such a plot could be explained as the sum of two curves: the first curve being steeper (more rapid decay) the second being shallower (less rapid decay), the region where both make a significant contribution accounting for the irregularity.

A biological interpretation might be that the steeper portion of the curve in the initial stage is due to the presence of a slightly damaged population of cells which thus have shorter lifetimes. The second half of the curve therefore represents the normal population decay. The greatest rate of loss was observed for the sample taken 1 hour after retransfusion, which would be accounted for by the very seriously damaged cells, ghosts etc, being removed from the circulation.

These observations are in contrast to those reported for previous preparations, including Jenner (1976)'s trials with minimum damage systems. The cells in some of these studies were completely cleared from the circulation (Jenner 1976; Tyrrell & Ryman 1976; Updike, Wakamiya & Lightfoot 1976; Chang 1976a; Chang 1976b; Chang 1977), with great rapidity. In the same studies those preparations that involved the mildest treatment showed a very sharp initial clearance of a significant proportion of the treated cells, followed by a removal of the survivors at about twice the rate for untreated cells.

As a control in the present study, FITC was encapsulated in resealed erythrocytes using the technique described by Tyrrell & Ryman (1976) except that the FITC was dissolved in eutonic K^+- reversed Hank's physiological medium and added to the cells prior to their complete haemolysis with water. The resulting preparation differed in several respects from that obtained by our developed minimum damage technique. The loaded cells were grey, having apparently lost most of their haemoglobin (and presumably other cell contents). Their survival time in the circulation was very short. In one hour only one third remained and after 12 hours none. At the latter time some macrophages were fluorescing, presumably due to engulfment of loaded ghosts.

Turner of Oxford University has recently (1980) used our encapsulation and labelling technique in an independent study. However, instead of using a manual count of fluorescing cells under the UV microscope, Turner had a fluorescent activated cell sorter, i.e. an automatic system which counts many more cells at great speed (in excess of 100 000 per minute). His results generally support those reported in this thesis for this particular question of survival of treated erythrocytes. He measured a 10 fold drop in the fluorescent emission of each cell after 18.5 hours in the circulation, but the remaining fluorescence was perfectly sufficient to label the treated cells. Encapsulated FITC treated cells were 11.4 % by number upon reinjection. This fell to 9.1 % in 41 hours. Assuming a perfect exponential decay, this would give a rate constant of 4.7×10^{-3} hours. For comparison, my mean results for a similar time are 7.3 % falling to 5.4 % in 48 hours giving a rate constant of 6.3×10^{-3}, which is reasonable agreement.

4.7 The effect of intravenous administration of corticosteroids encapsulated in intact RBCs on adjuvant arthritis in the rat

After the long term survival of the encapsulated preparation in the rat circulation had been demonstrated, the pharmacological effectiveness of the preparation was tested on the adjuvant arthritic rat.

Cortisol and prednisolone were used as the salts cortisol phosphate and prednisolone hemi-succinate since the amount of drug encapsulated generally depends on its solubility. Erythrocytes possess both phosphatase and esterase activities in their interiors.

In the first experiment the rat's own erythrocytes were used for encapsulating each drug and also for media only. In subsequent experiments mixed blood was used for convenience, since it was found that there was no adverse reaction and no difference in survival rate in vivo compared with rat's own RBCs. In fact, the highly inbred albino Wistar strain rats all have the same type A blood group (Ferris & Griffith 1963).

Throughout the experiments the doses of drugs used for encapsulation have been based on an assumption due to Jenner (1976) that equilibration of drug occurs

between the erythrocytes and the suspending medium at the point of haemolysis. However, the assays of amounts of drug encapsulated later showed that the encapsulation process is less efficient than Jenner suggests. It is therefore more accurate to describe the encapsulated dose as being nominal. However, the same method of dose estimation was used throughout the experiments, and these nominal doses are comparable to each other.

Since the actual amounts of drug encapsulated were in fact somewhat less than the nominal value assigned, the therapeutic effectiveness of the encapsulated preparations compares favourably with free drug dosage in that less drug is required to produce a better effect.

The initial experiment was run for 20 days. It was observed that chronic swelling occurred from day 10 and was well progressed by day 14. The effect or lack of effect of any treatment on this, the chronic phase, could therefore be readily observed on day 14. Because of the progressive crippling nature of the disease it was decided to terminate all subsequent experiments on day 14 in order to minimize animal suffering.

The results of the experiments comparing cortisol phosphate in the free and encapsulated forms are very clear. The encapsulated steroid was significantly more

effective than the free steroid injected subcutaneously in suppressing the adjuvant induced inflammation in both the acute and chronic phases of the disease. The onset of disease in the non-injected foot (also measured by swelling) was delayed by the encapsulated steroid for at least two days, and the foot swelling was greatly reduced compared to both control animals and those treated with a daily dose of free steroid.

In fact, in this experiment only, observations were made for evidence of any spread of the disease, and a subjective assessment of the severity was given a score from 0 to 3. In particular, lesions were examined on the front paws, ears and tail. A similar conclusion was reached as from the objective measurements: the encapsulated cortisol phosphate was a superior treatment to the same drug given daily subcutaneously.

The intravenous dose of cortisol phosphate given as two injections of an equivalent 10-day subcutaneous dose were ineffective against the inflammation. The steroid was probably very rapidly metabolized and excreted: the half-life of intravenously injected cortisol in man is 130 minutes (Scheuer & Bondy 1957). This means that if encapsulated cortisol phosphate was released fairly rapidly into the circulation then no anti-inflammatory effects would be observed. On the contrary, the effects suggest a genuine sustained gradual release of the drug.

Control encapsulation of media only proved that the erythrocytes themselves after the process of encapsulation do not contribute to the anti-inflammatory effect of the preparation. Neither do the operative procedures, i.e. cardiac puncture, skin incision and femoral vein injection (no counter-irritant action).

In a further experiment an attempt was made to encapsulate three different doses of cortisol phosphate to test whether there was any dose response in control of the disease. The previously used nominal dose of 10 mg (equivalent to 10 daily therapeutic doses of 1 mg) per kg was therefore increased 2-3 times.

However, there were practical difficulties in encapsulating the higher doses because the high drug concentrations exerted sufficient osmotic pressure to prevent the cells reaching the point of haemolysis even after the addition of substantial amounts of water. If the water additions had been carried on to the point of haemolysis, then the cells would have been exposed to a large extracellular volume, with consequent dilution of the intracellular components, resulting in a less viable cell preparation. Such a procedure could not properly be considered a minimum damage system.

Instead the swollen cells were returned back to tonicity (as with other preparations) in the knowledge that the haemolysis point had not been reached and that even if a part of the dose was encapsulated (due to some portion of the cells in fact having reached haemolysis) the amount was not comparable with a dose encapsulated in the normal way.

The results show that the encapsulated dose 10 was significantly anti-inflammatory as in the previous experiment. The two higher doses had an equal effect but only appeared to be significantly anti-inflammatory in the acute phase of the disease. In the chronic phase they were only mildly anti-inflammatory, for both the injected and non-injected foot. It can be assumed that the dose of cortisol phosphate contained by these preparations was actually lower than dose 10, for the reasons given above, accounting for the observed result.

There is a possibility which cannot be excluded that cortisol phosphate may be associated with the exterior of the membrane after it has been stretched, although normal erythrocytes incubated with cortisol phosphate did not show anti-inflammatory activity.

Overall, the chances are that the high dose is encapsulated into a relatively small proportion of cells, giving the preparation a total drug content less than those encapsulations with smaller nominal doses.

Thus it seems that a 10 day dose is close to the practical upper limit of the amount of cortisol phosphate which can be encapsulated into a viable preparation. Higher nominal doses will either not be entrapped due to osmotic pressure preventing pores opening, or if the mixture is diluted sufficiently for haemolysis then a less viable group of cells will result due to loss of cell contents into the excessive volume.

The dose response was demonstrated in the next experiment where the dose 10 was halved (=dose 5). As before the dose 10 significantly reduced inflammation; this group was repeatedly included in these experiments so that a direct comparison of responses could be made with a successful encapsulated treatment. The dose 5 was less anti-inflammatory, and only significantly different from control on day 3. A similar response was observed in the uninjected foot. In the same experiment the two doses were also simply incubated with erythrocytes for the duration of the encapsulation procedure, and washed afterwards. Both groups injected with such incubated preparations behaved as untreated controls did, suggesting that steroid attached to the outside of the cell did not contribute a significant source of anti-inflammatory activity.

The effect of administering encapsulated cortisol phosphate at different stages of the development of the disease was tested in a further experiment, and compared against nominally equivalent daily doses of the free steroid. It was found that once the arthritis had been allowed to develop past the acute stage, the course of the chronic stage could not be altered at all. Daily doses of cortisol phosphate from day 7, or an encapsulated dose on the same day, were equally ineffective in reducing inflammation.

When free steroid was given daily from the day of induction by adjuvant, there was a significant reduction in the amount of swelling observed in the acute phase. However, when treatment ceased the arthritis rapidly developed and was indistinguishable from untreated controls. On the other hand, encapsulated steroid administered on the day of adjuvant induction was effective in limiting the amount of inflammation throughout the experiment (acute and chronic phases) in both the injected and non-injected foot. This occurred even without a second encapsulated dose as had been given in the previous experiments.

Originally, it had been supposed that a 10-day dose given as an encapsulated preparation would not remain usefully active for much more than 10 days, so a second

'booster' dose was given on day 10. The above results do not support this idea, and it seems that loaded erythrocytes may be useful in prolonging pharmacological actions of drugs at doses much lower than the equivalent conventional therapy.

Another anti-inflammatory steroid, prednisolone, was also tested, as its sodium succinate salt. It was used encapsulated at two dose levels, in the free form, and incubated with erythrocytes. Prednisolone is known to be a more potent anti-inflammatory drug than cortisol, and the dose was appropriately reduced to make it comparable.

The results achieved were similar to those with cortisol. Daily injections of the free drug were found significantly anti-inflammatory, but the encapsulated prednisolone was superior in suppressing the adjuvant induced inflammation in both the injected and non-injected foot. A marked dose response was observed between the dose 5 and dose 10. Prednisolone incubated with the erythrocytes had some anti-inflammatory activity in the acute phase, but this effect was short-lived, and the chronic phase developed similarly to the untreated controls. Possibly prednisolone therefore has some (more than cortisol) limited attachment to the exterior of the cell wall.

Once again it can be seen that the amount of steroid required to produce an anti-inflammatory effect can be reduced by the encapsulation process. The same effect was observed with the encapsulated nominal 5 day dose as with daily injections of the free steroid.

Thus it was demonstrated that erythrocytes can be useful vehicles for the prolongation of the pharmacological action of anti-inflammatory steroids administered by the intravenous route.

It was also found that the preparations are active at lower doses than when given as free drug. In fact the assays of encapsulated cortisol phosphate indicate that about 10 to 20 % of the added drug was actually encapsulated. Although the assays give a somewhat varied result, the above experiments may be considered as a biological assay which proved the encapsulated preparations to be active and superior the the free drug dosage.

4.8 In vitro tests of encapsulated preparations

The use of dialysis sacs was employed as an analogue of the erythrocyte membrane to investigate if slow leakage through the cell wall was a likely mode for release of encapsulated material.

The experiments have shown that the free steroid salt crosses the dialysis membrane at a rate proportional to the concentration difference between the inside and the outside of the membrane, whereas the erythrocyte encapsulated preparation inside the dialysis sac acts as a slow release preparation, liberating a fairly constant amount of its load per hour. Although the true amount of cortisol phosphate encapsulated is less than its nominal value (see section 2.12.1), this lower concentration is probably not the sole reason for the release being at a relatively low and constant rate. The rate limiting step appears to be either the diffusion of cortisol itself across the erythrocyte membrane, or the conversion of cortisol phosphate to cortisol within the cell by the erythrocyte phosphatase.

In the invitro situation it is possible that a certain amount of haemolysis occurs, accounting for some of the drug release, though this would not be expected to the same extent in vivo.

Further in vitro experiments have shown that steroids actually stabilize the erythrocyte membrane against heat and mechanically induced lysis. This effect was biphasic when erythrocytes were suspended in various concentrations of the steroid in that stabilization occurs at the lower concentrations (up to 10^{-5} mol dm^{-3}) but the stabilizing effect falls off at very high concentrations. Encapsulated cortisol phosphate at a level similar to that used for intra-articular treatments of rabbits was found to stabilize the erythrocytes.

It is known that a variety of high lipid soluble or surface active compounds, such as steroids, will at low concentrations cause a stabilization of many types of membranes, including erythrocyte membranes, but can cause lysis at high concentrations (Seeman 1966; Seeman & Weinstein 1966; Lewis & Day 1972, 1975). The non-specific stabilization may be associated with an increase in the surface area to volume ratio of the erythrocytes, which may be due to an expansion of the cell membrane. Cells with a high ratio of area to volume tend to show a lower osmotic fragility and thus are associated with an increase in the critical haemolytic volume.

Although the in vivo survival time of erythrocytes with encapsulated cortisol phosphate did not differ from those encapsulating media only, there is a possibility that at certain steroid concentrations the survival time might well be improved, particularly in the intra-articular case.

The charge held by rat erythrocytes after various encapsulation treatments was tested by their electrophoretic mobility. Since the erythrocyte membrane negative charge diminishes as the cell ages in vivo (Harris & Keller 1970), the effect of encapsulation on this charge was investigated with a view to a possible "aging" effect the encapsulation might have on the erythrocytes. In fact the charge on freshly withdrawn rat erythrocytes, cells kept at room temperature, and cells after the process of encapsulation (media only and cortisol phosphate) were virtually identical (no significant difference). The only significant reduction in charge was observed in the preparation of erythrocyte ghosts.

Thus if the erythrocyte charge has any influence on the viability of the encapsulated preparation after retransfusion, the technique described in the present study had the advantage of retaining a virtually normal electric charge, whereas grey ghost preparations were found to have a significantly reduced charge.

The values of electrophoretic mobility obtained in this study for the normal rat ($U = -1.813\ \mu m\ s^{-1}\ V^{-1}\ cm^{-1}$) and normal human erythrocyte ($U = -1.506$) were slightly higher than a literature (Walter et al 1980) quoted value (rat $U = -1.08$; human $U = -1.23$). This is probably due to slightly different experimental conditions, since differing values are reported by other authors (Seeman 1965). However, a conclusive comparison between the tested groups can be made.

Walter et al (1980) report that the rat erythrocyte has a decreasing charge with increasing in vivo age (as reflected by electrophoresis) but in contrast human erythrocytes have the same electrophoretic mobility when young or old. The basis for this species difference is not known.

By thin layer chromatography examination of encapsulated cortisol phosphate, it was found that erythrocyte lysates are capable of hydrolysing the steroid ester, and that the site of this activity is predominantly the erythrocyte membrane, where acid phosphatase is mainly situated. In the dialysis sacs experiments with encapsulated cortisol phosphate, only free steroid was detected in the dialysate.

The possibility that the steroid esters are hydrolysed in the erythrocyte deserves further study. It suggests a means of encapsulating many insoluble drugs, via soluble derivatives such as phosphates. If the cell activity was inadequate to convert a particular derivative, it is even conceivable that suitable enzymes could be encapsulated along with the drug derivative. The successful encapsulation of proteins suggests that enzymes (in particular acid phosphatase) could be incorporated in order to increase the rate of conversion of cortisol phosphate within the erythrocyte and thus result in a faster rate of release of cortisol through the membrane. Possibly a greater loading of cortisol phosphate might also be achieved as the hydrolysis of the ester within the cell reduces the intracellular concentration, giving a greater concentration gradient favouring entry of more ester into the cell.

The microscopic examination of phagocytosis of encapsulated preparations helps to prove that the erythrocytes are not damaged by the encapsulation process in such a way as to be recognized by homologous PMNs as foreign cells. On the other hand, PMN engulfment of RBCs from different species indicates that the cells were alive and capable of phagocytosis at a very rapid (3 - 5 minutes) rate. (Complete ingestion by human PMNs of a

uric acid crystal was shown using a cine film to occur in 0.5 second, and degranulation in 5 seconds, by Rajan in 1975.) Some other factor was needed to induce phagocytosis of the homologous erythrocytes - such as the elimination of oxygen or cell aging. Whether such a situation occurs within the enclosure of the joint capsule can only be speculated, but the fact that the erythrocyte preparation is delivered into an unfavourable environment compared with the blood stream probably means that the erythrocyte membrane composition deteriorates and the preparation gets engulfed by resident and migrating PMNs. The contents of the erythrocyte preparation may leak out prior to or during the phagocytosis, but this phagocytosis would be the ultimate fate of the injected erythrocytes.

It was observed that in the process of phagocytosis some erythrocytes were engulfed entirely, while others were lysed, converting them into grey ghosts. The reason for differentiation into the two processes is not known, but possibly a recognition of the degree of damage shown by the erythrocytes to the PMNs is involved.

For convenience in photographing these events, rabbit PMNs and artificially damaged rabbit erythrocytes (by heat and oxygen exclusion) encapsulating preparations

were used to provide an illustration of the phagocytosis process. It was attempted to photograph the same field under the microscope at short intervals to obtain a sequence of the engulfment process.

4.9 Estimation of the concentration of encapsulated substances

The presence of haemolysate is the main drawback in estimating the concentration of a substance in the packed erythrocytes due to its interference with most assay methods for α_1AT and for cortisol. Thus the figures given for encapsulated α_1AT, obtained by TIC assay, must be viewed as an estimate rather than an absolutely precise measure. The results were adjusted to account for two known factors: the 50 % activity of the α_1AT used, and the interference by haemoglobin. The estimated α_1AT encapsulated in rabbit erythrocytes was then 21 % of the total added α_1AT, while 33 % was found to remain in the supernatant and 46 % was unaccounted for. It was fortuitous, but experimentally so, that both the α_1AT activity and the effect of haemoglobin interference were almost exactly 50 % in the standardizing tests made for the assay procedure.

The rocket immunoelectrophoresis of α_1AT should measure both active and bound α_1AT, and allowed a more direct comparison with a standard, so ought to be more reliable. According to a single assay, 5 % of the α_1AT was encapsulated, 92.5 % remained in the supernatant, and 2.5 % was unaccounted for.

Consideration of the therapeutic effectiveness of the preparations, and discussions with laboratory workers experienced in the assay procedures, suggest that the immunoelectrophoresis is likely to be a little conservative as an estimate, whereas the TIC is more probably optimistic in terms of amount encapsulated.

Encapsulated cortisol was assayed by the modified method of Peterson et al (1957) and Mattingly (1962), being the most widely used non-radioactive procedures for cortisol. However, the assay is designed for serum and plasma without the interference of haemoglobin. Therefore samples of encapsulated media only were used as blanks, and substracted from the results for actual encapsulations of the drug. The assay is in fact also specific for cortisol not cortisol phosphate. The samples were therefore hyrolysed first, which was found to be only about 50 % efficient, and the readings adjusted accordingly.

After correcting for these two limitations the amount of cortisol phosphate encapsulated into rabbit erythrocytes was found to be 11 % of the total addition. 47 % was present in the supernatant, 26.5 % in the washings of the resealed cells and 15.5 % was unaccounted for.

The fluorimetric assay of cortisol was also carried out in rat erythrocytes after first extracting the cortisol phosphate by chloroform to eliminate haemoglobin interference, followed by total enzymatic hydrolysis to convert the ester into cortisol. The results are in agreement with the previous assay in that 14.6% of the total added cortisol phosphate was encapsulated into erythrocytes. About half the encapsulated material was in the form of the free steroid and about half in the form of the phosphate. (Note the different species used.)

The amount used was the same as the nominal 10-day dose used for the rat adjuvant arthritis tests. Assay of the 5-day dose showed that only 6.1% of the steroid was encapsulated, and for the 20-day dose only 2% was encapsulated. This is in agreement with the effect these preparations had on the rat adjuvant arthritis, where the 10-day dose proved optimal.

Cortisol phosphate was also estimated by enzymatic hydrolysis then measurement of inorganic phosphate released. Allowance was of course made for the phosphate derived from the cells and the medium. According to this measurement, 18% of the cortisol phosphate was actually encapsulated, 88% was in the supernatant, a mass balance of 106%, which is reasonable for the expected accuracy.

The inorganic phosphate assay was thought to be relatively reliable, and may be commented on in a little more detail as follows.

The Hank's (modified) solution contained 43 ppm P. The supernatant should be virtually the same,as should the cell content apart from the added material. Within experimental error, the cortisol phosphate concentration within the cell was the same as in the supernatant, suggesting equilibrium or near equilibrium. Without added enzyme, the cortisol phosphate did not contribute to the measured inorganic phosphate in the supernatant. Without added enzyme, the added cortisol phosphate resulted in the erythrocyte internal inorganic phosphorus being elevated by 26 ppm. This is equivalent to 14 % of the encapsulated cortisol phosphate being converted by cell enzymes to free or protein bound cortisol.

There was obviously some variation between the methods of assay in the experimental results, but this was to be expected under the circumstances already mentioned. Although none could be totally relied upon, overall it can be seen that the amount of α_1AT encapsulated was of the order of 5 to 20 % of the total added. Similarly, some 10 to 20 % of the added cortisol phosphate could be encapsulated.

These figures can be compared with the maximum which might theoretically be achieved under ideal conditions. At the point of haemolysis, the extracellular and the intracellular volumes are approximately equal. If the substance to be encapsulated was evenly distributed throughout the combined volume, it can therefore be seen that 50 % would be within the cells. This is thus the absolute maximum which could be encapsulated. (Even under ideal conditions, a slight loss would be expected as the cell volume shrinks during resealing.) In fact, it has been reported that for ghosts under optimal conditions, 40 to 50 % take-up can be achieved (DeLoach & Ihler 1977; DeLoach, Harris & Ihler 1980).

With grey ghosts, all the cells are haemolysed and their interiors come into contact with the extracellular phase. However, in the minimum damage system to produce viable resealed cells, no more than 60 % actually reach the point of haemolysis. The intracellular volume of the remainder is thus unavailable. A calculation on this basis gives 37.5 % as the maximum encapsulation achieved assuming perfect equilibration. In fact, the kinetics of our system of encapsulation are not known. The assays therefore suggest a substantial portion of the possible encapsulation is achieved under optimal conditions.

It is possible that more material could be loaded into the cells by a suitable modification of the technique, though this might very well reduce the viability of the resealed cells. However, a long lifespan may not be strictly necessary for intra-articular adminstration. More important, the preparations were found to be pharmacologically active at lower doses than was at first assumed from the supposed efficiency of encapsulation. This could have the very beneficial effect of lowering the therapeutic dose required for intravenous injection, as well as the advantage of a slow release therapy.

4.10 Conclusion

It is probably true to say that the original hope was not fulfilled that erythrocyte-encapsulated α_1AT by intra-articular injection would prove to be a useful therapy for rheumatoid arthritis. However, when α_1AT was combined with cortisol phosphate in a free or encapsulated form a superior therapeutic effect compared to the separate substances was obtained. Therefore investigation of drug combinations for encapsulation is worthwhile with the aim of improving their therapeutic effect.

An unexpected benefit was the development of the encapsulation technique to the point where the loaded erythrocytes remain fully viable in the blood circulation. The present experimental work has demonstrated that erythrocytes can be a useful vehicle for the prolongation of the pharmacological action of anti-inflammatory steroids administered by the intravenous route. The technique could be applied to a wide range of drugs and their combinations apart from anti-inflammatory agents, and so is of potential clinical use. It is likely that intact erythrocytes would have some advantages over liposomes and the more damaged forms of erythrocyte preparation as agents for encapsulating and delivery of pharmacologically active substances. In fact, some commercial interest has already been expressed in the technique.

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