THE FIBROGENIC RESPONSE TO TISSUE DAMAGE

by

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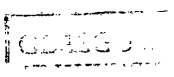

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DECLARATION

The work presented in this thesis covers a wide range of activities and methodology and could not have been attempted without the able assistance of those previously acknowledged. I wish to emphasise that all the ideas presented were developed by myself. I initiated and planned all experimental work. With the notable exception of electron microscopy, I instructed technical staff in all other techniques. I personally performed pilot experiments or confirmatory experiments in each area of work.

PRESENTATIONS AND PUBLICATIONS

Presentations :-

Presentation to Pathological Society - Summer Meeting 1977.
Collagen prolyl hydroxylase levels in experimental paraquat poisoning.
W.D. Thompson and R.S. Patrick.

Presentations to Pathological Society - Winter Meeting 1979.

Demonstration of macrophages in tissue sections by immuno-histochemistry and the in vivo formation of macrophage giant cells in damaged mouse liver.
A.S. Jack, W.D. Thompson P.G. Toner and R.S. Patrick.

The possible role of macrophages in hepatic fibrogenesis induced by acute carbon tetrachloride injury.
W.D. Thompson, A.S. Jack and R.S. Patrick.

Presentation to Pathological Society - Winter Meeting 1980.

Immunohistochemical demonstration of mouse macrophages by light and electron microscopy.
W.D. Thompson, J. Richmond and R.S. Patrick

Publications :-

Collagen prolyl hydroxylase levels in experimental paraquat poisoning.
W.D. Thompson and R.S. Patrick.
British Journal of Experimental Pathology, (1978) 59, 288-291.

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The collagen prolyl hydroxylase activity of hepatocytes and mesenchymal cells isolated from normal and regenerating rat liver.
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Investigative and Cell Pathology.

Publications in preparation :-

The mononuclear phagocyte system of the mouse as demonstrated by the immunoperoxidase technique using anti-mouse macrophage antiserum.
W.D. Thompson, A.S. Jack J. Richmond and R.S. Patrick.

Transient giant cell formation in experimental carbon tetrachloride liver injury.
A.S. Jack, W.D. Thompson, P.G. Toner and R.S. Patrick.

Collagen stimulating factors from lung in experimental paraquat poisoning demonstrated in vitro and in vivo.
W.D. Thompson and R.S. Patrick.

SUMMARY

This thesis is a study of the control of fibrogenesis following tissue damage. Existing knowledge of the relevant cells, macrophage and fibroblast, is reviewed. Previous work from this laboratory concerning factors extracted from damaged tissue which stimulate collagen synthesis in vitro is discussed and extended.

The distribution of macrophages has been studied in mouse tissue sections by immunohistochemistry using an antimacrophage antibody. The normal macrophage population has been demonstrated in a wide variety of mouse tissues, the shared common antigen confirming the unity of the mononuclear phagocyte system. The technique was employed at the electron microscopic level showing that staining of Kupffer cells in liver was located at the surface membrane. The technique was applied to animal models of liver and lung injury used in subsequent work. The sequence of events leading to transient giant cell formation after carbon tetrachloride liver injury is defined using immunohistochemistry and electron microscopy. The results support the concept that such giant cells arise by fusion of macrophages around necrotic debris.

The role of the macrophage in recovery from experimental liver injury produced by carbon tetrachloride was investigated. It was found that the healing process could be manipulated in various ways by impeding macrophage function with corticosteroid or carrageenin. Interference with normal macrophage function in clearing necrotic debris in turn interfered with the normal phase of increased fibrogenesis, as assessed histologically and biochemically. The findings support the initial hypothesis that macrophages influence fibroblast collagen synthesis at sites of damage.

Experimental paraquat poisoning provides a striking histological example of rapid fibrogenesis following extensive pulmonary alveolar epithelial destruction. Collagen prolyl hydroxylase activity, an index of collagen synthesis, was found to increase five-fold in rat lung with

parallel changes in serum but not in other organs. The pattern of biochemical changes has been related to the time-course of the histological changes. Collagen stimulating factors, previously described in experimental liver injury and healing skin wounds, have been demonstrated in paraquat lung injury. This finding suggests that such factors may be involved in the chronic inflammatory process at any site.

Arterial collagen synthesis is increased in experimental hypertension in the rat. The absence of a macrophage component and of necrosis in this model was the reason for its choice. The time-course of increased collagen synthesis in the aortic wall was defined by measurement of prolyl hydroxylase activity. Collagen stimulating factors were found to be present as the blood pressure rose to its highest level after six weeks. The implications of this finding are discussed with regard to the pathogenesis of hypertension and the possible source of the factors.

In attempts to quantify the effect of collagen stimulating factors, molecular filters rather than column chromatography were tried. The results with cultured fibroblasts as the test system for stimulation of collagen synthesis show that both the stimulating factors and toxic component are of higher molecular weight than previously described but can still be separated by the method. Initial results from intra-peritoneal injection of filtrates into mice show that the factors stimulate increased collagen synthesis <u>in vivo</u>, and are not merely an <u>in vitro</u> phenomenon.

Recent work is described concerning preliminary <u>in vitro</u> experiments with supernatant from cultured macrophages and fibrin. When applied to chromatography columns, this gives fractions which stimulate collagen synthesis in cultured fibroblasts. The pattern obtained resembles that of collagen stimulating factors as obtained from damaged tissue.

The major hypothesis that has emerged from the present work is that the enhanced collagen synthesis, characteristic of the chronic

inflammatory response is controlled by macrophages, and possibly endothelial cells. This is achieved by degradation of necrotic tissue and fibrin by enzymes from these cells producing small peptides with collagen stimulating activity.

PREFACE

This thesis is concerned with various aspects of the interaction of macrophage and fibroblast in the chronic inflammatory response induced by necrosis. In the introductory Chapter current knowledge of each cell type is briefly reviewed with emphasis on control of the cell functions described. Previous in vitro work on factors stimulating collagen synthesis is summarised with the exception of that related to macrophages which is postponed until Chapter 7. In Chapter 2, a new method of demonstrating macrophages in tissue sections is described and applied to two animal models used in subsequent chapters. The role of the macrophage in experimental liver injury is studied in Chapter 3 by manipulation of the course of events in the healing process in vivo by impeding macrophage function. In Chapter 4 another example of experimental injury, experimental paraquat lung damage, is studied for its intrinsic interest as a model of rapid fibrogenesis in the first part, and as a possible source of collagen stimulating factors in the second part. Experimental hypertension, where arterial collagen synthesis is increased in the absence of a macrophage component, is considered in Chapter 5. Various attempts to quantify the effect of collagen stimulating factors are described in Chapter 6. Chapter 7 is concerned with the development of an in vitro method of producing collagen stimulating factors from macrophages and fibrin.

Finally, the central theme of this thesis - how might macrophages control fibrogenesis ? - is discussed in the light of the insights gained from the various experimental approaches and a new hypothesis proposed.

CHAPTER 1

GENERAL INTRODUCTION

The reaction of living mammalian tissue to injury sufficiently severe to cause cell death involves the dynamic pathological processes of acute and chronic inflammation. These processes form a response which proceeds to repair the tissue and is accompanied by a variable degree of regeneration of specialised components. The acute inflammatory reaction is the immediate host response to injury. Increased blood flow, exudation of plasma proteins, and emigration of polymorphonuclear leucocytes characterise this response. Mild degrees of damage induce this transient reaction which then subsides with no deleterious effect on tissue architecture. With more serious damage this reaction merges after about 12 hours into the chronic inflammatory reaction which persists until tissue architecture is stabilised.

The chronic inflammatory response may be regarded as a system which has evolved to restore tissue integrity after damage of varying aetiology. If one were to devise such a system, imagining it as a design problem, it would surely need to be composed of the following elements : recognition of tissue damage ; removal of cell debris ; restoration of the physical defect by substances of sufficient structural strength.

These design imperatives can be readily seen to underly much current knowledge of inflammation as will subsequently be discussed. But there are further implications of such an apparently simple design. The body must recognise when removal of cell debris is complete and the mechanism for removal must cease. Structural repair must progress at a rate which matches removal of debris, and its cessation must shortly follow the completion of removal. This thesis is concerned with the cell types involved in these processes and especially with their interaction in the control of tissue repair.

Two cell types predominate in chronic inflammation - the macrophage and the fibroblast. Capillary proliferation is a less constant feature and since it is not found in the various animal models described later, this aspect is not considered further. There is good evidence, however, for the involvement of macrophages in this process (Polverini et al, 1977).

Cell death is the eventual product of a wide variety of pathological conditions and its common sequel, fibrogenesis, may result in untoward pathological consequences despite its basically reparative nature. These may vary from the simplicity of distortion from a skin wound contracture, to the complexity of acute viral hepatitis continuing into a chronic phase with hepatocyte necrosis associated with an immune reaction leading to chronic active hepatitis with fibrosis and eventually cirrhosis.

The Macrophage

Macrophages are highly mobile phagocytic cells which emerge from venules into necrotic tissue after about 12 hours. Although the morphology of this process has been studied for over a hundred years since the days of Cohnheim, the mechanisms responsible for cell adhesion to endothelium for example are largely unknown. The topic has been reviewed by Wilkinson and Lackie (1979) and it is obvious that there is similar ignorance concerning emigration from blood vessels. Once outside the vasculature, the better understood phenomenon of chemotaxis occurs, although much knowledge in this area comes from *in vitro* work. Chemotactic factors from complement activation, bacteria, and lymphocytes are recognised and there is evidence that necrotic cells may also release such factors (Bessis, 1973), though these have not yet been characterised. Partially denatured proteins are also chemotactic *in vitro*. More significantly perhaps, there is now evidence that fibrinopeptides B and Y are chemotactic (Kay et al, 1974 ; Mackenzie et al, 1975). Fibrin deposition is a major consequence of acute inflammatory exudation

and activation of fibrin-bound plasminogen is known to follow. Polymorphs, macrophages and endothelial cells secrete plasminogen activator. Native collagen, collagen chains and particularly collagen peptides are known to be chemotactic for macrophages but not neutrophils (Postlethwaite and Kang, 1976). Degradation of collagen at sites of inflammation by polymorph collagenase certainly occurs but although collagen peptides, like the other factors mentioned, are chemotactic, their effect on the control of recruitment of leukocytes from the vasculature is unknown. Chemotaxis itself is probably enhanced by prostaglandin E2. Prostaglandins are known to derive from various sources including blood vessels, polymorphs, lymphocytes, platelets and macrophages themselves (Johnston et al, 1979), and research in this field is accelerating.

Macrophages arise from emigration of blood monocytes deriving from myeloid precursors in bone marrow. The structure of the macrophage is considered in Chapter 2. This distinctive cell line forms the mononuclear phagocyte system first proposed by van Furth and Cohn (1968). The many properties of this cell type and its interrelationships with the immune system are reflected in the large literature which seems to have entered an exponential growth phase (2,000+ entries per year in Index Medicus). Most topics of importance are succinctly reviewed by Allison (1978).

Macrophages require to be "activated" before developing their full range of activities and a wide range of in vitro agents have been used for this purpose. Three factors are though to be the most relevant in vivo ; products of activated T lymphocytes, immune complexes and the complement cleavage product C3b. Macrophages may produce a variety of hydrolase enzymes which can be classed in 3 groups according to how production is affected by phagocytosis or stimulation (Gordon et al, 1974). Lysozyme is produced continuously even by non-activated cells. Plasminogen activator is an inducible enzyme promoted by stimulation of phagocytosis. Both of these are

unaffected by conditions causing increases in intracellular acid hydrolases. Prostaglandin E_2 is capable of regulating macrophage collagenase production in vitro (Wahl et al, 1977). These complex control mechanisms are readily studied in vitro but their in vivo role is largely a matter of speculation. It is at least established that these enzymes are packaged by the Golgi apparatus into primary lysosomes and secretory vacuoles. The primary lysosomes fuse with phagocytic vacuoles thus degrading the ingested material. The secretory vacuoles and possibly some primary lysosomes fuse with the plasma membrane releasing extracellular enzymes (Cohn, 1975 ; Dannenberg, 1975).

Apart from enzyme production and secretion, activated macrophages may release activated complement components, interferon, endogenous pyrogen, colony-stimulating factor, tissue thromboplastin and prostaglandins. They may ingest and kill micro-organisms and prevent multiplication of, or kill, tumour cells. Some of these functions are known to be aided by surface membrane receptors for the Fc component of IgG, immune complexes, C_3 component of complement, and denatured protein.

When one considers the ingestion and removal of necrotic cell material by macrophages, one might expect many of the functions described to come into operation, and for macrophages to be continuously recruited into the lesion so long as debris and phagocytic activity persist, as has been described by Spector (1969). Further than this, Ross (1968) considering wound healing observed "an optimal inflammatory response appears to be an important rapid non-specific stimulus for fibroplasia".

The Role of the Fibroblast

The fibroblast has long been regarded as the cell type in mammals which produces collagen. This is indeed the major synthetic product and as such indicates a degree of specialisation. Collagen is now a recognised constituent of a much wider range of structures than just the thick eosinophilic fibres of connective tissue (Miller, 1976). Langness and Udenfriend (1974) first showed that a variety of epithelial and non-epithelial cell lines were capable of collagen synthesis and since then practically every cell type examined has been found to exhibit at least minimal collagen synthetic activity. This is perhaps unsurprising as collagen is the most abundant protein and the major structural constituent produced, not only by mammals but by all animals, with the exception of single-cell protozoa and the arthropods which depend on chitin. Shape and mechanical functions are determined by the pattern of collagen deposition : properties determined by cellulose in the plant kingdom.

Accordingly, the fibroblast may be regarded as the cell type in which collagen production is least repressed. The fibroblast has been mainly studied in tissue culture and skin wounds. By light microscopy it is typically spindle-shaped, though triangular and stellate forms are seen during active proliferation in vivo and in vitro. Electron microscopic features of note are the prominent rough endoplasmic reticulum, and an unusual Golgi zone with saccules and vesicles throughout the cell, thought to provide the ability to secrete material from all parts of the cell surface (Ross, 1968). Cytoplasmic filaments are also prominent and fibroblasts, like many other cell types in addition to muscle, are now known to contain actin-myosin bundles. In his review of fibroblast functions Abercrombie (1978) states that the cell is capable of moving at 100 μm per hour but that the movement is random unless another cell is touched. Then that cell extension forms an adhesion and another part of the cell periphery will form the leading edge. This process results in a directed streaming of fibroblasts into unoccupied space. There is good evidence

that fibroblasts are contractile cells and that wound shrinkage is an active process attributable to this phenomenon (Gabbiani et al. 1973).

Evidence has recently been appearing suggesting that fibroblast movement in vivo might not be as aimless as in culture. Postlethwaite et al. (1976) showed a convincing quantitative chemotactic response in Boyden chambers to a heat-stable protein from lymphocytes exposed to either specific or non-specific mitogens in vitro. They used Ficoll-Hypaque separated peripheral blood mononuclear cells which they found were 24% monocytes by esterase staining. Separation of these cells from the lymphocytes abolished the effect but the authors did not report testing monocytes alone. Perhaps this intriguing effect is yet another macrophage function. The same group have described fibroblast chemotaxis towards type I II and III collagens and collagen-derived peptides and even di- and tri-peptides containing hydroxyproline (Postlethwaite et al. 1978). Using ^{14}C labelled α_1(I) chains, reversible binding to the surface of fibroblasts was demonstrated, and confirmed using an anti-α_1 (I) antibody for immunofluorescence (Chiang et al. 1978). These workers even estimated the number of binding sites - 16×10^6 sites per fibroblast using Scatchard plot analysis.

At this point the phenomenon of chemotaxis seems to merge again into cell adhesion and locomotion. Bornstein and Ash (1978) demonstrated both collagen and fibronectin by immunofluorescence forming a reticular pattern on the surface of fibroblasts and smooth muscle cells. When the cells were attached to a substratum, they found that they did not exhibit "capping", that is, no antibody-induced translational movement in the membrane. Since cells in mitosis did not stain, and since cells dissociated with trypsin only regained staining for both proteins some time after replating, they concluded that the surface collagen and fibronectin were synthesised from within rather than merely adherent. A separate group has claimed that a specific section of the α_1 (I) chain forms the binding site for fibroblast attachment to collagen (Kleinman et al. 1978). One may now speculate whether fibroblasts, normally adherent to surrounding collagen, become detached by proteases

including collagenase washing past from an inflammatory reaction and are provoked to move towards the site of collagen degradation, where the provision of new collagen would be appropriate.

Apart from collagen, fibroblasts synthesise and export the glycosaminoglycans of ground substance, elastin and fibronectin. The complement component C1q contains a collagen-like hydroxyproline-rich sequence (Calcott and Muller-Eberhard , 1972) and has been detected in fibroblasts (Al-Adnani and McGee, 1976).

Human skin fibroblasts in culture synthesise both type I and type III collagen (Gay et al. 1976). In tissue type I forms thick eosinophilic fibres and type III forms reticulin. In culture there appears to be rigid control of the proportions secreted by fibroblasts, the relative proportions remaining constant at different phases of growth (Hance and Crystal, 1977). This may not be so in pathological states as Narayanan et al. (1978) found largely type I collagen being secreted by fibroblasts from chronically inflamed gingiva. In experimentally injured liver and skin wounds, type III collagen predominates at first. (See Chapter 3). Deshmukh and Sawyer (1977) found that the type of collagen made by chondrocytes in culture was type I when attached but the normal type II when in suspension. A variety of other agents such as calcium concentration could also induce gene switching.

The detailed biochemistry of collagen synthesis and its disorders have been recently reviewed in some depth (Prockop et al. 1979 ; Minor 1980). The major steps are summarised in Figs. 1:1 to 1:3 and which have been adapted largely from these sources. Each type of collagen is composed of a particular combination of α-chains forming a triple helix. The chains are made of repeating triplets of glycine and two other amino acids with hydroxyproline often in the Y position. The formation and stability of the triple helix is now known to be dependent on the hydroxylation of proline which is a post-translational modification. Failure of hydroxylation due to vitamin C deficiency produces the clinical syndrome of scurvy as this vitamin is an essential cofactor. High dosage corticosteroid therapy also interferes with hydroxylation. Other defects affecting man and animals, often congenital, are increasingly being recognised throughout the synthetic pathway.

The enzyme responsible for hydroxylation is collagen prolyl hydroxylase and it is located within the endoplasmic reticulum where α-chains are hydroxylated as they are synthesised. This enzyme is one of the oxygenases, a group of enzymes with the unique requirements of α-ketoglutarate, ferrous ion, and molecular oxygen as cofactors. Hydroxyproline, long though to be exclusive to collagen, has now been found in small amounts in C1q, elastin and acetylcholinesterase. It remains valid, however, that the activity of the enzyme in a tissue parallels the rate of collagen synthesis in a wide variety of normal and pathological tissues (Cardinale and Udenfriend, 1974). The special requirements of this enzyme and its key role in collagen synthesis combine to offer a sensitive and meaningful assay (Appendix No. 3).

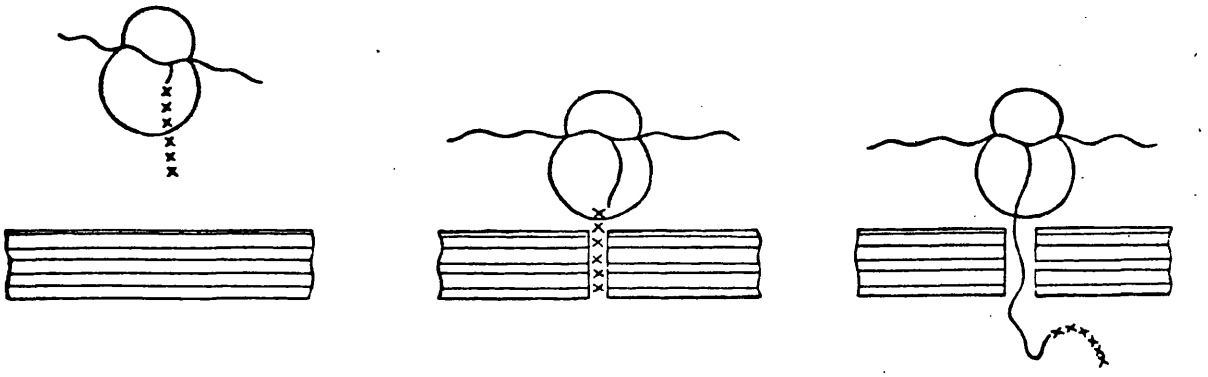
Collagen which has not been denatured is resistant to proteolytic digestion and this process must be initiated by a collagenase. This offers the opportunity for an assay of production based on the specificity of collagenase digestion and release of radio-labelled peptides (Appendix No. 4).

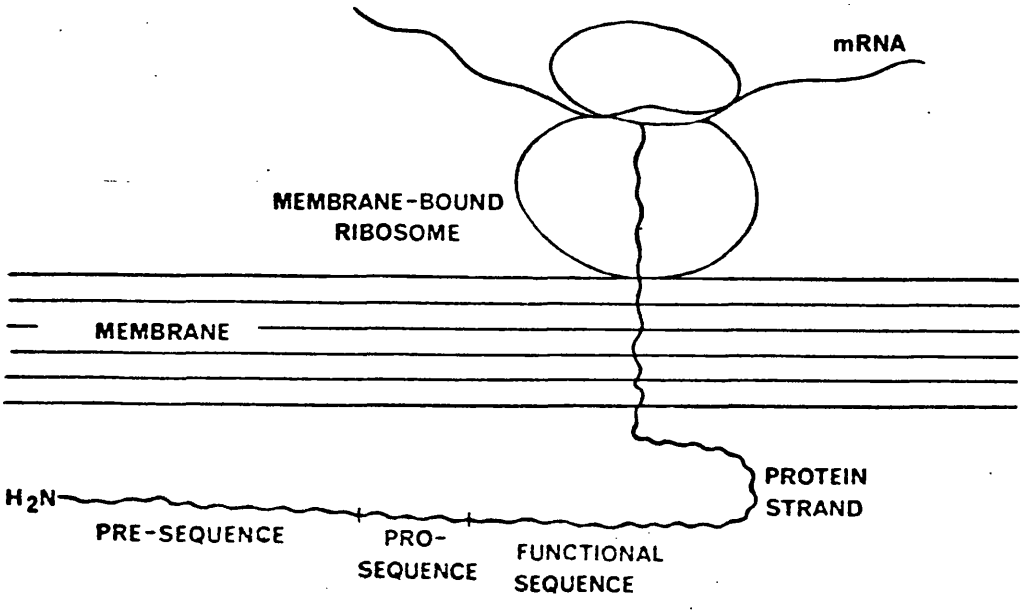
The appearance of collagen in a lesion has long been thought to be a balance between synthesis and degradation. However, it has been shown in experimental liver injury that the deposition of collagen is a consequence both of increased production and reduced destruction of collagen (Ruiz-Torres et al. 1976). Collagenase is produced not only by inflammatory cells but by fibroblasts themselves and collagenase is now recognised to be bound, probably in inactive form to its substrate collagen (Montfort and Perez-Tamayo, 1975) in all tissues, rather in the way that plasminogen is bound to fibrinogen. The enzyme may be present as an enzyme-inhibitor complex or in a "pro" form and the regulation of its activity is unknown (Perez-Tamayo, 1978). Reynolds et al. (1977) postulate that it is activated by proteases in inflammation. There is some evidence of its involvement in growth (Klein and Chandrarajan 1977) and less appears bound to collagen once experimental carbon tetrachloride cirrhosis becomes irreversible (Montfort and Perez-Tamayo, 1978).

Apart from its likely role in controlling extracellular collagen turnover, collagenase or other proteases may have an intracellular function since collagen has now been convincingly shown to be degraded intracellularly. Bienkowski et al. (1978) found that about 30% of collagen chains synthesised are reduced again to small peptides by fibroblasts and then released into the medium. No degradation occurred in the medium. They point out that although it has been shown that intracellular degradative processes have a control role in regulating the quantity of proteins that function within cells, such degradation has not previously been considered as a general mechanism for regulating export of proteins. By substituting a proline analogue in culture, degradation was doubled. This finding suggests a way in which collagen prolyl hydroxylase might control effective collagen secretion by conferring stability on the triple helix and immunity from intracellular degradation.

Synthesis of extracellular proteins

Fig. 1:1 Collagen is a protein synthesised for export. The signal hypothesis suggests that ribosomal assembly of amino acids in the cytoplasm begins with a "pre-sequence". This attaches the ribosome tp the endoplasmic reticulum as shown and is subsequently removed even before the chain is complete. There is recent evidence that there may be no need to postulate a pore for transport of the protein across the membrane. The ribosome becomes sufficiently firmly attached that the growing peptide chain is propelled across, possibly leaving the pre-sequence embedded in the membrane (reviewed by Freedman, 1980).



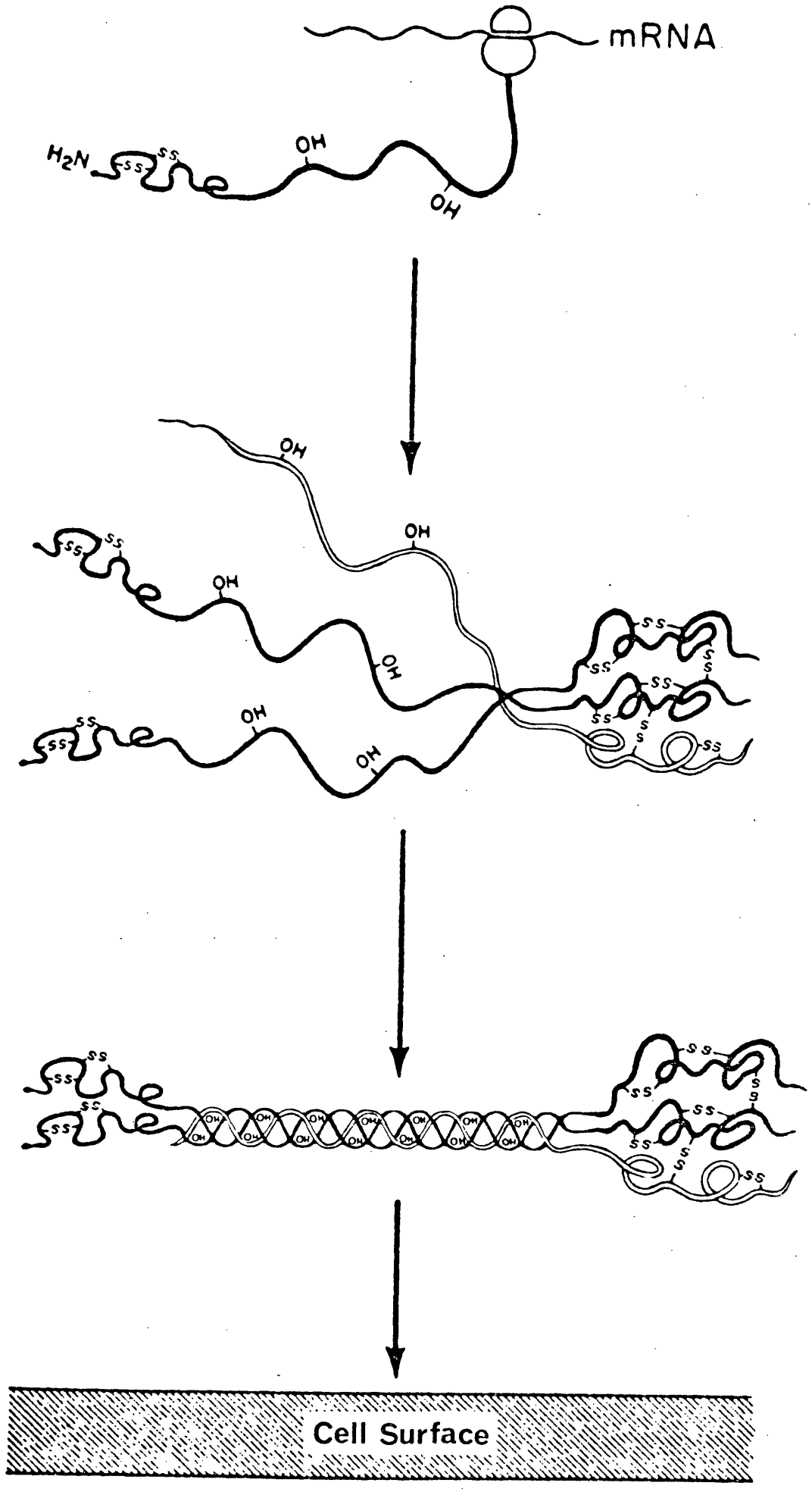


Synthesis, processing and secretion of collagen

Fig. 1:2 Different types of tissue structures are composed of different types of collagen of at least 5 major kinds differing in the α chain composition of the triple helix. Following transcription of the genes for each different procollagen chain, the messenger RNA is translated on the rough endoplasmic reticulum. As the chains enter the cisternae various post-translational modifications occur. Hydroxylation of proline and lysine residues by prolyl 3- or 4-hydroxylase and by lysyl hydroxylase requires the presence of free oxygen, α-ketoglutarate, ascorbate and ferrous ion.

Hydroxylation of proline residues is an essential requirement for triple helix formation which may begin even before detachment of nascent prepro-α-chains from ribosomes. Interchain disulphide bond formation occurs between the C-terminal propeptides. As lysyl residues are hydroxylated, sugar residues are added by galactosyl and glucosyl transferases. The propeptide regions are glycosylated by glucosyl and mannosyl transferases.

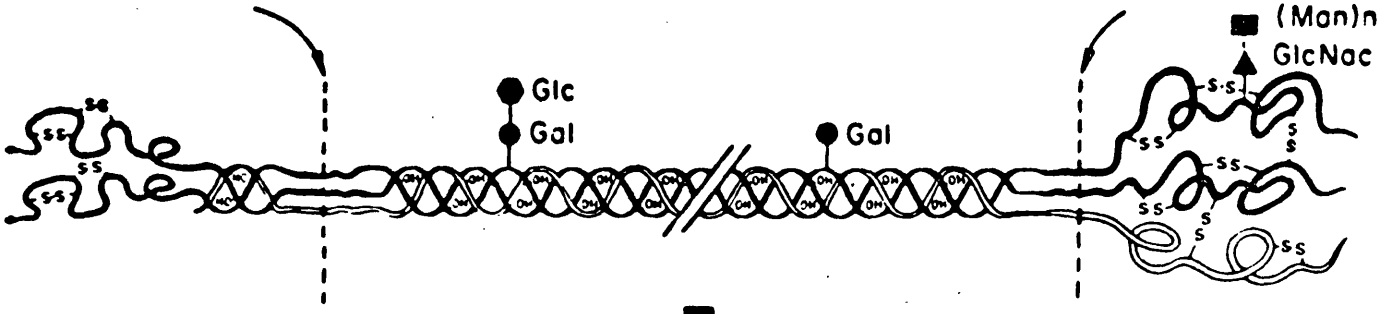
The procollagen molecules are transported in vesicles from the RER to the Golgi complex. The procollagen molecules are aligned in condensed secretion granules which are transported to the cell surface for release into the extracellular space. This transport mechanism is poorly understood but may be disrupted by agents which disrupt microtubules or microfilaments,or which inhibit energy production.

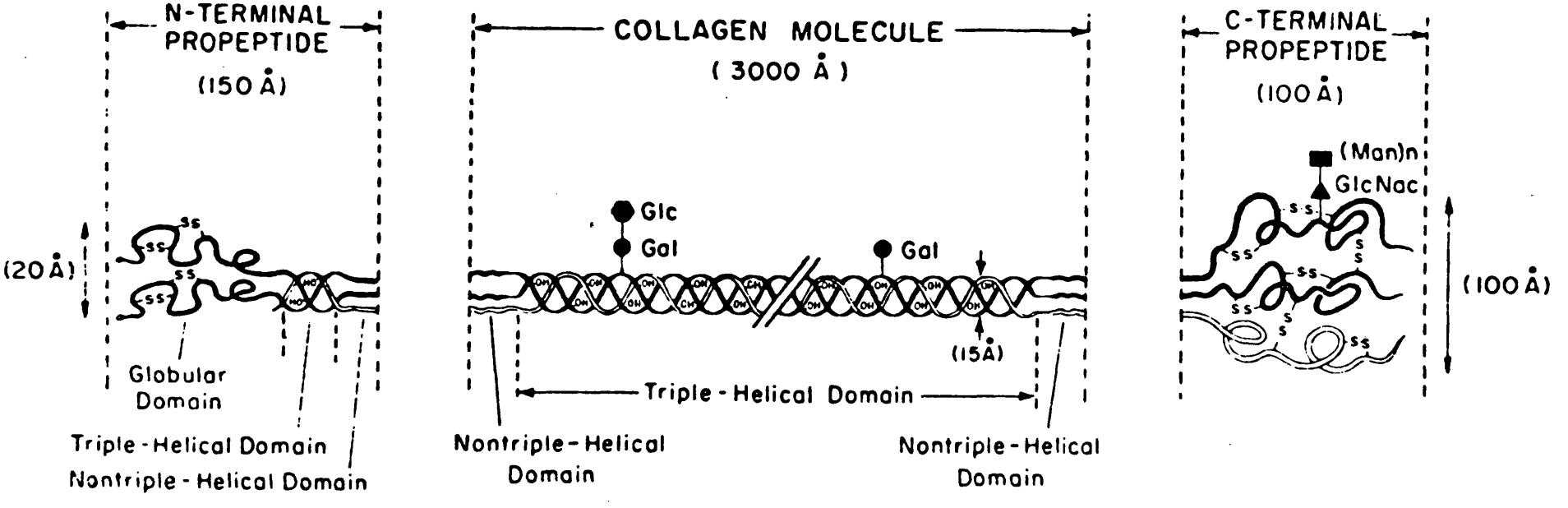

mRNA
H_2N
OH
OH
OH
OH
OH
OH
OH
OH
Cell Surface

Extracellular Processing of Collagen

Fig. 1:3 Procollagen is converted to collagen extracellularly by removal of C- and N-terminal propeptides by two corresponding enzymes. The resultant collagen molecules aggregate spontaneously into fibrils. Additional tensile strength is conferred by cross-linkage of the fibrils by covalent bonds of various types catalysed by lysyl oxidase, a copper-containing enzyme.

PROCOLLAGEN MOLECULE





The control of fibroblast activity

Knowledge from tissue culture

The idea,that the inflammatory cells in chronic inflammation might elicit the growth of reparative tissue,has been in existence for some time. However a firm basis for the idea was not established until Carrel (1922) in a series of fascinating experiments utilising the then new tissue culture techniques, found that leukocyte extracts increased fibroblast growth in culture as did embryonic tissue juices. Peritoneal inflammatory exudates and extracts of healing wounds had a similar effect. He concluded that leukocytes were capable of bringing growth activating substances to tissue cells. Practically all work in this area continues to be concentrated on wound healing and on tissue culture or other in vitro approaches.

As the proliferation of fibroblasts in wounds begins to decline, this is followed by collagen deposition. Collagen forming fibroblast cell lines also grow through a proliferative phase into stationary phase when collagen is deposited extracellularly. These observations have led to the awareness of separate influences controlling proliferation and increased collagen synthesis. Growth is the aspect which has received the greatest attention. This effort has produced many facts but few established concepts as yet.

Virtually all animal cells require serum for proliferation in culture in addition to nutrients and buffer salts. Serum contains high molecular weight growth factors and low molecular weight nutrients. The distinction between mitogens, nutrients,and survival factors is frequently unclear because of their interaction in vitro. In vivo validation of the role of putative mitogen is impracticable because of endogenous factors. The presence and activity of many factors at extreme dilution has also hindered research. Gospodarowicz and Moran (1976) have evaluated the many supposed growth factors extracted from blood, tissue, and tissue cultures. After reviewing the appalling complexity of problems of interpretation and circular argument, they conclude that if strict criteria are adopted, most

factors such as the serum fractions, somatomedins and insulin, etc., are imperfect mitogens although they may initiate DNA synthesis in concert with other factors. Of all the factors only Fibroblast Growth Factor (FGF) and Epidermal Growth Factor (EGF) met all criteria.

FGF is a polypeptide (13, 400 daltons) isolated from pituitary and EGF (6, 045 daltons) has been isolated from submaxillary gland but detected also in serum and urine. It is far from clear what their physiological role might be and no specific role in inflammation is apparent. Platelet factor which contributes much to the superior growth-promoting properties of serum compared with plasma and has similarities to FGF is the only likely candidate so far for a role in wound healing and regeneration since platelets and serum might gain unusual access in such a situation. Wound healing is rather a special case, however, and one can immediately think of many pathological instances of cell necrosis with fibrogenesis such as acute myocardial infarction where platelets and haemorrhage are not so prominent. Proteases including thrombin have stimulatory effect on cell division which appears to be a potentiation of other factors. This could be an effect on surface receptors and it is possible to imagine a possible role in vivo in inflammation. (Chen and Buchanan, 1975). No single mechanism for growth control has yet appeared but it is entirely possible that such a mechanism exists (Gospodarowiez and Moran, 1976) although whether this would be utilised in healing does not necessarily follow.

The relevance of all this complexity to the present work is the conclusion that tissue culture as a test system for potential mitogens special to inflammation, is fraught with problems because, although it is easy to grow fibroblasts no one knows what the normal milieu for "resting" fibroblasts should be. Cultures which reach saturation density used to be thought to be cell contact inhibited but it is now realised that this "density dependent regulation" is an artefact (?) of culture due to limitation of access to serum in the medium (reviewed by Holley, 1975). Intense stirring of the medium, for example, can

re-start cell multiplication. Another well known phenomenon of tissue culture has recently been challenged. The Hayflick effect whereby diploid non-transformed cells in culture can only divide up to above 60 times is now found to be attributable to diluting out a small proportion of stem cells. It is thought that in vivo these stem cells continually give rise to progeny which exhibit a limited replicative capacity (Holliday et al. 1977). The fact that the number of divisions achieved in vitro diminishes with the age of the donor indicates that the Hayflick effect still has some deeper relevance to the phenomenon of aging.

The problems concerning control of cell growth also apply to collagen synthesis. Should one test a substance for its ability to stimulate collagen synthesis during early, mid or late log phase or at confluence ? Would this substance normally be present anyway, or might its presence in inflammation be restricted to the relevant phase of maximum collagen synthesis ? Collagen deposition by diploid cells in culture occurs towards confluence. The biological assays now available permit more detailed study of this process generally in transformed fibroblasts which are technically easier to culture because for one thing, they do not deposit an adherent matrix of collagen fibres making passage difficult. L929 mouse fibroblasts, for example, are a popular choice and the failure of collagen to be deposited despite being the major protein synthesised is attributable to this cell line's inability to produce procollagen peptidase.

Fig.4:11 in Chapter 4 illustrates that collagen chain synthesis in L929 cells is actually maximal during log phase. This anticipates the late increase in prolyl hydroxylase activity which is due to conversion of the previously synthesised components to active tetramer formation. Comstock and Udenfriend (1970) showed that this activation could be induced at any phase by lactate in concentrations known to appear at late log phase. Hunt et al. (1978) showed that such high lactate levels did occur in wounds by the use of implanted diffusion cylinders. In these circumstances lactate appears to substitute for ascorbic acid which also activates prolyl hydroxylase.

Ascorbic acid tends to deteriorate and disappear rapidly from tissue culture medium at 37°C (Peterkovsky, 1972). If ascorbic acid is constantly supplied, then proline hydroxylation is maximal at all phases of growth. While collagen chain synthesis is unaffected, the secretion of collagen, presumably in degradation-resistant triple helix form, is also maximal at all phases if ascorbic acid is supplied (Cardinale et al. 1975 ; Blanck and Peterkofsky, 1975). In the absence of ascorbic acid, cultured fibroblasts show accumulation of cytoplasmic procollagen demonstrable by immunohistochemistry (Gay et al. 1976). It is now thought that ascorbate is a specific reductant required to reactivate the prolyl hydroxylase after oxidation of enzyme-bound iron (Myllyla et al. 1978). The problem of course, is to know just what limiting effects might apply in vivo.

It is interesting to note that Mussini et al. (1969) found an increase in prolyl hydroxylase activity maximal at day 5 not just within rat skin wounds but in the adjacent uninjured skin. This latter effect must be elicited from non-proliferating cells presumably as a result of diffusion of some stimulus. This effect also suggests that the phase of cell growth in vivo may not be crucial to the expression of enhanced collagen synthesis.

Collagen stimulating factors

Previous work from this laboratory has demonstrated in simple extracts of experimentally injured liver and skin the presence of factors which stimulate collagen prolyl hydroxylase activity and collagen synthesis in fibroblast cultures and liver slice organ cultures (McGee et al. 1973 ; McGee, 1973 ; O'Hare, 1977 ; Fallon, 1977 ; McGee and Fallon, 1978). The methodology is described in Chapter 4 where it is applied to paraquat lung injury. Their major findings are briefly summarised here.

Gel chromatography on a column of Sephadex G-25 of supernatant from crude homogenate of mouse liver acutely damaged by carbon tetrachloride showed at least 4 zones or peaks of stimulatory activity

when fractions were incubated with replicate fibroblast cultures and compared with control cultures. When the collagen synthesis assay was used, no stimulation was observed with control normal liver. The collagen prolyl hydroxylase assay though technically superior and more sensitive showed a small peak of activity with normal liver corresponding to the 2nd peak from damaged liver. This was subsequently shown to contain ascorbate in sufficient concentration to account for the minor effect. The pattern of peaks from injured liver using both assays appeared identical and up to 4 or 5 fold increases were observed. The pattern was consistently similar and repeatable allowing for the vagaries of the test system. Certainly no similar pattern was obtained from control material.

A similar pattern of at least 5 zones of activity was obtained from healing mouse skin wounds. Selected column fractions from both damaged livers and wounds also stimulated prolyl hydroxylase in mouse liver slices in organ culture. Similar results were obtained using guinea pig and dog liver slices. These findings indicated that the factors were neither organ or species specific. It was also found that any cultured cell line that expressed prolyl hydroxylase could be stimulated.

Using fractions from the first two peaks from injured liver it was shown that the effects were not additive suggesting that they acted at the same site. A relatively smaller increase in general protein synthesis was also stimulated by these factors but the same increase was obtained from normal tissue. This suggests a specific effect on collagen synthesis.

The first peak of activity is of molecular weight 5,000 daltons or above and the other peaks less than 5,000 daltons. The factors were stable at 56^{o}C for 30 minutes. At least the first two peaks are trypsin-degradable suggesting a peptide structure. Trypsin exposure did not affect the minor ability to stimulate protein synthesis. The factors deteriorated on storage in 1 or 2 days at 4^{o}C and were not stable at -20^{o}C or in liquid nitrogen. Lactate, ascorbate, and

bradykinin were excluded as unlikely to contribute to the stimulation of collagen synthesis. The possibility of proteolytic production of the factors during homogenisation was excluded by inclusion of protease-inhibitors. Prostaglandins E_2 and F_{2a} were both detected in the second peak and a similar concentration of only E_2 produced a two-fold increase in collagen synthesis. A lower concentration of E_2 and the same concentration of F_{2a} were found in normal liver. Since prostaglandins are not trypsin degradable but peak 2 activity appears to be, it appears that the major effect is due to one or more peptides. No effect on DNA synthesis or cell turnover has been found. In conclusion these factors appear to have a selective stimulatory action on collagen synthesis involving prolyl hydroxylase activation, which is (thankfully) distinct from a growth factor effect.

These experiments showed a convincingly repeatable effect but by their design were not amenable to statistical analysis. For good technical reasons only a limited number of assays (up to 20) could be performed as any one experiment. Only 1 or 2 buffer controls were included. Material from normal tissue could not be processed and tested simultaneously with damaged tissue. These points are not so much criticisms as limitations of the system. For example the more cultures used as controls the less are available to show peaks of activity. Also, by the very nature of the effect obtained, less sensitive non-parametric statistics are required. The pattern of peaks obtained does not lend itself to quantitation allowing mapping of the time-course of events. While the effect was demonstrable on liver slices in organ culture, the possibility of a really convincing <u>in vivo</u> effect was not explored by "adding back" to the animal. Some of these problems have been considered in work on paraquat lung injury in Chapter 4.

The possibility of macrophage involvement in the production of these factors was briefly considered in the previous work but the cell source, if any, of these factors has not been defined. The possible influence of macrophages on fibrogenesis is considered further in Chapter 3 regarding the role of macrophages in liver injury.

Review of *in vitro* evidence of macrophage factors affecting collagen synthesis is postponed until Chapter 7.

Chapter 2

THE IDENTIFICATION OF MACROPHAGES IN TISSUE BY IMMUNOHISTOCHEMICAL STAINING

Macrophages in tissue can be recognised by light microscopy with conventional staining methods. Individual cells are seen to be larger than polymorphonuclear leukocytes, to possess large indented nuclei, and to show P.A.S. - positive, diastase - resistant, cytoplasmic staining. While these features may suffice for everyday purposes, they are non-specific and a selective staining method for light microscopy would be a desirable tool but as yet such a method appears not to exist.

More exact identification is obtainable by electron microscopy utilising such features as phagocytic vacuoles and lysosomes and the characteristic surface projections of the activated macrophage (North and Mackaness, 1963). Even these features are not entirely exclusive (Spector, 1969).

Ingestion of particular material such as carbon can provide good identification but this requires previous injection, and the material will only gain access to cells in contact with the circulation. The cells are in any case rendered abnormal by the procedure itself.

Various histochemical methods demonstrating acid phosphatase, esterases, lysozyme and peroxidase activity are non-specific and may necessitate fresh tissue. Surface receptors such as the Fc receptor may be utilised to form rosettes with antibody-coated red cells (Fig 2:1). Such methods require unfixed cells or tissue and are again non-specific.

That such difficulties are real rather than theoretical may be judged from the recent work of Nash et al (1980), who attempted to quantitate the macrophage component intermixed with mouse mammary tumour. They reported that no histochemical technique was satisfactory, and obtained better results with Fc mediated phagocytosis of red blood cells by fresh tumour. Phagocytosis is not exclusive to the macrophage

and the most generally accepted definition of the mononuclear phagocyte system is based on glass adherence (van Furth and Cohn, 1968) - a functional property irrelevant to histological sections.

Cohen (1973) demonstrated the presence of the plasma protein α_1-antitrypsin in pulmonary macrophages by immunofluorescence using a specific antibody. More recently this antiprotease has been demonstrated in peripheral blood monocytes by the immunoperoxidase technique and the presence of this granular cytoplasmic staining in the neoplastic cells of certain types of small intestinal lymphoma has been held as evidence of macrophage lineage (Isaacson et al, 1979). Since albumin and immunoglobulin were not stained, it is stated that the presence of α_1-antitrypsin is not likely to be due to uptake from the plasma and therefore must be due to synthesis. This is a totally premature conclusion in the absence of positive evidence of synthesis as against the mere presence of a protein. A specific uptake of α_1-antitrypsin is feasible. This is supported by Cohen's observation (1973) that macrophages from a subject totally deficient in α_1-antitrypsin (homozygote ZZ) were initially negative with immunofluorescence but became positive after incubation with normal serum. This method of demonstrating macrophages is obviously promising but it is not stated whether polymorphs also stain and the distribution of staining throughout the body is not described as yet. The ubiquitous nature of α_1-antitrypsin, an acute phase reactant protein of relatively low molecular weight (45,000 daltons) and its presence in hepatocytes (where there is good evidence of synthesis) would be expected to limit the application of this technique particularly at inflammatory sites and in the liver.

There is nothing new about the raising of an antibody to a preparation of macrophages. This type of approach using antileucocyte and antineutrophil antibody has provided a basic tool for the experimental study of acute inflammation since the days of Metchnikoff (reviewed by Simpson and Ross, 1971). Similarly antibodies to macrophages have been used to inactivate macrophages in wounds (Leibovich and Ross,

1975) and to provoke giant cell formation by macrophage fusion (Chambers, 1977).

Such an antiserum was raised to mouse peritoneal macrophages with the original intention of modifying the course of events in experimental carbon tetrachloride liver injury. This has not yet been attempted as the application to immunohistochemistry has proved so distractingly successful.

The following work is described in three sections. The first describes the preparation of the antibody and the results with normal mouse tissues, some unexpected, are displayed. The second illustrates an experimental application to giant cell formation in carbon tetrachloride liver injury. This phenomenon is also considered in its own right with additional electron microscopic findings. Finally, the implications of the technique and current work with human macrophages are briefly discussed with regard to potential applications.

1) THE PREPARATION OF ANTIBODY AND RESULTS WITH NORMAL MOUSE TISSUES

Preparation of antibody :

The method of Liebovich and Ross (1975) was followed. Rabbits received repeated ear vein injections of mouse macrophages obtained by peritoneal lavage. Each mouse received 0.5 ml of sterile mineral oil by intraperitoneal injection 3 days before the cells were recovered. Each intravenous injection consisted of approximately 50 million cells suspended in isotonic saline without adjuvant and four such injections were given over a 3 month period. The rabbits were bled one week after the last injection. The same procedure was used to produce an antibody to rat peritoneal macrophages.

The macrophages obtained by this method contained prominent cytoplasmic vacuoles of oil. The purity of the macrophages in the peritoneal washings was about 90% as assessed by light microscopy of cytospin preparations stained by haematoxylin and eosin and by Giemsa. Obviously blood stained samples were rejected but subsequent cytospin preparations of acceptable samples showed variable contamination with small numbers of red cells, polymorphs, lymphocytes and mesothelial cells.

The antiserum obtained was absorbed to remove unwanted anti-erythrocyte activity, following complement inactivation by heating to 56^{o}C. Heparinised whole mouse blood was washed 3 times in 0.01 M phosphate-buffered saline pH 7.2 (P.B.S.). The final centrifuged pellet was mixed with twice its volume of the undiluted rabbit antiserum and incubated for 30 minutes at 37^{o}C, followed by removal of cells by centrifugation. The intensity of macrophage staining appeared unaffected by this procedure.

A minor degree of staining of mouse polymorphonuclear leukocytes was inconstantly obtained in subsequent immunoperoxidase work. Although not always a problem this could be removed by

absorption. Mouse polymorphs of rather variable purity (60% to 80%) were obtained by peritoneal lavage following two intraperitoneal injections of sterile 3% proteose peptone in P.B.S. as described by Humphries (1955). Higher purity (80% approximately) was obtained by lavage 6 hours following a single injection. Antiserum diluted 40 times with P.B.S. was incubated for 30 minutes at 37°C with polymorphs which were subsequently removed by centrifugation. Complete removal of polymorph staining amongst the cells within large blood vessels in sections was achieved at the proportions of 2 million cells per ml of diluted antibody whilst retaining macrophage staining.

Staining of Sections for Light Microscopy

Paraffin sections were obtained from tissues fixed for 24 hours in 0.12 M phosphate buffered formalin, pH 7.2. Endogenous peroxidase activity was suppressed by treatment for 30 minutes with methanol containing hydrochloric acid and hydrogen peroxid e. This was followed by a standard immunoperoxidase enzyme bridge method (see Appendix No. 1) based on Mason et al (1969) Sections were counter-stained with haematoxylin or methyl green. Anti-macrophage antibody was generally used at a dilution of 1:40 with P.B.S.

Control sections were treated with normal rabbit serum in place of specific antiserum and also with antiserum absorbed with intact mouse peritoneal macrophages prepared as previously described. 0.5 ml of antiserum diluted 1:50 with P.B.S. previously incubated for 30 minutes at 37°C with 10 million cells produced no staining. The cells used for absorption stained intensely if subsequently subjected to the rest of the immunoperoxidase procedure.

Preparation of Liver for Electron Microscopy

Mice were killed by cervical dislocation and the abdomen opened at once. The liver was immediately injected with several ml of 0.12 M phosphate buffered formalin at 4°C which caused swelling and blanching.

Tissue was then taken from adjacent to the needle track and small blocks of 1 cubic mm size were trimmed and fixed overnight at 4° in the same fixative.

On the following day an immunoperoxidase procedure suitable for electron microscopy based on a method by Wilson-Kimball and Nakane (1978) was commenced (See Appendix No. 2). The procedure was adapted to make it as comparable as possible with the method at light level. It is noteworthy that the immunoperoxidase stained blocks if examined by light microscopy either before embedding or after embedding but before cutting, showed the same pattern of exclusively Kupffer cell staining as with the conventional method. Glutaraldehyde was found to abolish antigenicity with the conventional method and so was not used for electron microscopy. This is a recognised problem with glutaraldehyde as is the creation of diffusion barriers at membranes (Kraehenfuhl and Jamieson, 1974).

Consideration of the Results with Normal Mouse Tissues (Figs 2:2 to 2:9)

The cell line of mononuclear phagocytes is supposed to consist of monoblasts, promonocytes, monocytes and macrophages (van Furth and Cohn, 1968). The present results from staining a large variety of mouse tissues with anti-macrophage antiserum confirm the unity of this cell system.

Cells of monocyte morphology alone stained amongst cells within blood vessels using the fully absorbed antiserum. Monocytoid cells and myeloid but not erythroid precursors or megakaryocytes appeared to be stained in femoral marrow obtained by extrusion under pressure of injected fixative. However the preparations so far obtained are of poor quality and are not illustrated. Decalcification destroyed antigenicity preventing examination of osteoclasts.

Although the monocytic origin of macrophages elsewhere appears to be relatively undisputed, the origin of the Kupffer cell continues to excite controversy as extensively discussed in contending articles at a recent symposium (Kupffer Cells and Other Liver Sinusoidal Cells,

edited by Wisse and Knook, 1977). The best-argued case to my mind was presented by van Furth et al. (1977) both from the point of view of reviewing the evidence and in the presentation of new data regarding the kinetics of Kupffer cells in the normal animal and after administration of zymosan. Interestingly, their conclusion that the Kupffer cell is of marrow origin despite the ability to divide locally to a limited extent, is consistent with our results. As illustrated in Fig. 2:2 and Fig. 2:8 Kupffer cells stain positively at both light and electron microscopic levels. Endothelial cells and perisinusoidal fibroblasts (Ito cells, or lipocytes) do not stain.

Immunoperoxidase electron microscopy of Kupffer cells showed dense surface deposition of reaction product coating surface macrovillous projections and the surface generally. In addition many small outer cytoplasmic vesicles, most probably pinocytotic vesicles, stained positively. This is in contrast to endogenous peroxidase staining which is localised to the nuclear envelope and endoplasmic reticulum (Deimann and Fahimi 1977), and which can also be found in endothelial cells in the same locations (Shohr et al. 1978). It is of interest to note that Kupffer cells have a characteristic fuzzy outer coat thicker than any other cell coat with the exception of that on intestinal epithelial cells (Wisse, 1974). This coat is about 700 Å thick and is demonstrable only by special techniques. This material is also present in pinocytotic vesicles and membrane invaginations (reviewed by Wisse, 1977). The nature and function of this material is unknown and it is tempting to suppose that it is this material which is being stained and that this is responsible for the unique facility of macrophages for rapid glass-adherence.

Cells staining in lymph nodes were distributed in a predictable fashion occupying the subcapsular sinus and the sinuses of the medulla. Occasional cells were scattered through the centres of germinal follicles. In the thymus most macrophages were observed in the cortical region in fair numbers and one wonders what their function can

be at this site. In the spleen the majority are in the red pulp.

There has been some controvery over the nature of epidermal Langerhans cells with some authors considering them to be of macrophage type (Stingle et al, 1977). No positively staining cells were observed in epidermis however. Similarly negative results were obtained in the renal glomerulus where mesangial cells are supposed to be macrophages by some authors (Mauer et al, 1972).

Cells which stain positively can be seen in the stroma of all tissues examined corresponding to the traditional fixed tissue histiocytes but their numbers vary greatly according to the site. In the brain they are seen in small numbers in the choroid plexus plus occasional single perivascular cells elsewhere being presumably microglia. The pancreatic stroma also has a scanty population and in the kidney they appear to be confined to interstitial tissue adjacent to blood vessels. Larger numbers are seen in relation to various surfaces as might be anticipated ; they are plentiful in dermis, in the lamina propria of the ureter and bladder, in the lamina propria of small intestinal villi and to a lesser extent in that of colon. In lung, macrophages were prominent in interstitium and in alveoli. At this latter site where macrophages lie free of surrounding tissue contacts and become rounded, the staining pattern of the cell is more obviously related to the surface.

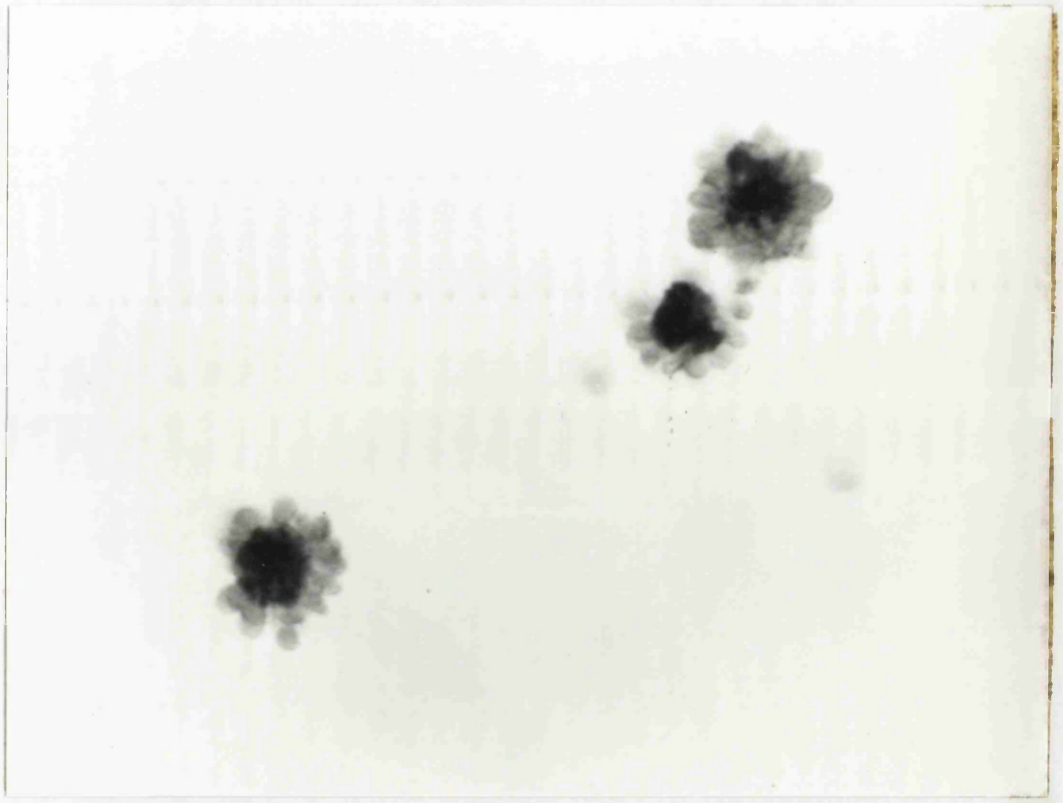
Moderate numbers of macrophages are seen in fallopian tubes, peritoneal surfaces, adipose tissue, cardiac and skeletal muscle and salivary glands. A curious finding was the presence of macrophages in endocrine areas. Macrophages were seen within pancreatic islets, parathyroid and thyroid glands and particularly amongst leydig cells in the testis. Do they ingest endocrine secretory products at these sites ? Again macrophages were prominent in an abdominal sympathetic nerve ganglion and in the medulla (though not cortex) of the adrenal. Does this indicate a "mopping-up" of neural secretory peptides ?

Later in the year after this work was presented to the Pathological

Society, a French group reported similar results with anti-rat peritoneal macrophage antiserum. Their aim was to confirm the monocytic origin of brain macrophages and the only other tissues examined were thymus and spleen. They used frozen sections and indirect immunofluorescence and the results are of predictably limited quality. It is interesting to note that they used their antibody at a similar dilution and found no cross-reaction with neutrophils, platelets or lymphocytes (Persson and Ronnback, 1979).

In general the findings correspond to the usual description of the mononuclear phagocyte system. The paraffin section orientated methodology allows further assessment than previously possible of cell lineage in disputed areas and has disclosed some additional curious anatomical findings. The nature of the macrophage surface material stained is uncertain but the macrophage "fuzzy coat" seems a more likely candidate than specific known receptors such as the Fc receptor which can be found on other cell types.

Fig 2:1 Mouse peritoneal macrophages containing phagocytosed carbon particles and rosetted with sheep red cells coated with anti-red cell antibody. Wet preparation, unstained. X 1,260



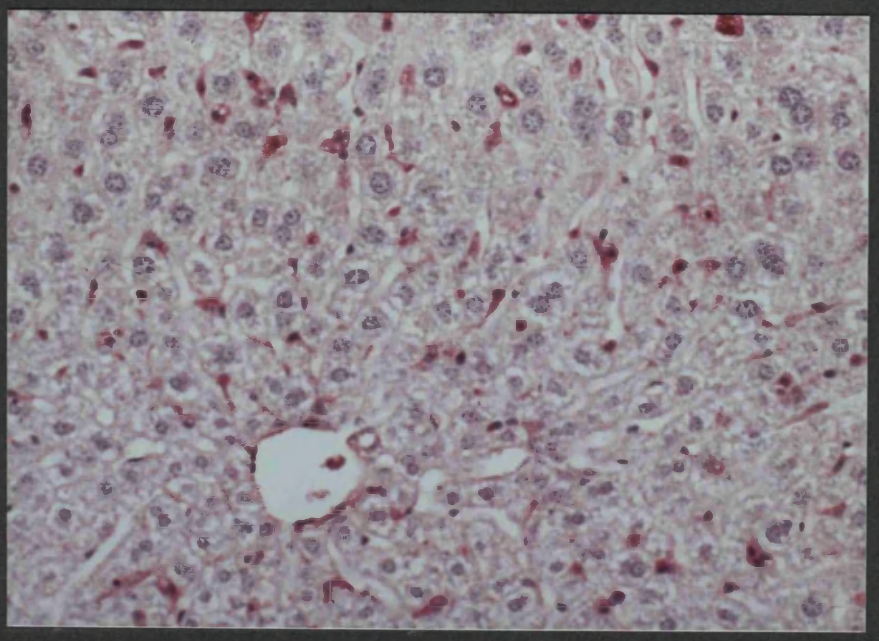


Fig 2:2 A. Immunoperoxidase demonstration of macrophages in normal mouse liver using antimacrophage antibody. X 280

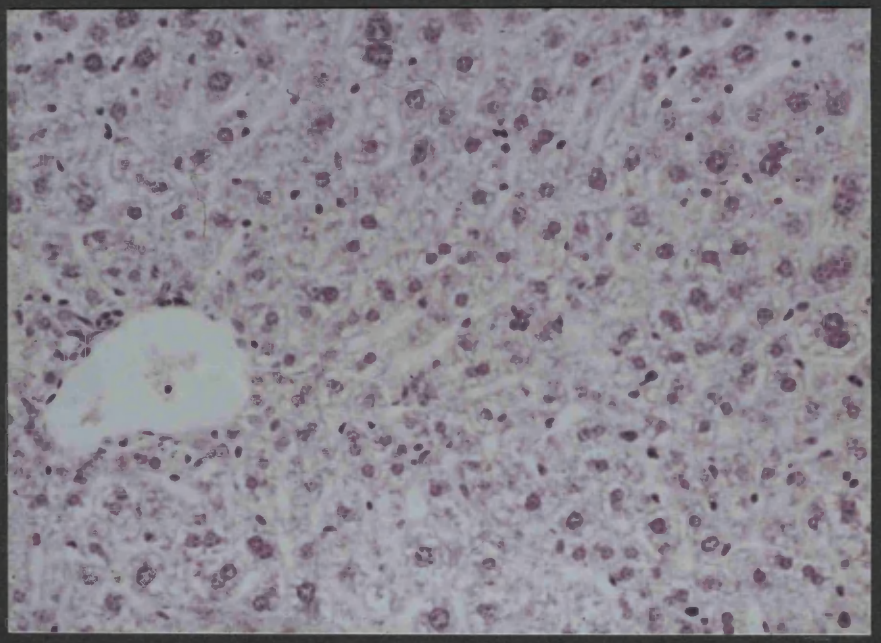
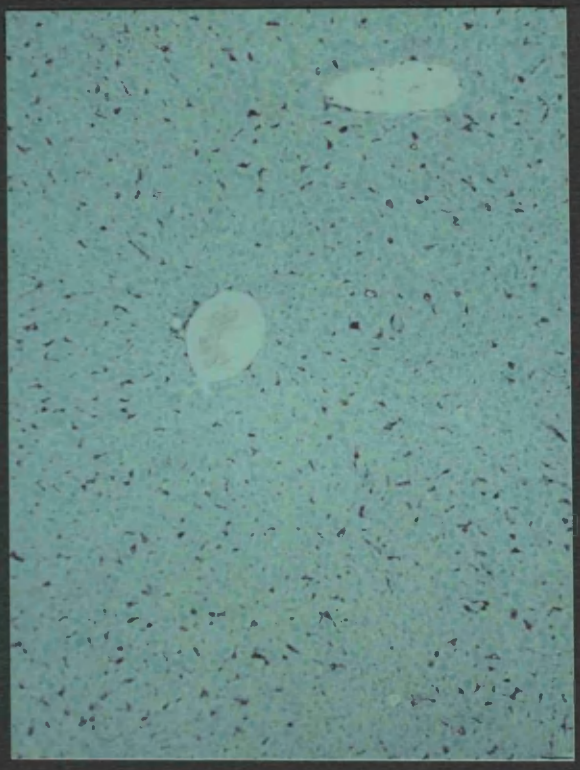
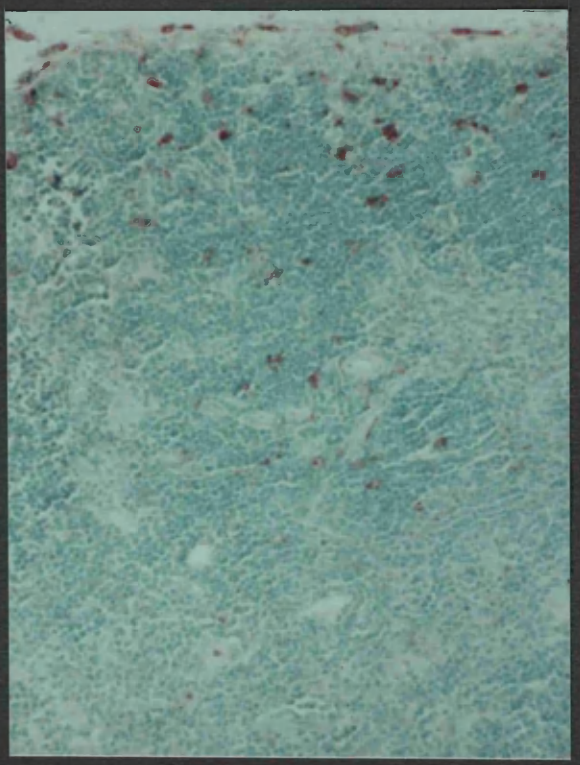


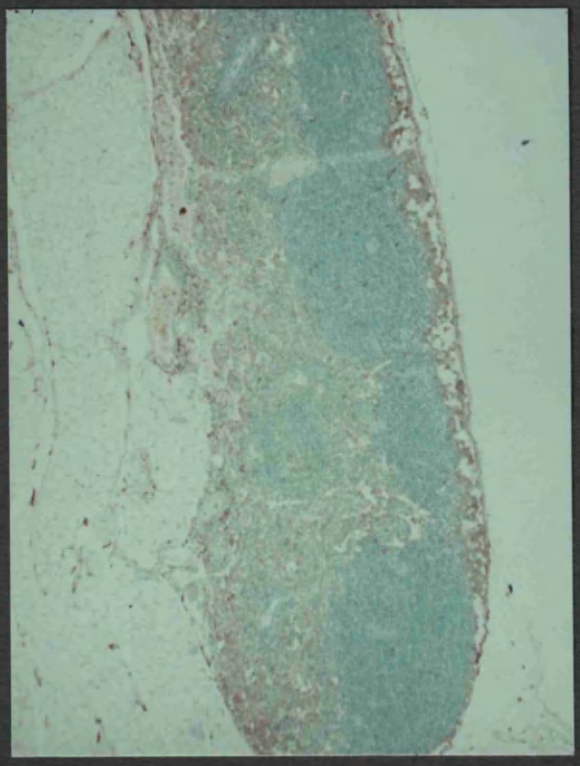
Fig 2:2 B. The same procedure as A except for absorption of antibody with peritoneal macrophages. X 280



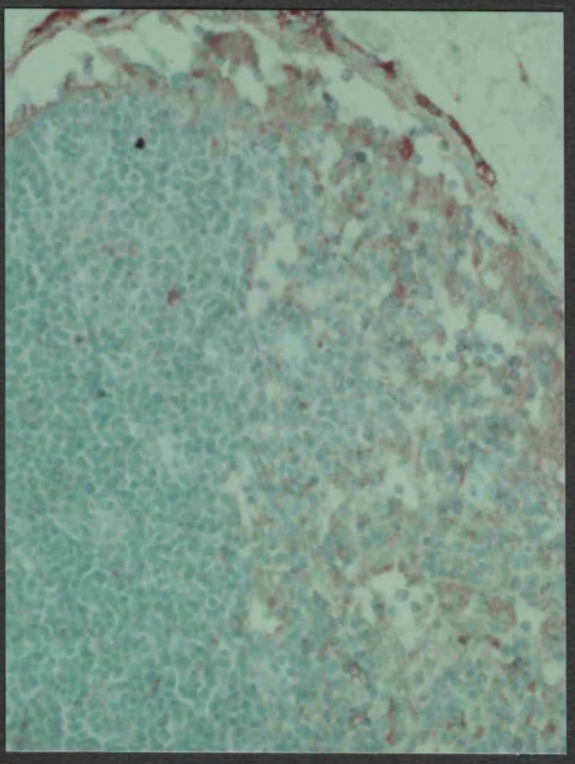
A) Normal liver. Kupffer cells positive. X 70



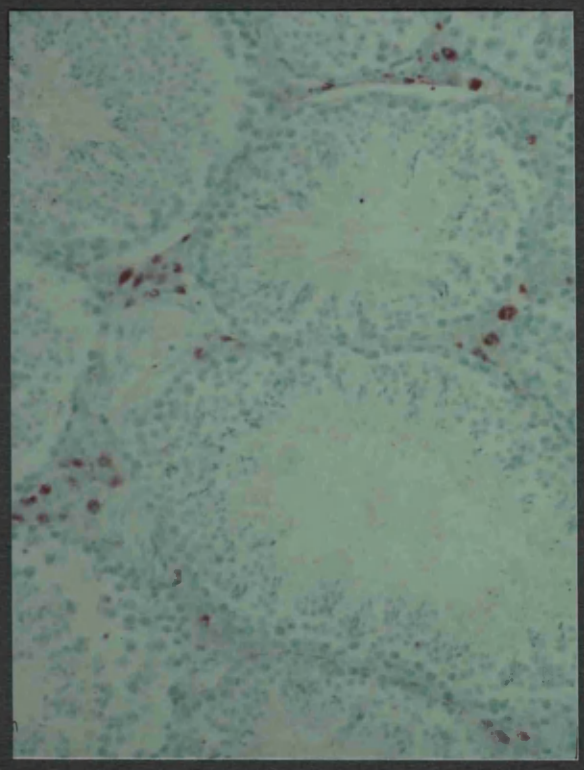
B) Thymus. More macrophages in cortex than medulla. X 175



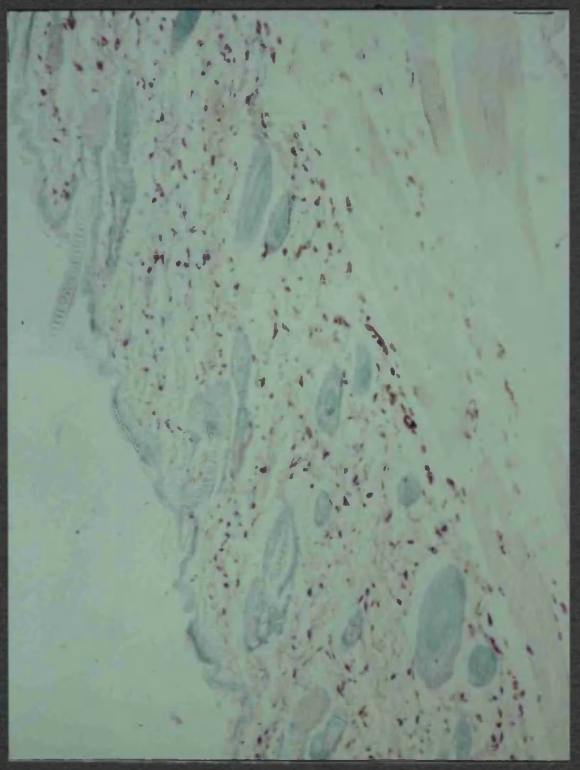
C) Lymph node from mesentery. Many macrophages in subcapsular and medullary sinuses. X 70



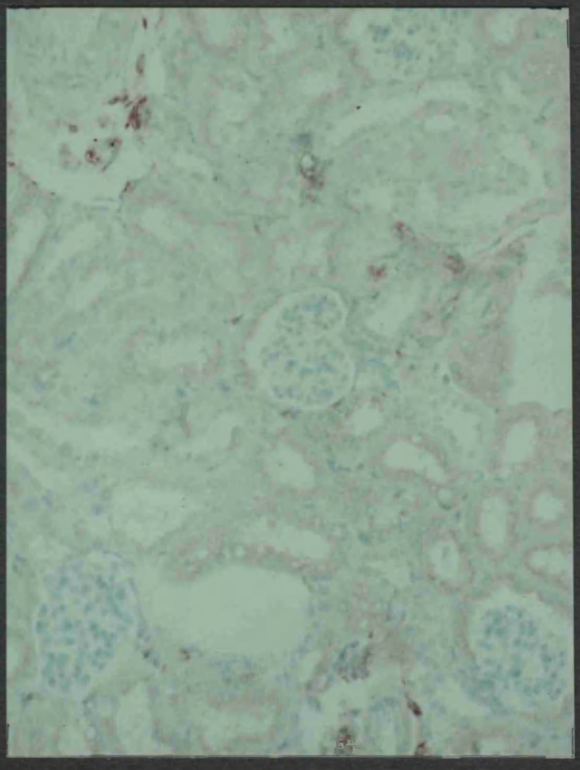
D) Lymph node. Higher magnification to show occasional macrophages within germinal follicles. X 280



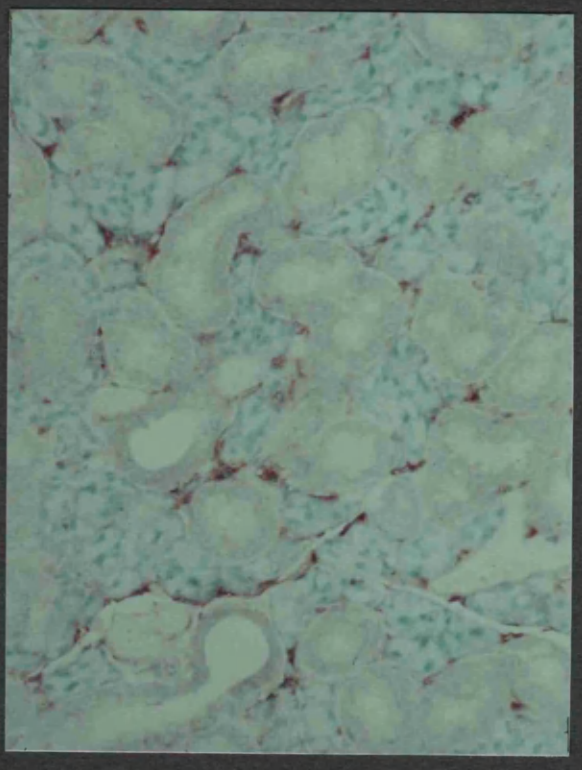
A) Testis. Many macrophages amongst Leydig cells. X 175



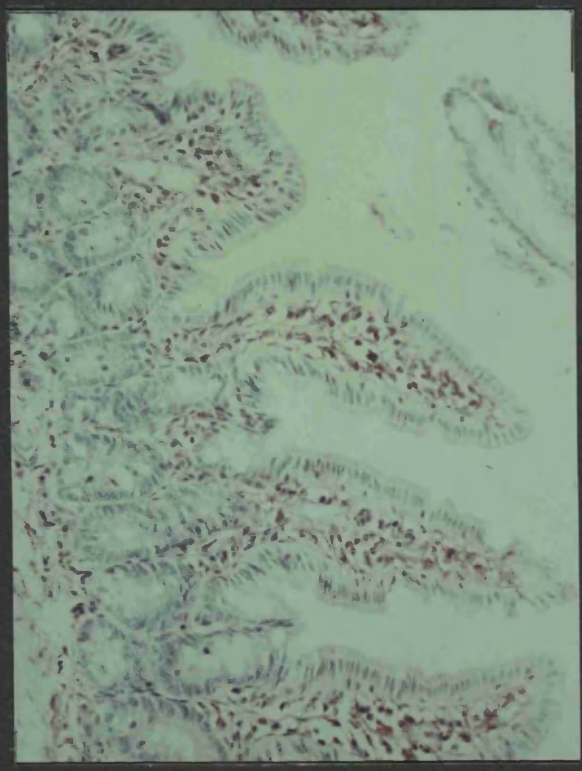
B) Skin. Many macrophages in dermis. None in epidermis. X 70



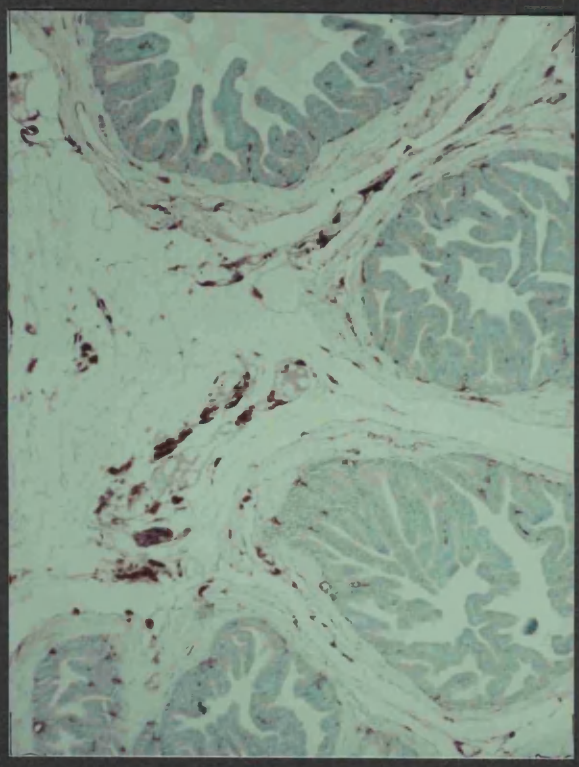
C) Kidney. Some perivascular macrophages. Glomeruli negative. X 175



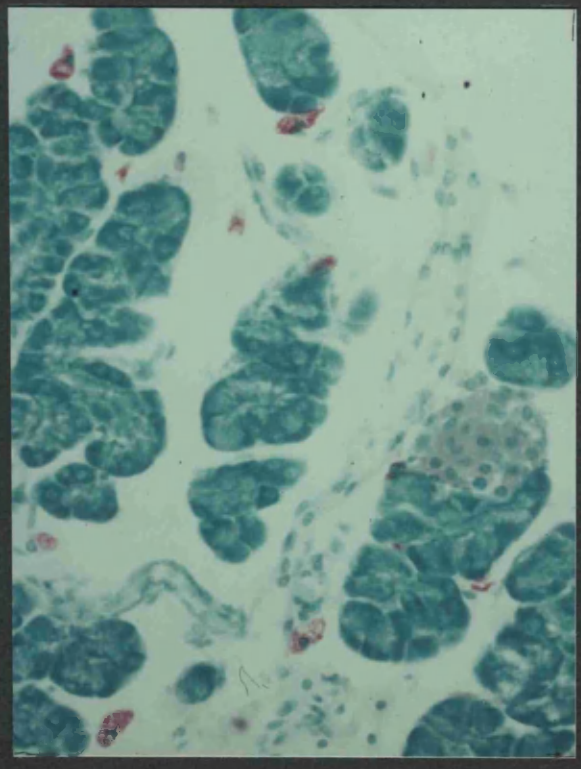
D) Salivary gland. X 175



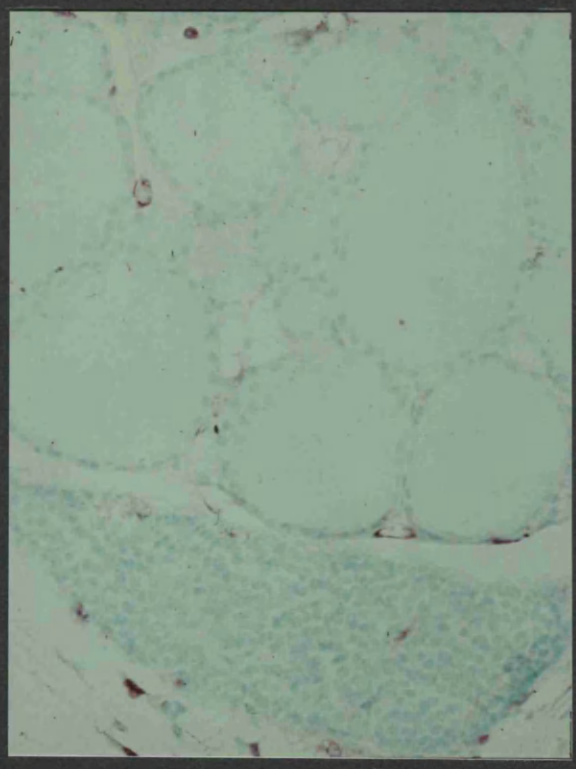
A) Jejunum. Macrophages prominent in the lamina propria. X 175



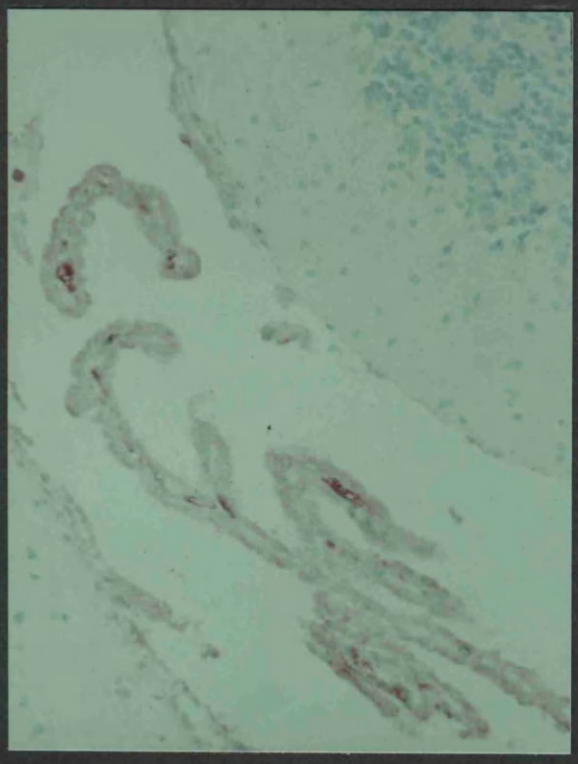
B) Ileum. Macrophages less prominent in the lamina propria. Many present in mesentery. X 70



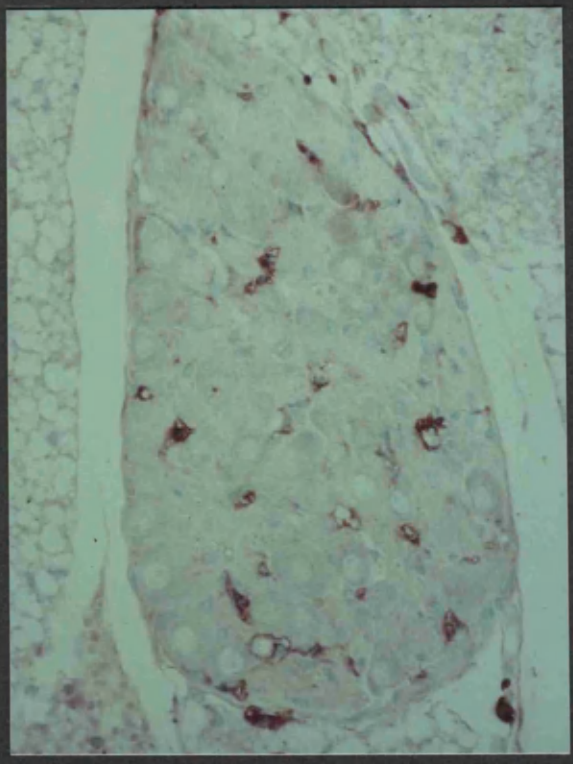
C) Pancreas. Macrophages in interstitium. None in islets. X 175



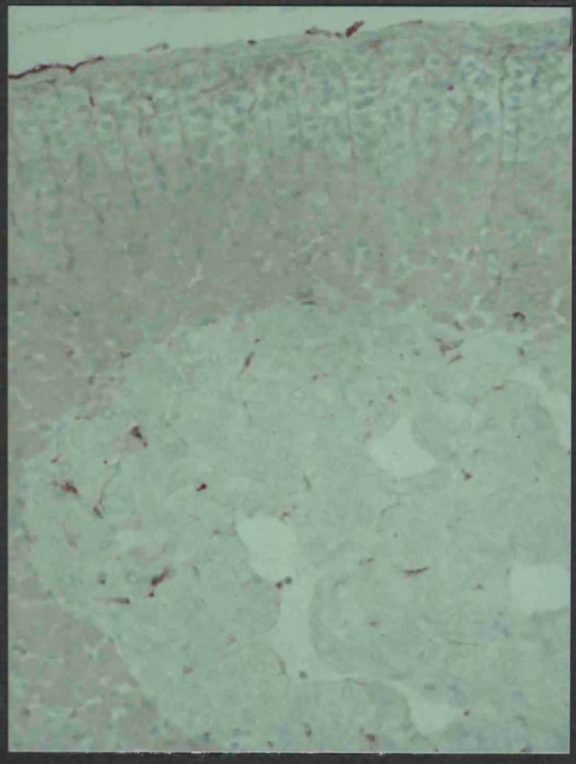
D) Thyroid and Parathyroid. Macrophages within both glands. X 175



A) Brain. Occasional macrophages in ependyma. X 175

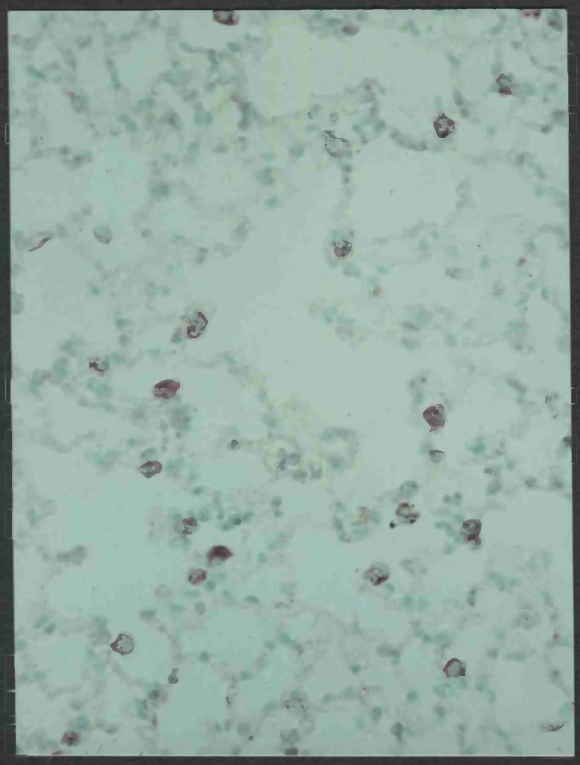


B) Nerve ganglion from mesentery. Macrophages amongst ganglion cells. X 280

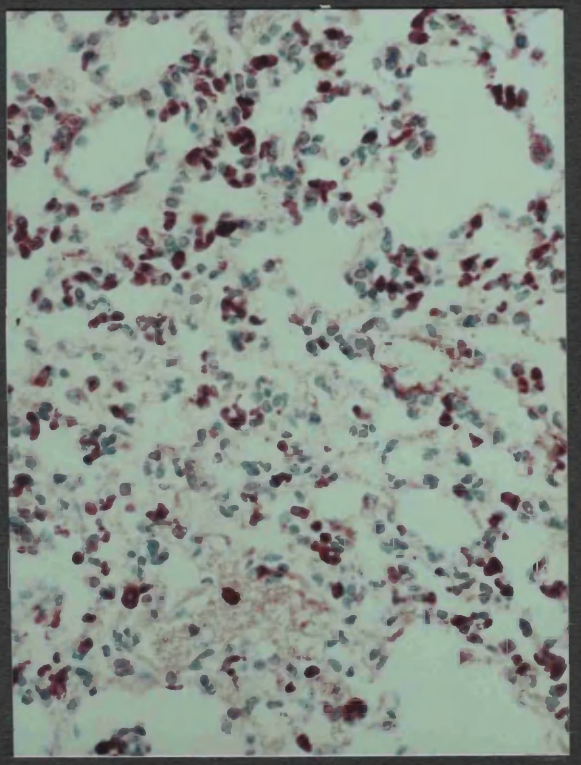


C) Adrenal. Macrophages, mainly in medulla. X 175

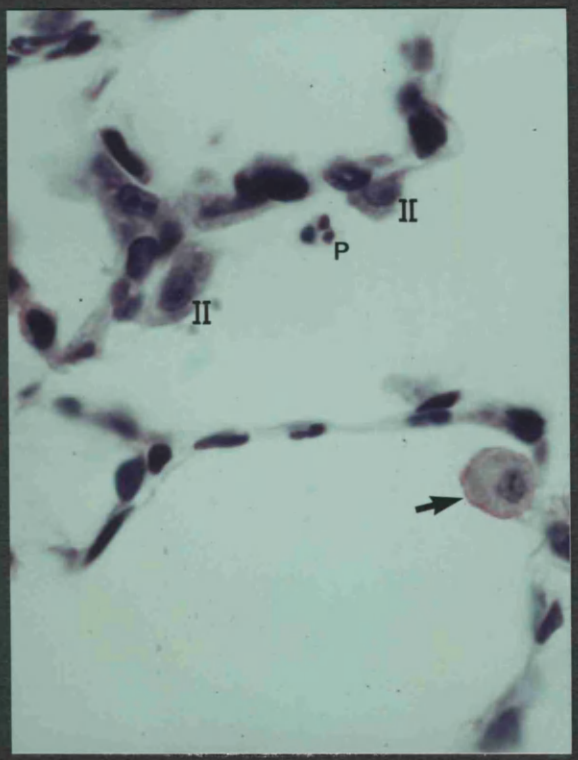
D) Cardiac muscle. Surprising number of interstitial macrophages. X 175



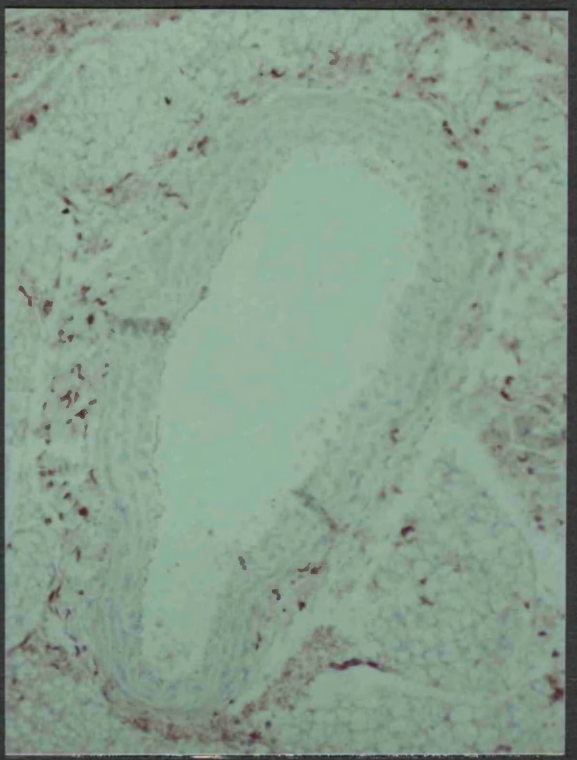
A) Normal rat lung. Interstitial and alveolar macrophages. X 175



B) Paraquat injured lung after 2 days. X 175



C) 4-day paraquat lung. Mildly damaged area. Negative polymorph, fibroblasts & regenerating type II cells. Rim of staining around ➔ macrophages. X 700



D) Large blood vessel to show adventitial macrophages. X 70

Fig 2:8 Immunoperoxidase labelling of Kupffer cell at ultramicroscopic level with antimacrophage antibody.

The Kupffer cell in a sinusoid shows some cytoplasmic projections and shows granular surface and some cytoplasmic staining. An adjacent perisinusoidal fibroblast (Ito cell) contains a typical fat globule (—>) and is unstained, as are portions of hepatocyte cytoplasm. Unstained, X 31,850

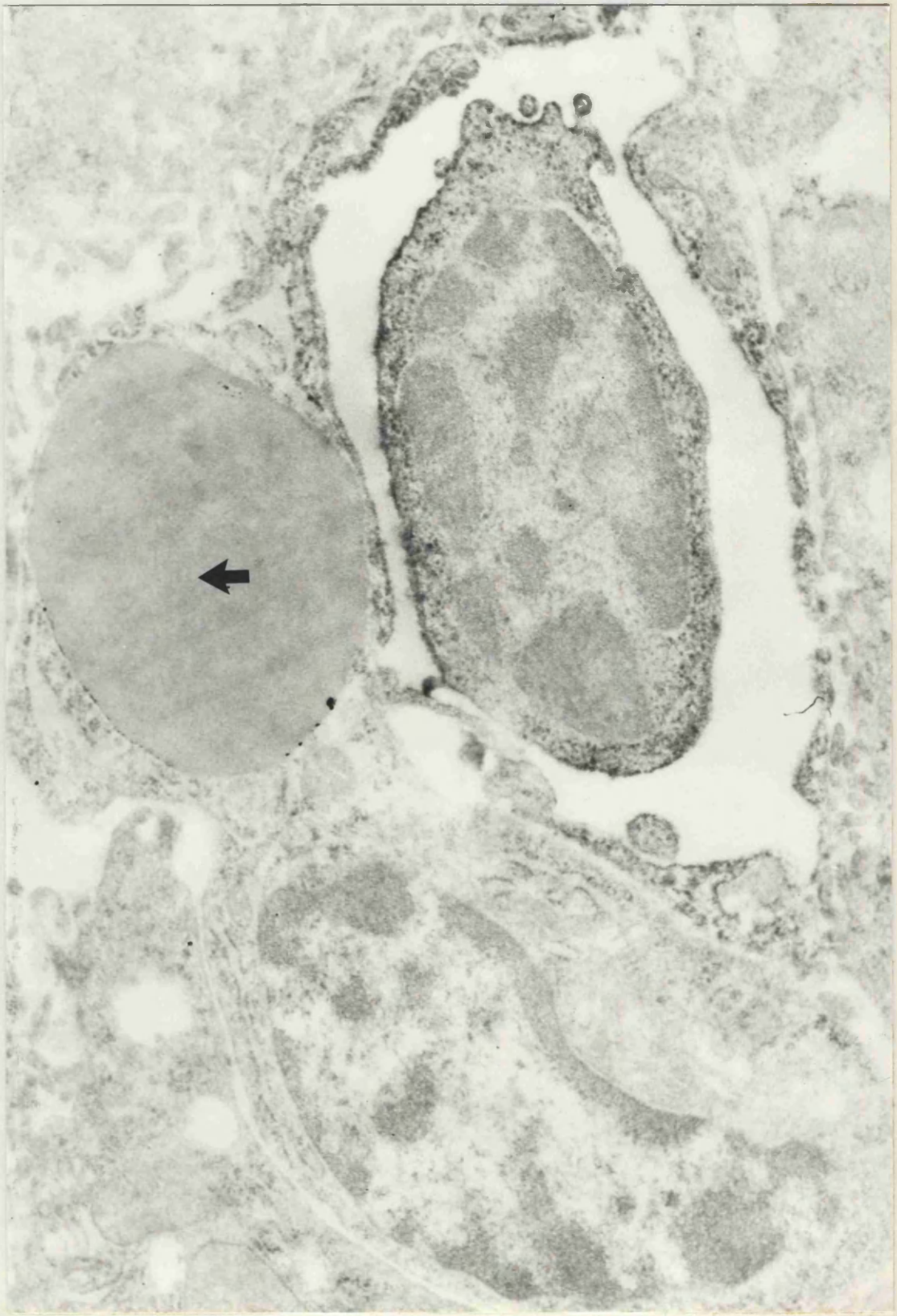
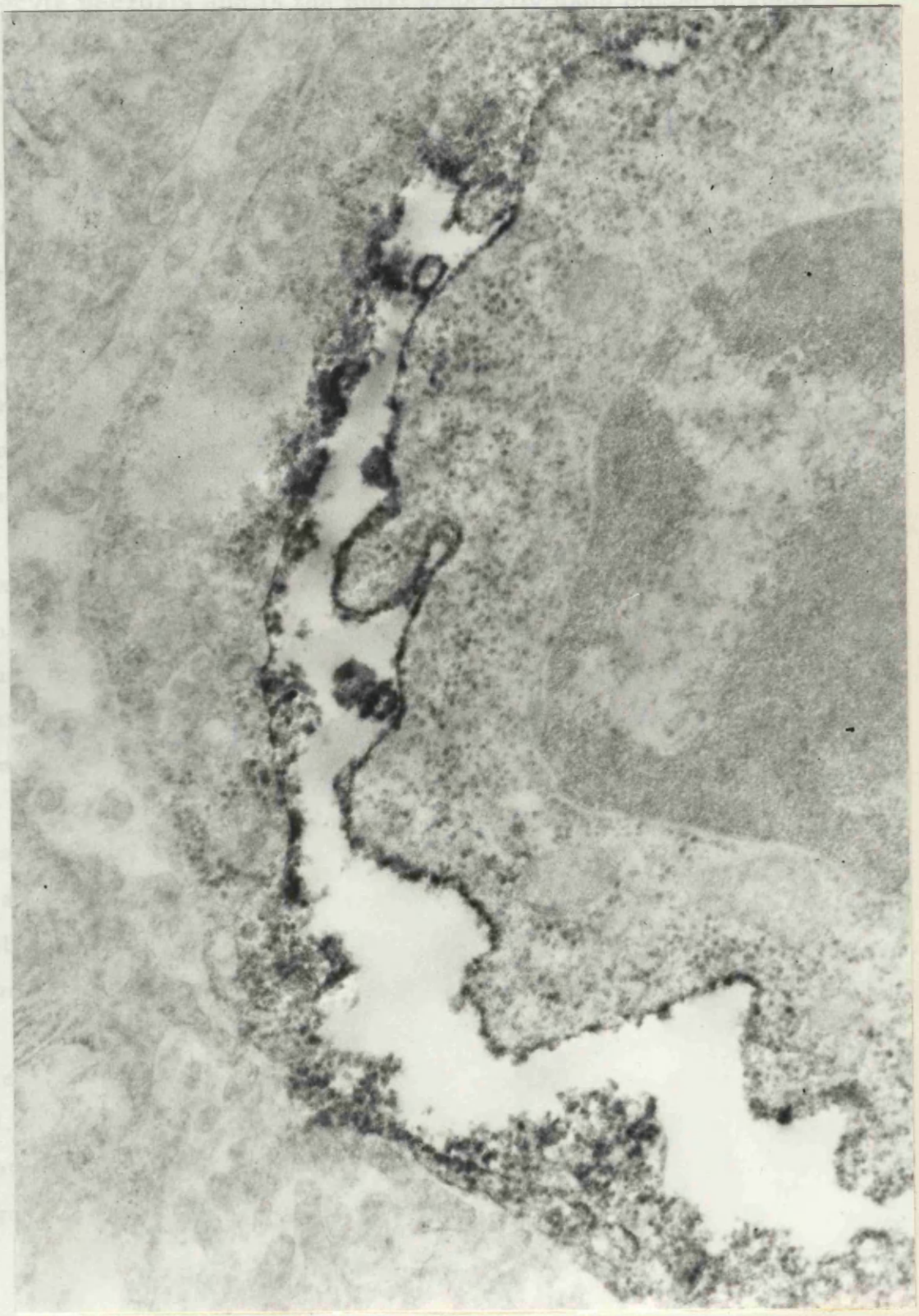


Fig 2:9 Immunoperoxidase labelling of Kupffer cell membrane at ultramicroscopic level. The granular staining is largely located on the cell membrane which appears to have a configuration matching the endothelial surface from which it appears to have been torn. Some outer cytoplasmic staining of small vesicles, probably pinocytotic vesicles, is present.

Unstained, X 51,250



2) GIANT CELL FORMATION IN CARBON TETRACHLORIDE LIVER INJURY IN THE MOUSE

Carbon tetrachloride poisoning produces centrilobular hepatocyte necrosis and is the most generally used model of acute toxic liver injury. Repeated dosage results in cirrhosis (See Chapter 3). By 3 days after poisoning most of the necrotic debris has been removed and there is a considerable mesenchymal infiltrate around the central veins. Using the immunoperoxidase technique this can be seen to be composed largely of macrophages (Fig. 2:10A). After a week the liver has usually returned to normal.

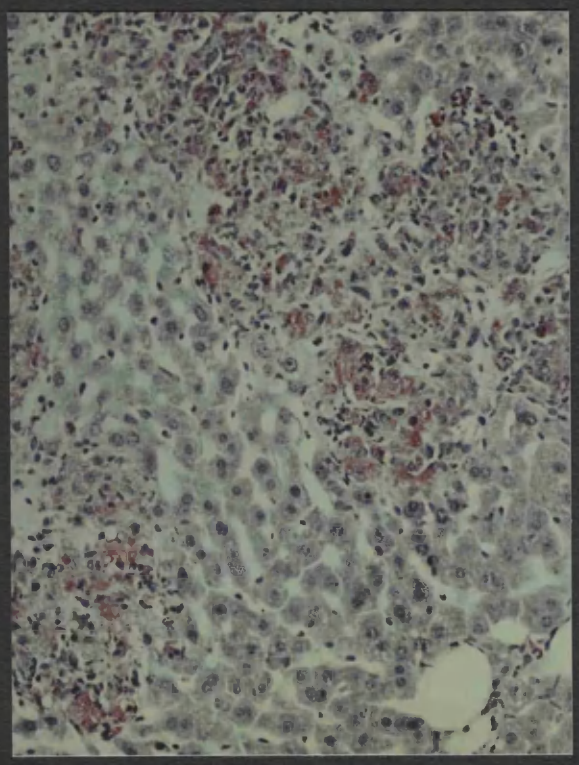
Careful examination of the infiltrate after about 4 days shows the presence of giant cell formation. This becomes a more prominent feature amongst the shrinking mesenchymal infiltrate after 5 days in only a third of animals (Figs. 2:10 C & D) and disappears by day 6. In occasional animals there is persistence around foci of calcification for up to 2 weeks. This,usually very transient,phenomenon of giant cell formation seems to have escaped comment by previous investigators who have tended to mention only muntinucleated regenerating hepatocytes (assuming the two cell types not to have been confused). Immunoperoxidase staining confirms the macrophage nature of the debris-related giant cells.

Previous experimental work on macrophage giant cells or polykaryons has been carried out <u>in vitro</u> or has used inert materials such as silica or glass to cause giant cell formation. The present model offers an opportunity to study giant cell formation <u>in vivo</u> in response to necrotic tissue of endogenous origin.

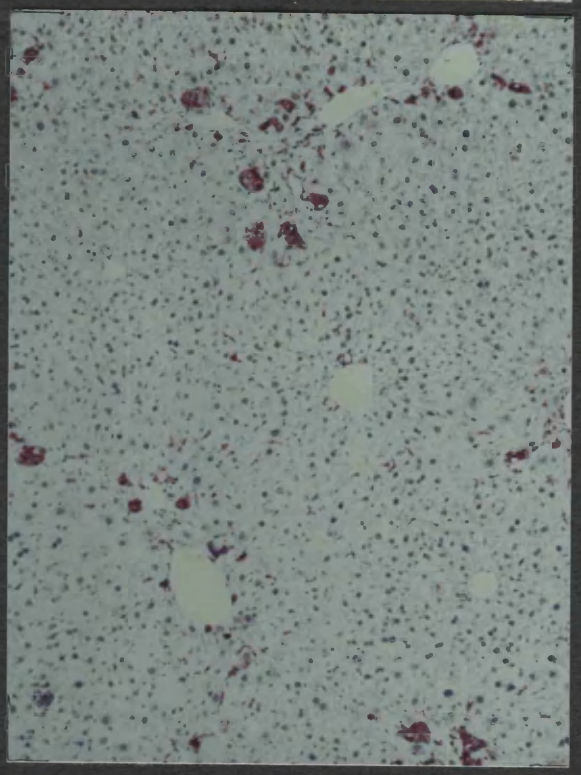
It has been proposed that giant cells may form when several macrophages attempt to ingest a single particle (reviewed by Chambers, 1978). In liver, macrophages are seen to adhere to the surface of necrotic hepatocytes and a sequence of events can be traced, recognisable at light level but more clearly seen with electron

microscopy, leading to giant cell formation (Figs. 2:11 to 2:15). As a necrotic cell is progressively eroded by phagocytic activity, so macrophages are observed to fuse with their neighbours forming what appears as a ring in cross-section but what must be a sphere in three dimensions around a progressively diminishing lacuna of necrotic debris. Close apposition of membranes of interdigitating lamellipodia appears to precede fusion. At light level this debris, initially negative with the immunoperoxidase technique, becomes positive as it is enclosed and this may indicate accumulation of macrophage derived antigens or penetration by many macrophage filipodia. Giant cells with multiple peripheral nuclei measuring up to 50 μ in diameter are the end result of this remarkable process.

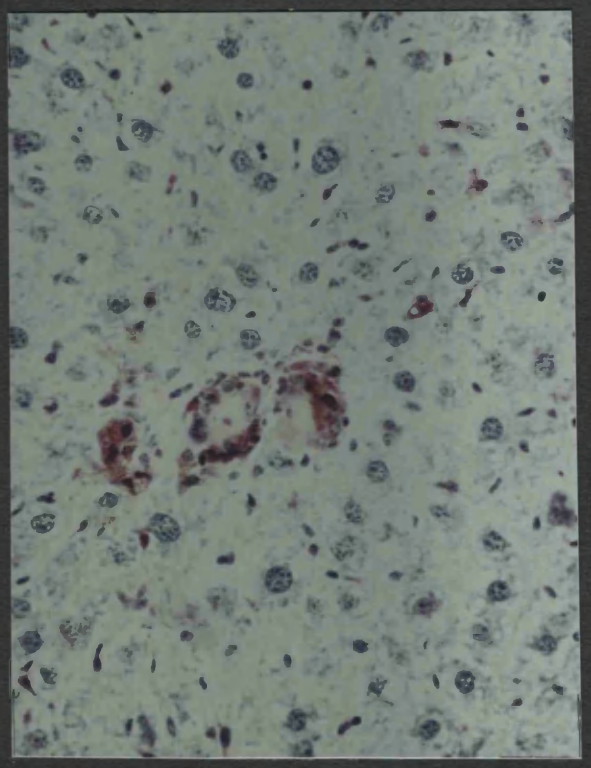
Little is known of the regulation and fate of macrophages (Adams, 1976). The function of giant cells is also uncertain although there is some evidence of cytoplasmic reorganisation in the longer lived Langhans type suggesting some secretory activity (Cohn and Kraus, 1980). On the basis of chromosomal abnormalities it has been suggested that they are the terminal stage in the evolution of the macrophage granuloma (Mariano and Spector, 1974). This is supported by our observation that the giant cells are a normally transient phenomenon and are the last type of macrophage to disappear as healing is completed. It may be that macrophage fusion and giant cell formation, so prominent a feature of granulomas, form an extreme example of a phenomenon applicable to chronic inflammation in general and that minor transient examples of fusion have been overlooked. Some signal or mechanism for self destruction must occur when material available for phagocytosis or resistant to dissolution as a result of calcification is no longer present. It is known that scattered pigmented macrophages can persist for many years at the site of an incident of damage or infarction. Do they persist because they have missed a phase of fusion with other macrophages and have been denied the opportunity to achieve oblivion ?



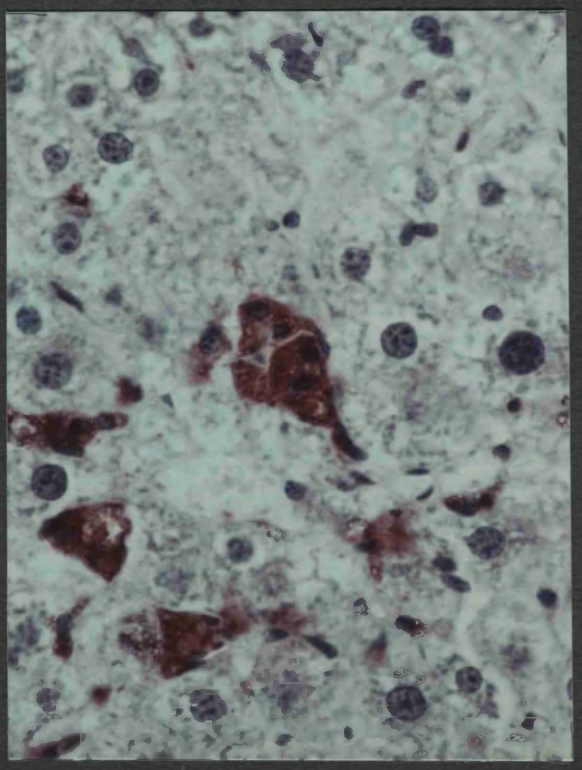
A) Liver 3 days after CCl_4. Centrilobular necrotic areas heavily infiltrated by cells nearly all stained by ab. X 175



B) Liver 6 days after CCl_4. Architecture restored except for occasional foci of giant cells. X70



C) Liver 6 days after CCl_4. Ring forms of fusing macrophages. X 280



D) Liver 6 days after CCl_4. Aggregates of macrophages showing varying amounts of fusion. X 448

adherent to a necrotic mouse hepatocyte (H). The macrophages are identifiable by their many cytoplasmic projections and many organelles including lysosomes. The hepatocyte shows degenerating cytoplasm with central clumping of organelles. The hepatocyte nucleus is not in the plane of section.

X 10,750

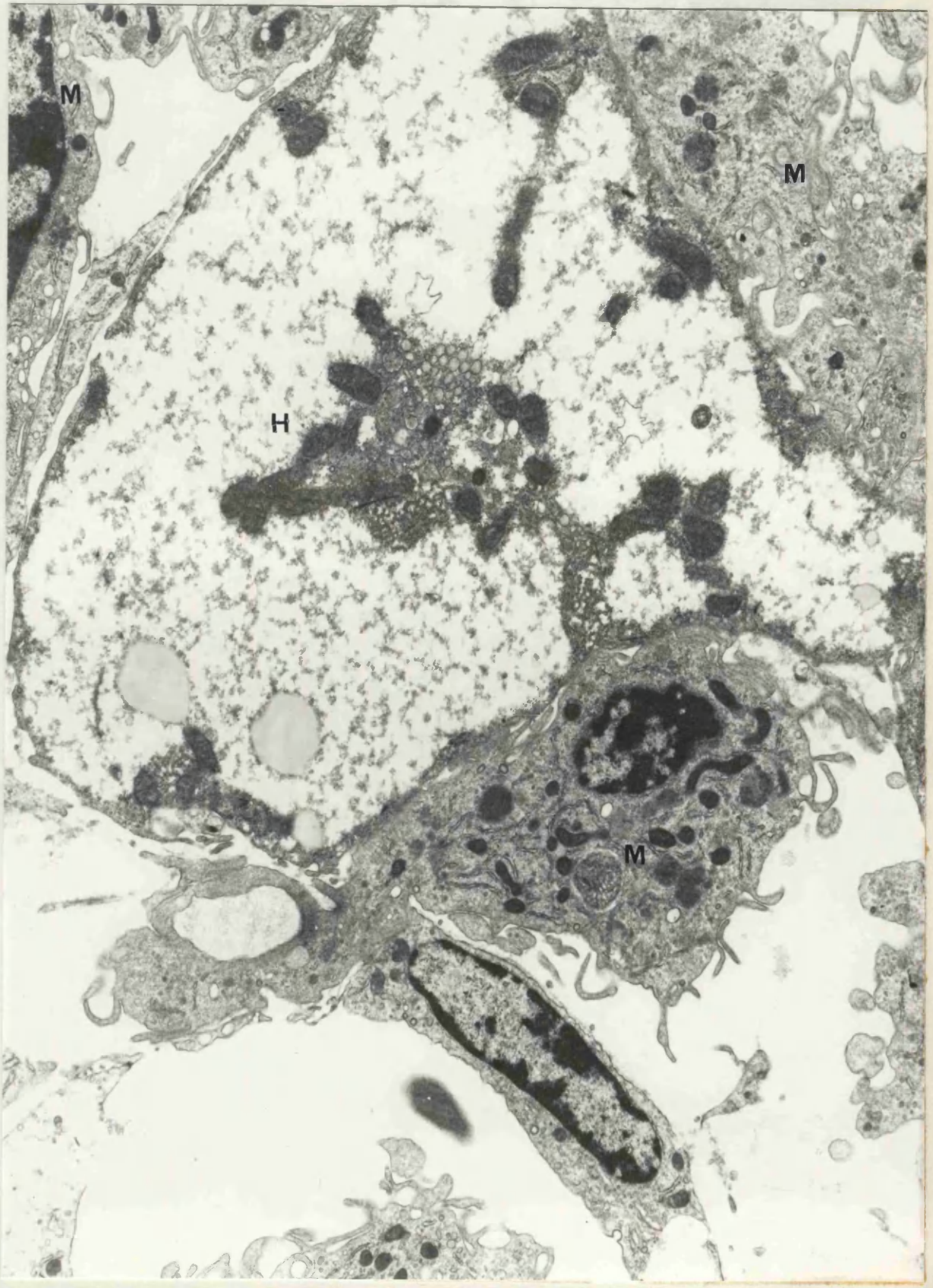


Fig 2:12 A. Progressive removal of necrotic hepatocytes by macrophages. Filipodia (—>) are particularly prominent.

X 2,780

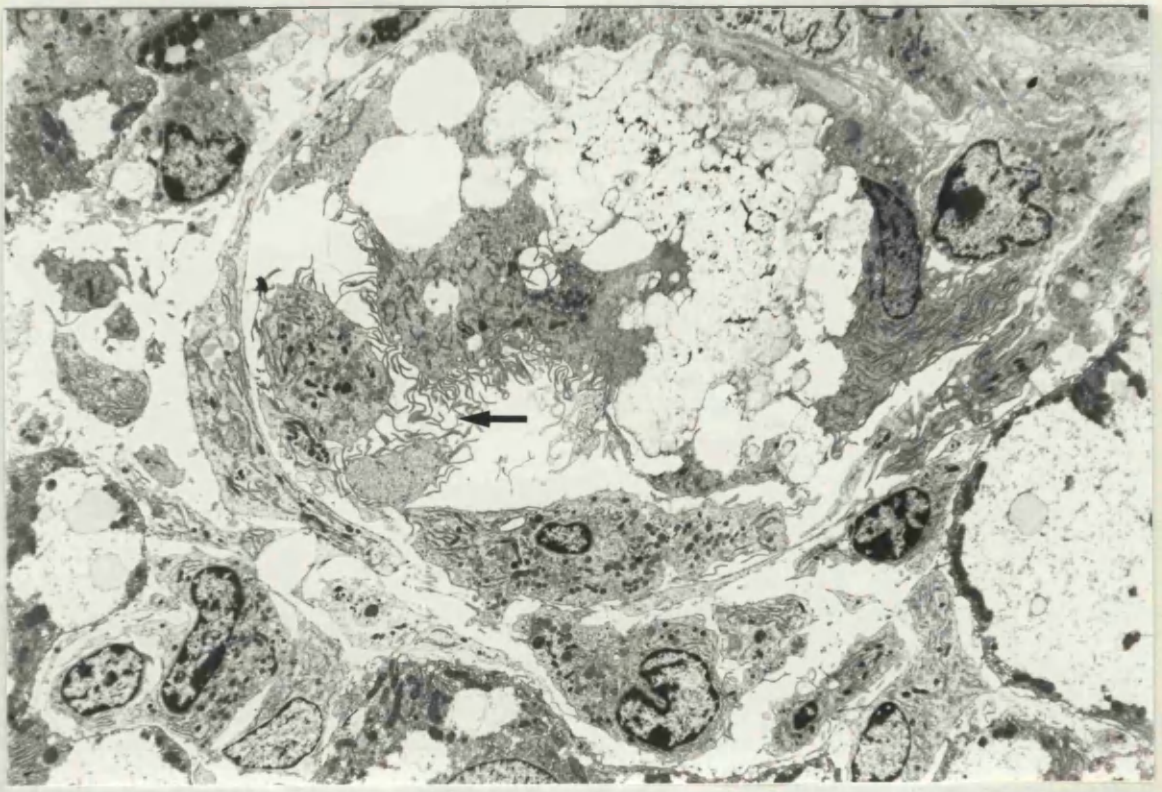


Fig 2:12 B. Aggregation of macrophages around residual debris. The area indicated (—>) is seen at higher magnification in the next Figure.

X 3,360

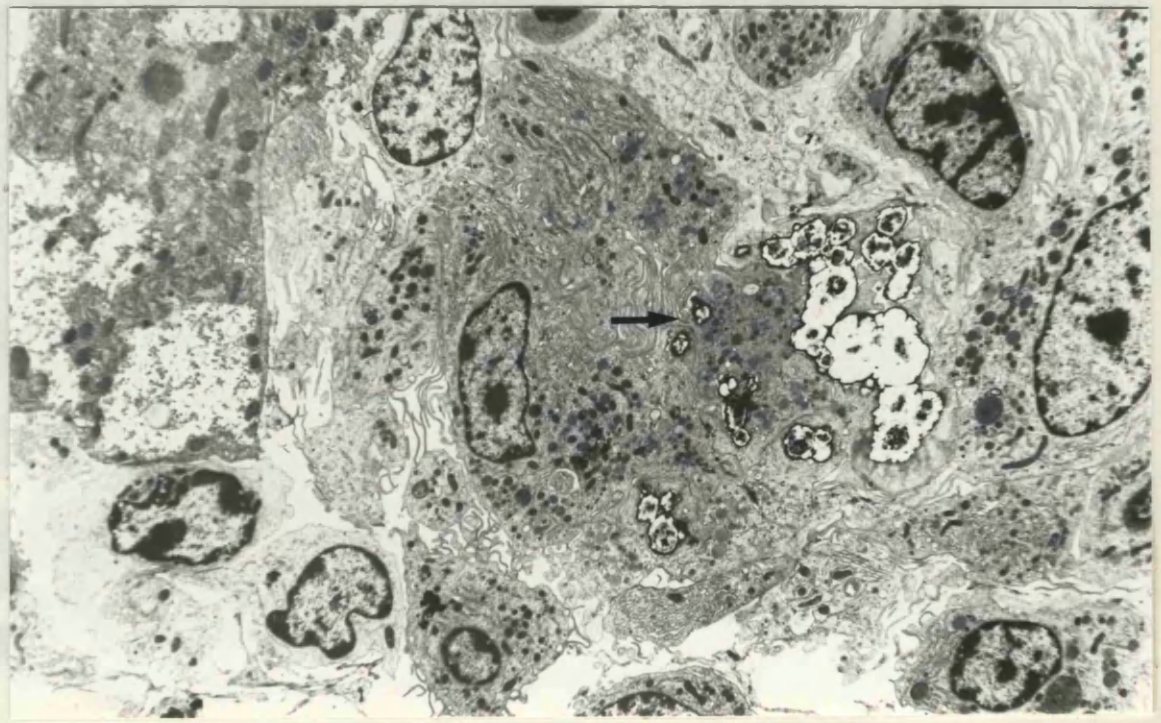
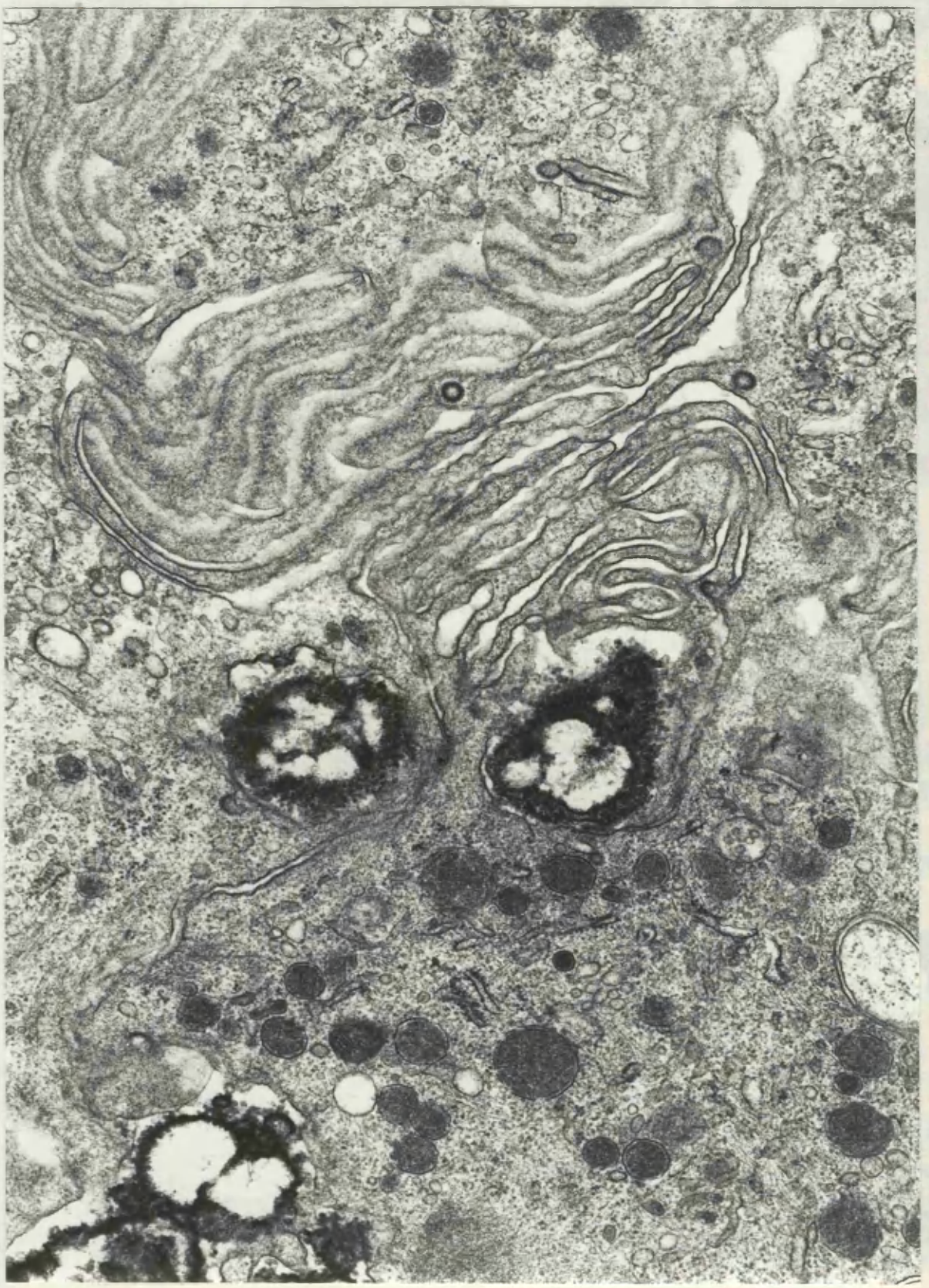


Fig 2:13 Interdigitating lamellipodia of fusing macrophages. Phagolysosomes persist containing debris.

X 32,500



An example (—>) shows long cytoplasmic extensions, rough endoplasmic reticulum, and absence of filipodia unlike macrophages. Collagen fibres appear later next to such cells.

X 6,720

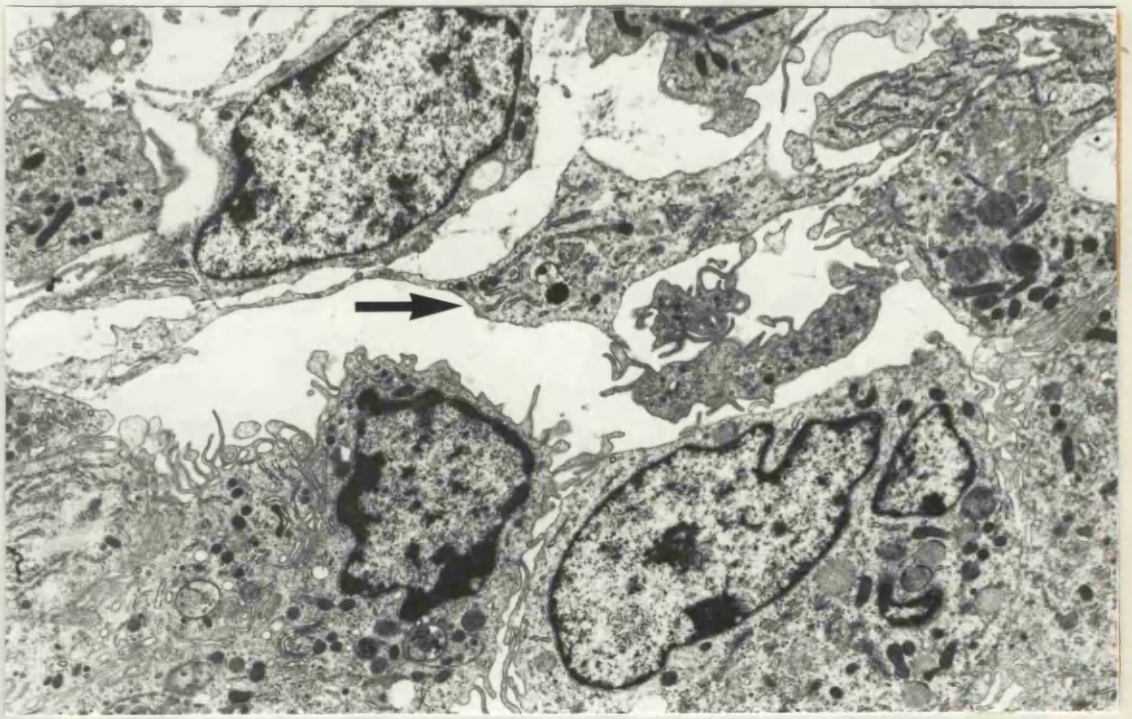


Fig 2:14 B. Completed fusion of adjacent macrophages. The area indicated (—>) is shown at higher magnification in the next figure.

X 3,360

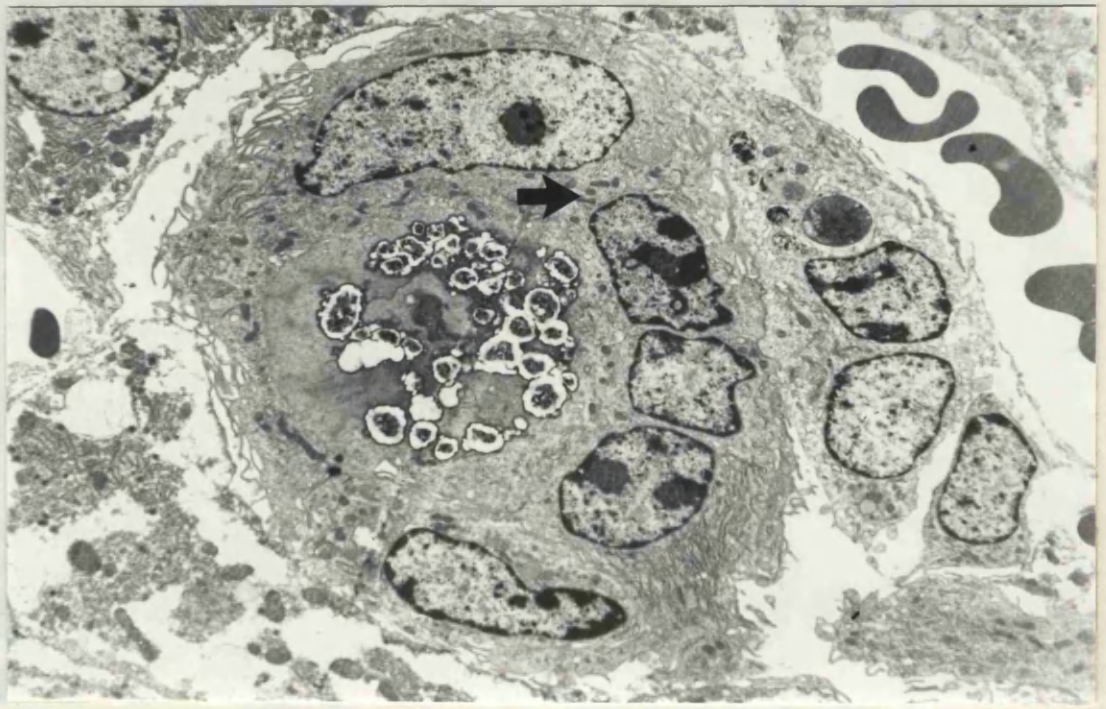
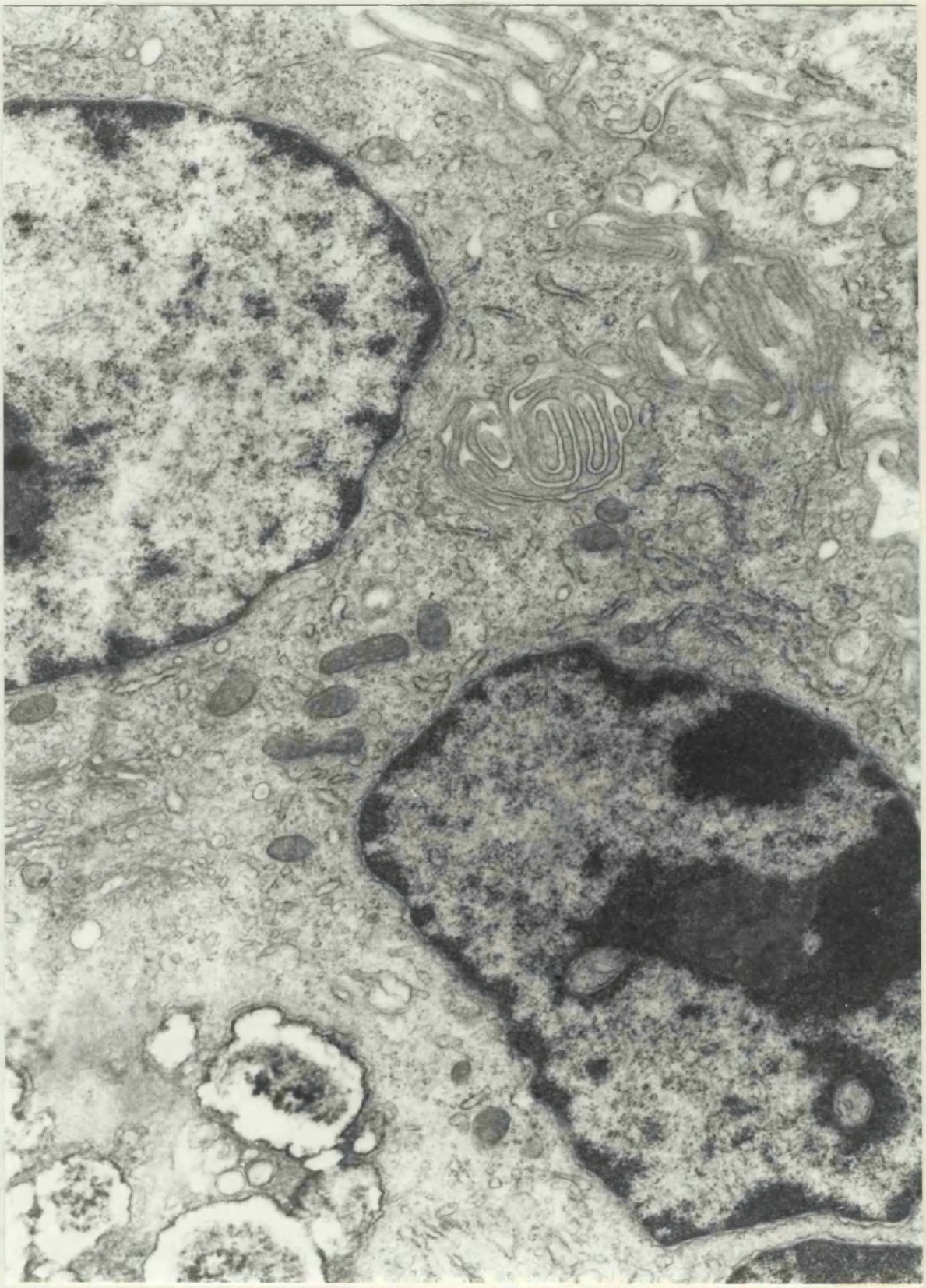


Fig 2:15 High power view of area of fusion. A close search of this area including tilting the specimen failed to reveal any persistent cell membrane between nuclei.

X 22,750



3) THE POTENTIAL OF AN ANTI-HUMAN MACROPHAGE ANTISERUM

The antisera to mouse and rat macrophages cross-react with the other animal's cells but neither stains human macrophages.

An anti-human macrophage antiserum could obviously be put to similar uses as the mouse macrophage antiserum in the study of chronic inflammation. Another potentially fruitful area would be in identifying cell types and cell origin in the solid tumours of lymphoid origin, namely the Hodgkin's and non-Hodgkin's lymphomas.

The nature of the Reed-Sternberg cell and the Hodgkin's cell itself have long been disputed and are currently controversial. The conflicting evidence appears evenly weighted between a monocyte and a B lymphocyte origin (reviewed by Stuart, 1978). A specific stain for macrophages should offer additional information for or against a monocytic origin allowing study of paraffin section quality slides and review of past cases. This assumes that neoplastic macrophages might retain surface antigens to which the antiserum was raised.

The designation of "histiocytic lymphoma" has proved the hardest category of non-Hodgkin's lymphoma to define by other than subjective impression. There is considerable current interest in this entity and its separation from other large cell lymphomas and plasmacytoid lymphomas particularly as it affects the small intestine (Isaacson et al, 1979). Another area of interesting application of such an antiserum, be the results positive or negative, would be the "fibrous histiocytomas" particularly of malignant type. Even experts in the field of soft tissue tumours admit the unsatisfactory ambiguity of such terminology (Mackenzie, 1975).

There seem to be two approaches to the problem of cytochemical identification of tumour cell type. On the one hand, a neoplasm may express embryonic antigens absent or nearly absent in the adult such as carcinoembryonic antigen or α-foetoprotein. Alternatively, a neoplasm

may retain the ability to synthesise proteins characteristic of the organ or tissue such as thyroglobulin or mucin. The latter approach is the one adopted in the use of an antimacrophage antiserum. Assuming this is successful one wonders how many other cell types might be labelled by surface antigen characteristics.

Enticed by such diverse potential applications, I recently decided to prepare such an antiserum. This has not proved straightforward and only very weak staining has so far been obtained which cannot be illustrated. The preliminary results are sufficiently promising to justify a brief description of the rather different methods used for purification of human macrophages.

Materials and Methods

Peritoneal dialysis fluid from the Glasgow Royal Infirmary Renal Unit was used as the source of human macrophages. The initial problem of centrifugation of large volumes (1 to 2 litres) of fluid to obtain relatively few cells was solved by borrowing a large capacity centrifuge head from the blood transfusion laboratory. Only 40 to 60 million cells are usually obtained and there is always some degree of blood contamination. Also patients on chronic dialysis have a low grade peritonitis and polymorphs form a major proportion of the cells. The bizarre finding of red cell rosetting of macrophages on two occasions was attributable to the patients' methyldopa medication.

Further purification of macrophages was obviously desirable and the method of Ulmer and Flad (1979) for separation of human monocytes from blood was adapted for peritoneal cells. A more recently described method is also now available (Pertoft et al, 1980).

The dialysate was centrifuged at 70 g for 15 minutes and the cells scraped off the bottom of the 500 ml bottles with a plastic "policeman". The cells were resuspended in a known volume of P.B.S. and counted. The cells were pelleted again at 70 g for 15 minutes and the supernatant discarded.

A discontinuous (step) gradient was set up in 50 ml plastic centrifuge tubes using the recently introduced gradient material Percoll (Pharmacia) in varying dilution with sterile isotonic saline. The gradient was formed in 5 ml layers from the bottom of the tube commencing with the lowest density of the following : 1.004, 1.062, 1.064, 1.066, 1.068, 1.070, and 1.080. The final gradient (1.080) was admixed with the cell pellet before application to the bottom of the tube.

The gradient was centrifuged at 390 g for 30 minutes and stopped without braking to avoid vibration. Cells were recovered from the interfaces of layers either by direct pipetting with a "hoovering" action, or by displacement with 60% sucrose using an M.S.E. displacement stand and extraction cap assembly. The 50 ml centrifuge tubes were adapted by adding a section of polycarbonate ultracentrifuge tube with Araldite to fit the extraction cap. Fractions were collected with an L.K.B. fraction collector loaded with tubes already containing formal saline. Good quality cytocentrifuge slide preparations were obtained by this immediate fixation.

Results and Discussion

From the 40 to 60 million cells including cells from blood contamination, about 2 to 5 million cells were recovered from the second interface from the top of the gradient tube. These cells largely had the morphology of macrophages as expected from the method used, with about 2% contamination by mesothelial cells readily identifiable with a Giemsa stain. The major contaminant was polymorphs varying from 5% to 20%. The cells present in the lower layers were confirmed to be lymphocytes and at the bottom polymorphs and red cells. These lower cells were irrelevant to the project except to confirm that the technique was working. It is noteworthy that, although large numbers of polymorphs were successfully separated away from macrophages by a distance of about 8 cm, there

was still a problem of polymorph contamination. From the cytospin preparations it is likely that this is due to clumping and adhesion between these two cell types. Brief treatment by an enzyme such as trypsin or neuraminidase would probably reduce cell stickiness but there would seem to be an unacceptable risk of destroying cell surface proteins and receptors as can occur with insulin receptors on adipocytes (Cuatrecasas, 1971). This might remove the very surface antigenicity required to raise a suitable antiserum.

Polymorph contamination has to be accepted with a view to future selective removal of anti-polymorph activity by appropriate absorption of the antiserum. Preliminary results using 1:10 diluted antiserum show weak staining of polymorphs and macrophages in sections of human acute appendicitis with reassuring faint selective staining of macrophages in the centre of germinal follicles. Delays have been caused through death of rabbits. The relatively low yield of cells obtained from dialysate compared with that from inflamed mouse peritoneum previously described accounts for the slow rise in antibody titre.

Chapter 3

THE ROLE OF THE MACROPHAGE IN THE FIBROGENIC RESPONSE TO ACUTE CARBON TETRACHLORIDE LIVER INJURY

Carbon tetrachloride is especially toxic to hepatocytes and has been in use for many years as the standard toxin for inducing experimental cirrhosis. Surprisingly the body of literature on the histopathology of the process is rather small. This toxin in single dosage has also provided a standard model of acute liver damage for much biochemical investigation but the sequence of histopathological events again seems neglected. With regard to the role of macrophages in damaged liver, there has been a concentration of interest on the immunology of liver disease on the one hand and the origin and physiology of Kupffer cells on the other. This is all the more surprising in view of the growth of gastroenterology into a biopsy-orientated clinical specialty. The study of chronic inflammation as it affects the liver and in particular the activity of macrophages in damaged liver has hardly begun. And yet large numbers of patients with chronic liver disease are treated empirically with drugs intended to suppress the chronic inflammatory process.

The mode of action of carbon tetrachloride on the hepatocyte is becoming clearer (reviewed by Dianzani, 1976). Carbon tetrachloride is converted to the free radicals $CCl_3^{\cdot}$ and $Cl^{\cdot}$ in the smooth endoplasmic reticulum by the microsomal drug metabolising system. Peroxidation of the lipid component of cell membranes is the major consequence and the other major features of protein denaturation, enzyme inactivation, and triglyceride accumulation are thought to be secondary effects. Much is known of the intricacies of the biochemical derangements which affect the hepatocyte but it seems sufficient to state that the result is fatal.

Swelling of endoplasmic reticulum and mitochondria are seen by electron microscopy as early as one hour after carbon tetrachloride (Bassi, 1960) and with concomittant loss of glycogen and RNA , progressing to extrusion of cell cytoplasm and organelles (Ashworth et al, 1963 ; Reynolds, 1963). A variety of associated histochemical alterations have been reported (Cotutiu and Streja, 1966). Subsequent histological events were comprehensively described by Cameron and Karunaratne (1936), and since then significantly added to by only Stowell and Lee (1950) and Leduc and Wilson (1958). Briefly, centrilobular hepatocytes show hydropic change from 5 hours onwards and by the second day are necrotic with degenerating, pyknotic nuclei. A few polymorphs are present but are not a prominent feature. Liver sinusoids containing endothelial and Kupffer cells remain undamaged. Between the second and third day necrotic tissue is quite suddenly invaded by macrophages and fibroblasts, surrounded by a border of large, pale, foamy hepatocytes. Mitoses are seen in hepatocytes throughout the surviving liver and not just adjacent to the damaged area. Mitoses are found as early as 12 hours and reach a maximum after 2 or 3 days, continuing up to 5 days. Necrotic tissue largely disappears by 4 days followed by mesenchymal cells which disappear with as spectacular rapidity as they arrived. Normal liver architecture is restored typically within one week.

Repeated dosage with carbon tetrachloride produces hepatic cirrhosis and it is accepted that this is due to new synthesis of collagen and not collapse of reticulin as discussed by Rubin and Popper (1967). The sequence of events and mechanisms involved are more readily studied following a single dose. This acts as a stimulus to increased synthesis of hepatic collagen and glucosaminoglycans (Patrick and McGee, 1967 ; McGee and Patrick, 1969). This phenomenon is transient and is followed by a phase of increased collagenolytic activity as architecture becomes restored (Okazaki and Maruyama, 1974). Increased collagen synthesis is accompanied by elevated levels of collagen

prolyl hydroxylase (Takeuchi et al, 1967 ; McPhie and Short, 1975 ; Risteli et al, 1978) and hepatic fibroblast activity. These cells are derived from perisinusoidal fat-storing cells (McGee and Patrick, 1972 ; Kent et al, 1976). This particular cell type, although histologically unobtrusive, is present in considerable numbers - 1 for every 20 hepatocytes in human liver (Bronfenmajer et al, 1966) and was first described by Ito (1952). The lipid vacuoles are Vitamin A and this is now recognised as an (unexplained) feature of fibroblasts throughout the body not just in the liver (Yamamoto et al, 1978).

The fibrogenic response begins after 3 days, contemporaneous with the removal of necrotic debris by macrophages ; a temporal relationship which seems more than coincidence. The role of activated macrophages in providing a stimulus to fibroblast activity with the production of new connective tissue has been suggested from the results of a variety of in-vitro experiments with silica (Heppleston and Styles, 1967 ; Aalto et al, 1976) and from studies of experimental cutaneous wounds (Leibovich and Ross, 1976). With the notable exception of the paper by Paumgartner et al (1968) on phagocytic activity in experimental liver injury, there have been no investigations directed towards the response of macrophages to hepatic necrosis. Kent et al (1964) found however that massive iron-loading accelerated the induction of carbon tetrachloride cirrhosis and speculated that iron-laden macrophages may have been responsible.

It seemed likely to me that macrophages were concerned in the fibrogenic response to necrosis, and that experimental interference with macrophage activity might lead to interference with fibroblast activity. The following work describes the effects of macrophage depletion or altered functional activity on the responses of mouse liver to a single episode of acute carbon tetrachloride injury.

MATERIALS AND METHODS

Young male albino mice of the CFLP strain weighing 20-25 g were used in all experiments. Carbon tetrachloride, four parts, was mixed with liquid paraffin, six parts, and administered by oesophageal tube. Each animal received a single dose (0.15 ml) of this mixture.

Cortisone acetate (Sigma) was given by intramuscular injection on 3 consecutive days, each daily dose being 2.5 mg suspended in 0.1 ml isotonic saline. One group of mice received the first of three doses on the day preceding carbon tetrachloride intoxication and a second group received the first dose on the 3rd day after poisoning. A control group received cortisone alone.

Iota carrageenan (Sigma Type V) 1 g was dissolved in 100 ml warmed isotonic saline immediately before use. A single dose (2 ml) was given by intraperitoneal injection immediately after carbon tetrachloride poisoning. Small blocks of liver were fixed in 10 per cent formalin and paraffin sections stained by haematoxylin and eosin and for reticulin fibres by Gordon and Sweet's method. Macrophages were demonstrated using the immunoperoxidase technique with an antibody to mouse peritoneal macrophages prepared in a rabbit as described in the previous chapter. Some sections from carrageenan-treated mice were also stained with Alcian Blue and by the PAS-diastase technique.

The remainder of each liver was frozen quickly and stored at $-20^{\circ}C$ prior to enzyme analysis. All assays from any one experimental group of mice were performed together and in duplicate. For this procedure 0.2 g liver was ground in a tight-fitting glass homogeniser together with 2.0 ml 0.05 M-tris buffer at pH 7.2 to which were added 0.25 M-sucrose, 10^{-5} M-EDTA, 10^{-3} M-dithiothreitol and 0.1 per cent triton X-100. Prolyl hydroxylase activity was assayed in 50 µl aliquots of these homogenates (i.e. 5 mg liver) using the tritium release method of Hutton et al (1966). (See Appendix)

In addition to these studies on liver, similar enzyme measurements were made on mouse peritoneal macrophages and on mid-log phase cultures of L 929 mouse fibroblasts. The former were obtained from washings of peritoneum 3 days after the injection of mineral oil. L 929 fibroblasts were obtained from Gibco Biocult (Paisley, Scotland) and grown in standard MEM and glutamine with the addition of 10% foetal calf serum and ascorbate. Each cell concentration for enzyme assay was 1 million per ml.

RESULTS

Experiments with carbon tetrachloride alone

Sub-lethal centrilobular zonal necrosis of liver develops in all animals and there is histological evidence for the commencement of repair within 48 hours. By the 3rd day after poisoning the damaged areas are heavily infiltrated with mesenchymal cells which stain by the immunoperoxidase technique using antimacrophage antibody (Fig 2:10). Necrotic debris is mostly removed by the 4th and 5th days when the numbers of macrophages diminish. This phagocytic activity is accompanied by regeneration of surviving parenchyma, while a transient increase in reticulin fibres within the damaged zones may be observed. In many animals recovery of normal liver structure is obtained within a week, but, in a minority (less than 10%), small groups of cells including multinucleated forms which stain with the anti-macrophage antibody may remain for a few days longer, and prominent reticulin fibres usually persist around these. Eventually, however, there is complete restitution of the liver including a return to a normal reticulin pattern. These features have been studied in over a hundred mice.

A detectable increase in hepatic prolyl hydroxylase activity was noted on the 3rd day after poisoning. By the 5th day this activity was several times the normal control level, towards which there was a gradual return thereafter (Fig 3:1). This experiment and subsequent experiments have been performed at least twice with comparable results.

Cortisone experiments

Mice given cortisone alone for 3 days have increased amounts of liver fat and glycogen, but there is no hepatocyte necrosis or hepatitis and no corresponding increase in prolyl hydroxylase activity ; indeed there is usually a transient fall of up to 50 per cent in the normally low level of this enzyme, with return to normal 2 days after the last

dose (Table 3:1).

When given around the time of carbon tetrachloride administration, the hormone tends to intensify the necrotic injury and there is a notable delay in the healing response. The livers of these mice 3 or 4 days after poisoning have histological features similar to the 1-day lesion in animals not given cortisone (Fig 3:2 A & B) while the macrophage response is not well established until the 6th or 7th day (Fig 3:2 C). The increase in hepatic prolyl hydroxylase activity was likewise delayed, the peak being reached about the 8th or 9th day after poisoning (Fig 3:1). No increase in reticulin at the normal time was found (Fig 3:3 A & B) and the increase was similarly delayed (Fig 3:4 A & B). In these experiments, therefore, it would appear that the elevation of prolyl hydroxylase was closely preceded and may have been determined by macrophage activity in the liver.

This simple association was not upheld by the results of those experiments where cortisone was given on the 3rd, 4th and 5th days after carbon tetrachloride, when the mesenchymal response is already established. This treatment fails to retard the healing process which proceeds in the normal way. It was effective, however, in aborting the rise in prolyl hydroxylase which even fell temporarily below the starting level (Fig 3:1). Reticulin deposition was also markedly reduced.

Carrageenin experiments

Kupffer cells are prominent in the livers of mice which received iota carrageenin alone. Many contain small vacuoles of acidic and neutral mucopolysaccharide and all stain with the antimacrophage antibody. Hepatocyte degeneration or necrosis are not seen but minor damage may have occurred as mitotic activity in these cells is prominent. There was neither elevation nor depression of hepatic prolyl hydroxylase activity in mice receiving carrageenin alone (Fig 3:5).

Unlike cortisone, carrageenin does not aggravate the carbon tetrachloride-induced lesion nor delay the initial macrophage

infiltration and ingestion of necrotic tissue. Nevertheless, there appears to be significant functional interference with these cells as, in many mice, they remain in exaggerated numbers for 2 weeks or more after the time of injury. There is no notable increase in reticulin fibres around persistent macrophages.

In the animals so treated there was almost complete suppression of the prolyl hydroxylase response in the liver, and this observation has been confirmed in three separate experiments (Fig 3:5). As in the delayed cortisone experiment, the level of enzyme does not, therefore, correlate simply with the presence of macrophages in the liver ; however, these cells appear to have undergone some functional disturbance from carrageenin injection.

Experiments with macrophages and cultured fibroblasts

The mouse fibroblasts had 8 times more prolyl hydroxylase activity (1, 722 DPM per 10^6 cells) than peritoneal macrophages (205 DPM per 10^6 cells). The complete results are displayed in Fig 3:6.

DISCUSSION

Macrophages are constantly present in all types of human chronic inflammatory liver disease leading to fibrosis and eventual cirrhosis. Acute carbon tetrachloride liver injury offers a convenient experimental model to study the role of macrophages in fibrogenesis in vivo. Macrophages form a major component of the mesenchymal response (Paumgartner et al, 1968) which is such a prominent feature by the 3rd day after injury. This has been illustrated and confirmed by our own immunohistochemical observations (see also Chapter 2). This reaction precedes and therefore may influence the increasing prolyl hydroxylase activity which is itself followed by collagen chain synthesis (McGee et al, 1973).

The peak of maximum enzyme activity has been attained consistently on the 5th day after carbon tetrachloride in the present work. The original work by McGee and colleagues demonstrated a rise a day or two earlier but McGee attributes this to the use of another strain of mouse (personal communication) as subsequent work in his department at Oxford showed a later peak. The present time-scale is supported by the work of Risteli et al (1977) on rat liver. This is also found in the present work on rat paraquat lung injury (chapter 3), and that of O'Hare, (1978) where the peak of prolyl hydroxylase in mouse skin wounds was on day 5. Maximum activity at day 5 has also been reported for rat skin wounds (Mussini et al, 1967). In fact the consistency of this observation in rodent liver, skin and lung injury of varying causation points to a common pattern of biological response which owes little to the initial mode of injury.

The source of hepatic prolyl hydroxylase is controversial. Hepatocytes possess some of this enzyme activity (Ohuchi and Tsurufuji, 1972). This feature is greatly exaggerated in human hepatocellular carcinoma (Keiser et al, 1972) and experimentally induced hepatocellular carcinoma in rats (Baillie et al, 1975). An

increase has also been found in hepatocytes regenerating after partial hepatectomy (Patrick et al, 1980). However a much greater level of activity and order of increase was observed in the mesenchymal cell fraction.

The enzyme elevation in liver injury is more readily attributed to the striking increase in cells with the features of fibroblasts which appear rapidly in the damaged areas (McGee and Patrick, 1972). Mesenchymal cells isolated from liver homogenates possess prolyl hydroxylase and the capacity for collagen synthesis. These functions are both enhanced in cells from carbon tetrachloride liver. Such mesenchymal cells obtained by the method of Roser (1968) form a mixed population which includes many macrophages as well as cells with the ultrastructural features of fibroblasts (Shaba et al, 1973). Macrophages have recently been reported to possess prolyl hydroxylase activity (Myllyla and Seppa, 1979). The data shown in Fig 3:6 comparing cultured mouse fibroblasts and peritoneal macrophages suggest that this activity is likely to make only a minor contribution compared to that of fibroblasts.

It is known that cortisone depresses both macrophage infiltration and parenchymal regeneration of liver following carbon tetrachloride injury (Patrick, 1955) and it is evident from the present work that hepatic prolyl hydroxylase is depressed also in these experimental animals (Table 3:1).Inhibition of regeneration is presumably an effect of the hormone on DNA synthesis as previously shown in mouse liver following partial hepatectomy (Lahtiharju and Teir, 1964). Suppression of hepatocyte proliferation may contribute to the fall in prolyl hydroxylase but is probably unimportant for the reasons already stated.

Although knowledge of steroid hormone action at the cell biological level is now extensive (reviewed by Chan et al, 1978), much of the basis for the anti-inflammatory action of glucocorticosteroids in tissue remains uncertain (Stevenson, 1977). Apart from a possible local depressing effect on Kupffer cell proliferation and activation,

steroids are likely to inhibit the recruitment of monocytes to the sites of liver damage from the circulating blood, as in other types of experimental injury (Thompson and Van Furth, 1970). The delay before the rise in prolyl hydroxylase and appearance of reticulin could be a consequence of the delay in appearance of macrophages in the necrotic tissue when cortisone is given at the time of poisoning. Both features are suppressed and subsequently recover in unison. It is also possible, however, that these are two independent effects of steroid administration which happen to be manifest concurrently. There is good experimental evidence that cortisone can depress directly collagen formation in vitro and in vivo (Kruse et al., 1978) ; moreover, there is a reduction of both prolyl hydroxylase and soluble collagen in normal liver following steroids (Cutroneo et al, 1971). This is confirmed by the results for animals receiving steroids alone (Table 3:1). Nevertheless the point of the carbon tetrachloride plus steroid experiment is that the stimulus for fibrogenesis is postponed and remains associated with macrophage activity.

Cortisone given after the establishment of the healing response did not reduce the numbers of macrophages in the damaged liver or impair their phagocytic activity. This is in keeping with the studies on healing cutaneous wounds by Leibovich and Ross (1975), which indicate that antimacrophage serum as well as steroid is necessary to inhibit the activity of the few macrophages already present in tissue and at the same time completely inhibit fibrogenesis. The present experiment shows that cortisone cut short the beginning of the fibrogenic response whilst permitting continuing macrophage activity proceeding to resolution of the lesion. These experiments with cortisone do not finally resolve the question as to whether it acted directly on cells with fibroblastic activity, whether it acted directly by a selective effect on macrophage activity independent of their phagocytic activity, or whether it rendered fibroblasts refractory to a macrophage stimulus. The last possibility seems the most likely to me since the stimulus to fibrogenesis did not reappear and since the

steroid-alone experiment confirms temporary suppression of collagen synthesis.

The results of the experiments using delayed administration of steroids are also of interest in showing that the temporary fibrogenic response which follows acute liver injury, whatever its cause and significance may be, is unnecessary for complete recovery of the damaged organ within a normal time interval. A somewhat similar experiment with carbon tetrachloride but with continuous administration of steroid and resolution of the lesion over the normal time scale in spite of a reduced inflammatory response has been reported some time ago (Hoffman et al, 1955). The results obviously baffled the authors as no explanation or speculation is offered.

A more selective agent was sought to interfere with macrophage function. Unlike cortisone, carrageenin is not known to inhibit fibroblastic activity and the absence of a depressant effect on normal liver prolyl hydroxylase would support this. The hope was that experiments with carrageenin as a macrophage inhibitor would provide less enigmatic results. Iota carrageenin appears to be the least toxic of the available types (Thomson and Horne, 1976), and in the conditions of our experiments it did not cause liver cell necrosis. Certain other types of carrageenin cause a florid acute inflammatory response at the ingestion site leading on to a persistent granulomatous reaction and would obviously have been unsuitable. A toxic effect on Kupffer cells which readily ingest it has been noted (Fowler and Thomson, 1978), and it appears from our results that it retards considerably the normal functional activity of macrophages in the damaged liver. It seems reasonable to assume that this phenomenon may be implicated in the failure of the prolyl hydroxylase response in mice given carrageenin.

These various manipulations of the basic carbon tetrachloride liver injury model may be individually inconclusive. Together they provide a body of evidence that normal macrophage function is linked to fibrogenesis, and fail to disprove the hypothesis that macrophage

activity influences and perhaps controls fibroblast activity in damaged tissue.

Fig 3:1 Mean levels of hepatic prolyl hydroxylase activity in mice given carbon tetrachloride alone ; carbon tetrachloride plus early cortisone administration ; carbon tetrachloride plus delayed cortisone administration. Each time point represents the mean of at least four observations. The hatched figures represent the periods of cortisone administration. The results are expressed as D.P.M. $\pm$ S.E.M. per 50 µl liver homogenate, being the actual experimental data.

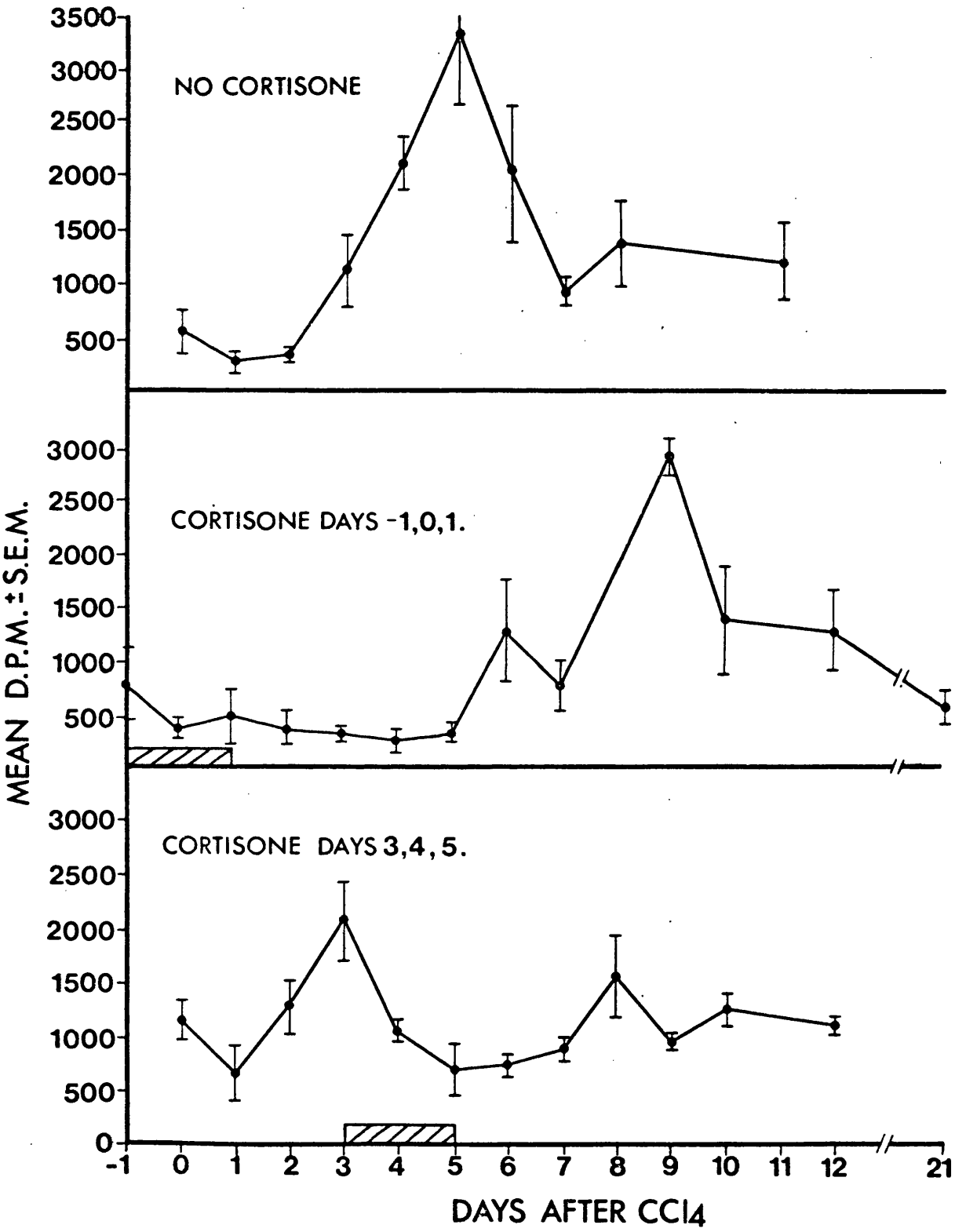

NO CORTISONE
CORTISONE DAYS -1,0,1.
CORTISONE DAYS 3,4,5.
MEAN D.P.M. ± S.E.M.
DAYS AFTER CCl4
3500
3000
2500
2000
1500
1000
500
0
-1
0
1
2
3
4
5
6
7
8
9
10
11
12
21

TABLE 3:1

Effect of cortisone on hepatic prolyl hydroxylase

Hepatic prolyl hydroxylase values for groups of 5 male CFLP mice (littermates of weight 25 - 30 g) given 2.5 mg cortisone acetate subcutaneously for 3 consecutive days. Each value is the mean of duplicate estimates of 50 µl of liver homogenate. A significant fall in level of approximately 50% is seen on Day 2 (Student's t test : $t = 3.38$; $2P<0.01$) returning to normal values on Day 4 (t not significant)

Day	DPM/50 µl	Mean DPM $\pm$ S.E.M.
0	409	463 $\pm$ 57
	317	
	401	
	557	
	630	
2	234	250 $\pm$ 27
	198	
	278	
	340	
	198	
4	697	553 $\pm$ 56
	641	
	574	
	405	
	445	
6	357	378 $\pm$ 26
	361	
	317	
	384	
	474	
10	319	363 $\pm$ 60
	453	
	178	
	530	
	332	

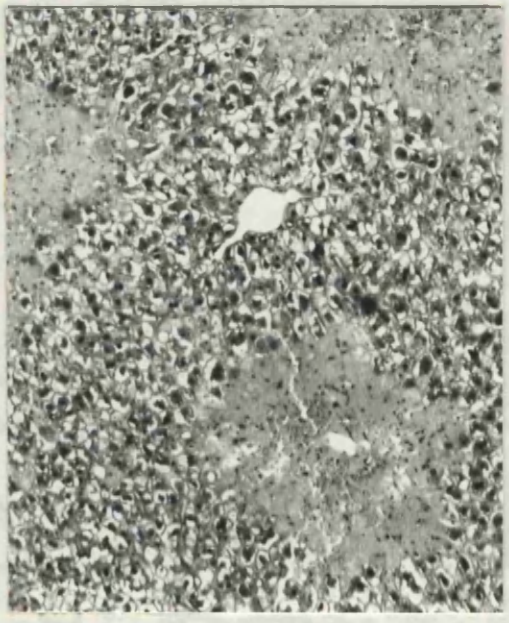


Fig 3:2 A. Hepatic centrilobular zonal necrosis in a mouse receiving cortisone and 3 days after carbon tetrachloride. Surviving hepatocytes are swollen with lipid and glycogen. Haematoxylin and eosin (HE). X 96

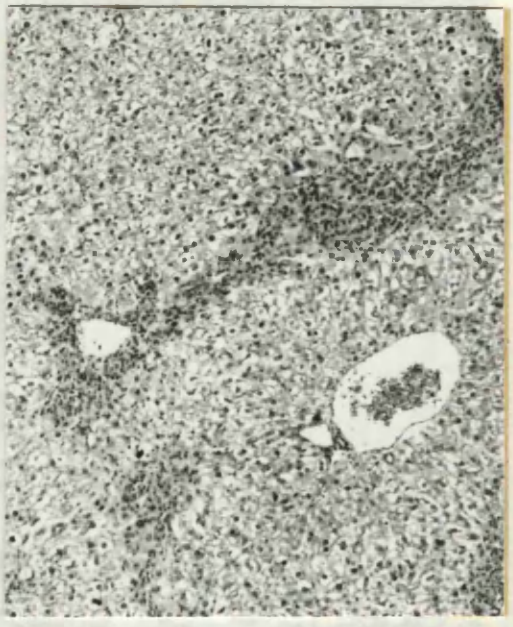


Fig 3:2 B. Three-day carbon tetrachloride lesion in mouse not given cortisone, for comparison with Fig 3:2 A. HE X 68

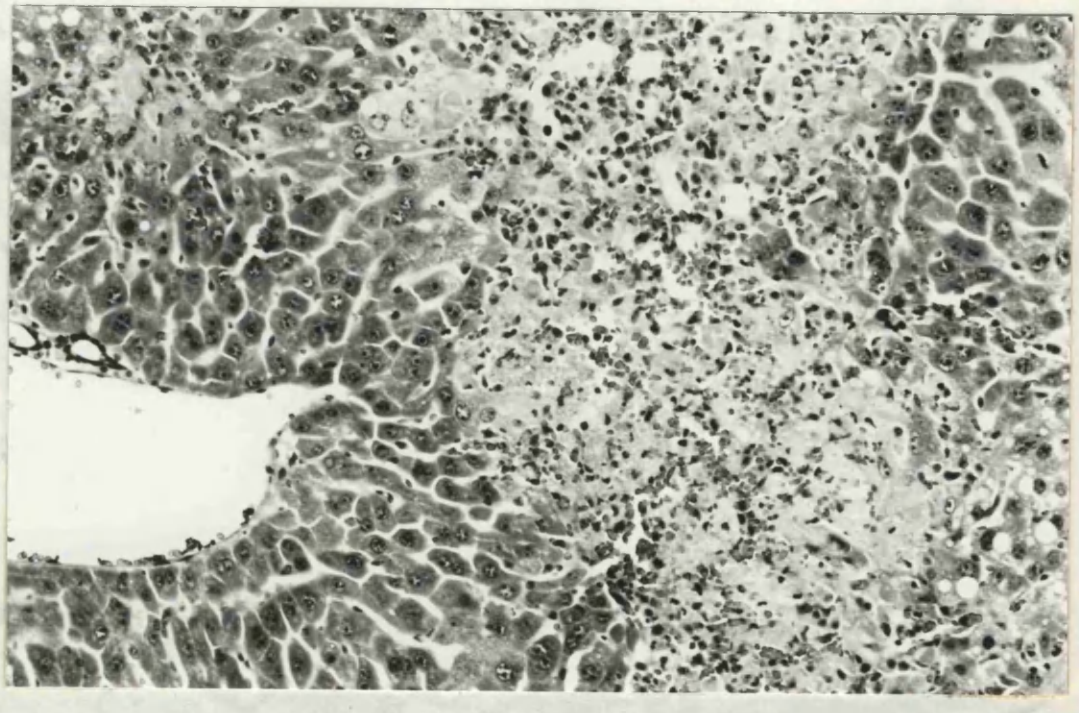


Fig 3:2 C. Centrilobular zonal necrosis in mouse liver 1 week after the administration of carbon tetrachloride and cortisone. HE X 225

Fig 3:3 A. Reticulin pattern of mouse liver 5 days after CCl_4.

X 225

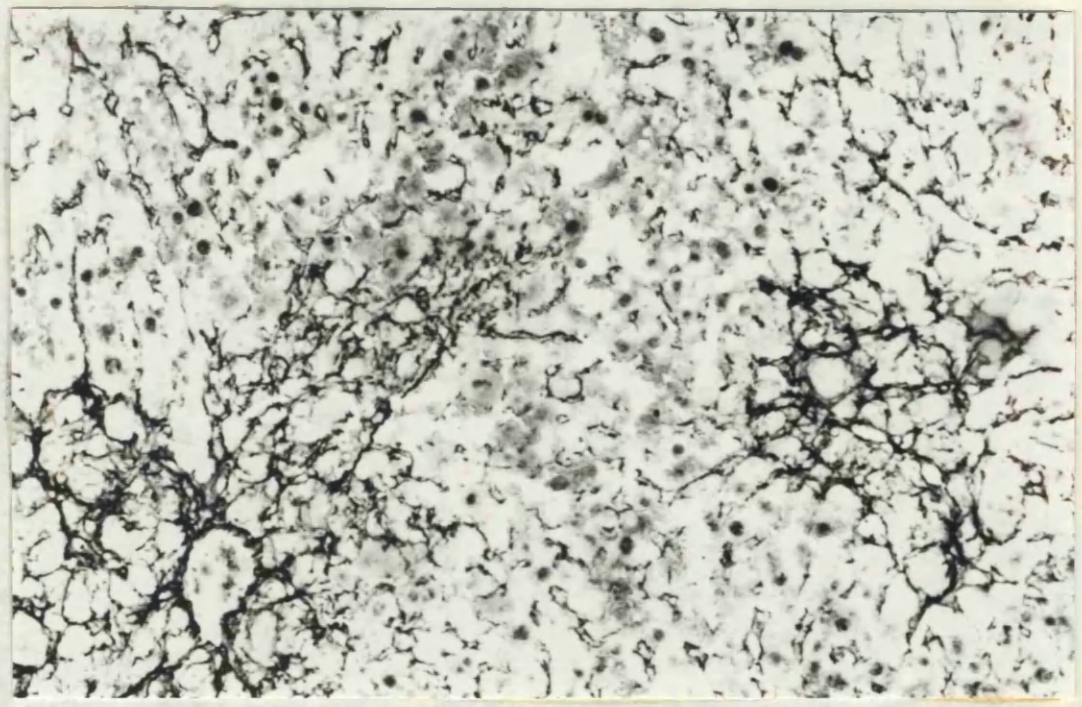


Fig 3:3 B. Reticulin pattern showing the effect of combined early cortisone and CCl_4 after 5 days. No increase is apparent.

X 225

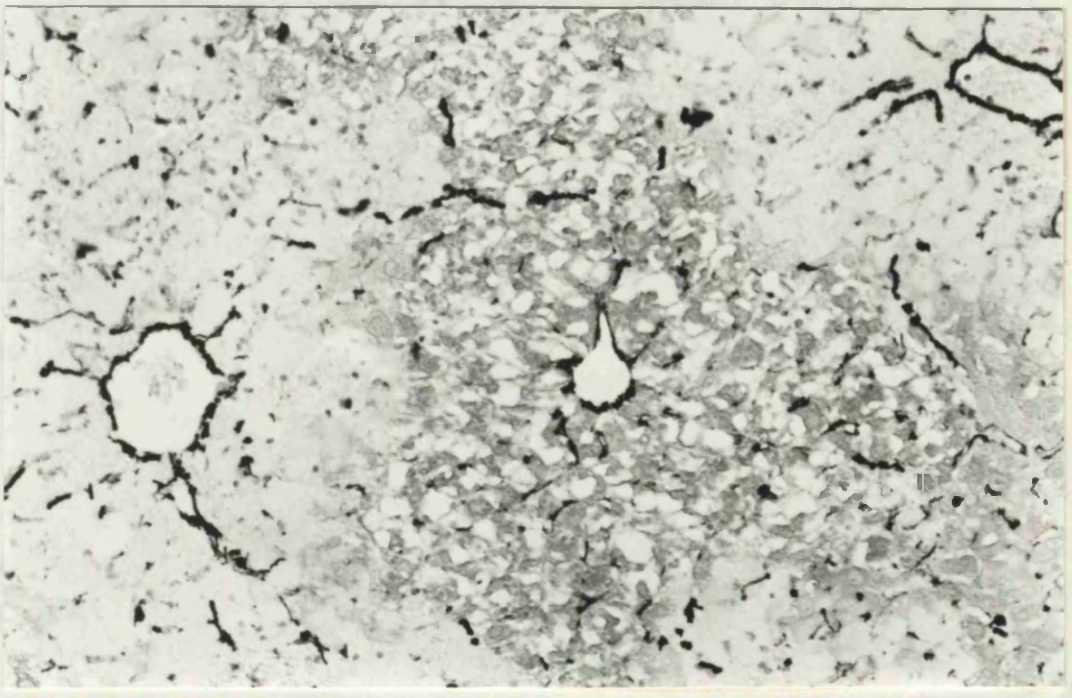


Fig 3:4 A. Reticulin pattern of mouse liver 9 days after CCl_4. The increased reticulin has disappeared. X 225

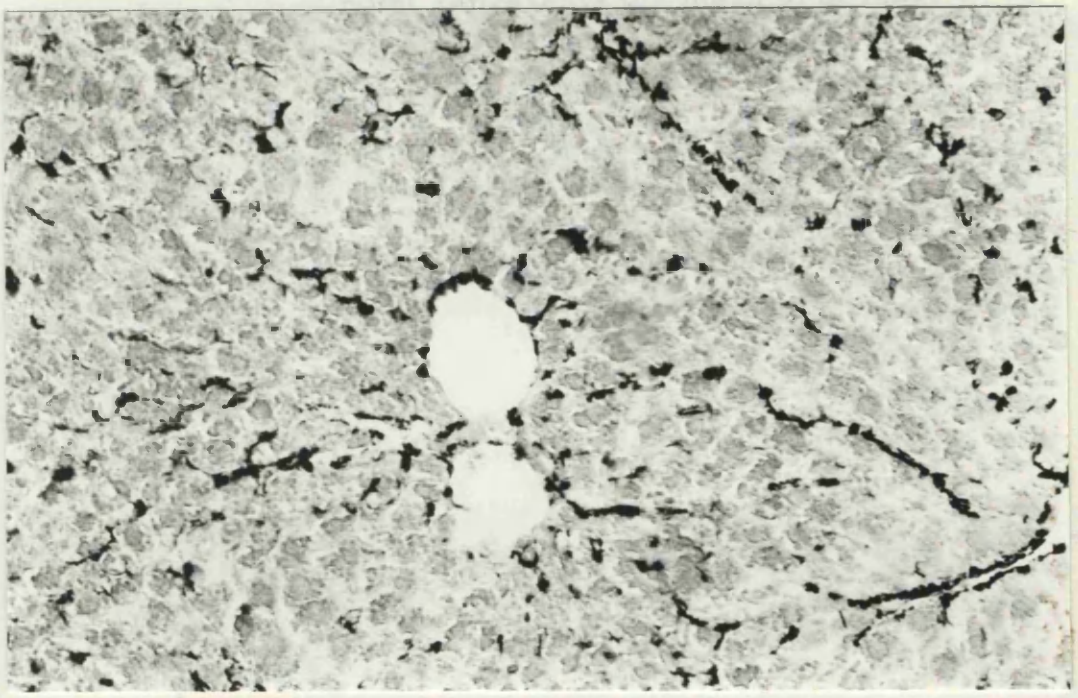


Fig 3:4 B. Reticulin pattern showing the effect of combined early cortisone and CCl_4 after 9 days. An increase is now apparent. X 225

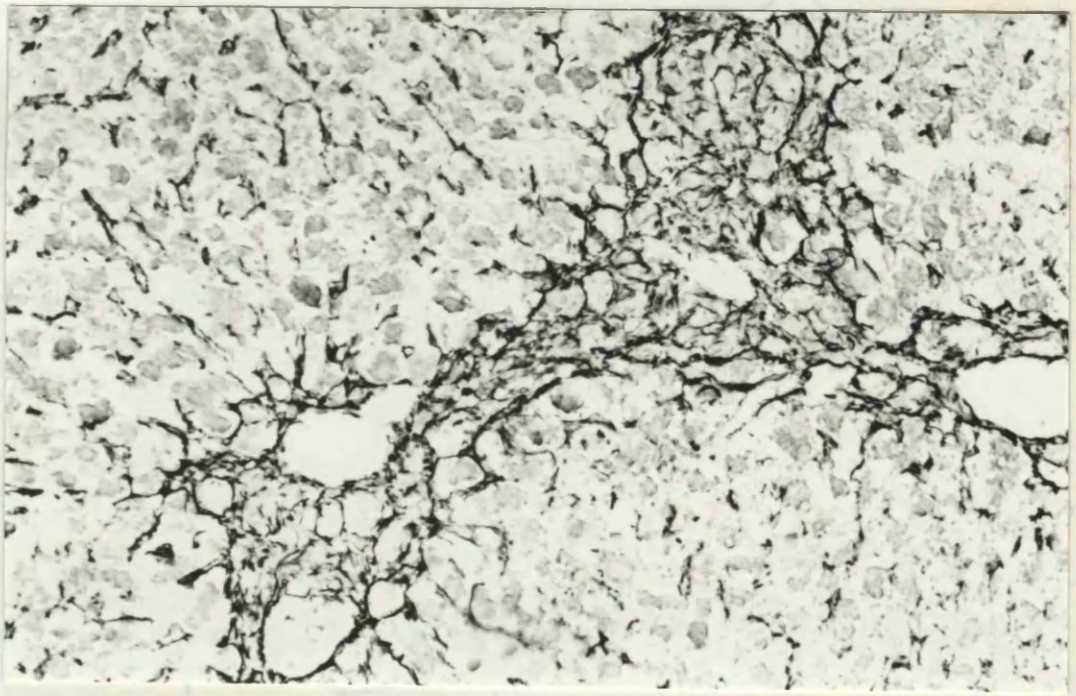


Fig 3:5 Mean levels of hepatic prolyl hydroxylase activity in mice given CCl_4 alone ; CCl_4 plus carrageenin ; carrageenin alone. Each time point represents the mean of at least 4 observations.

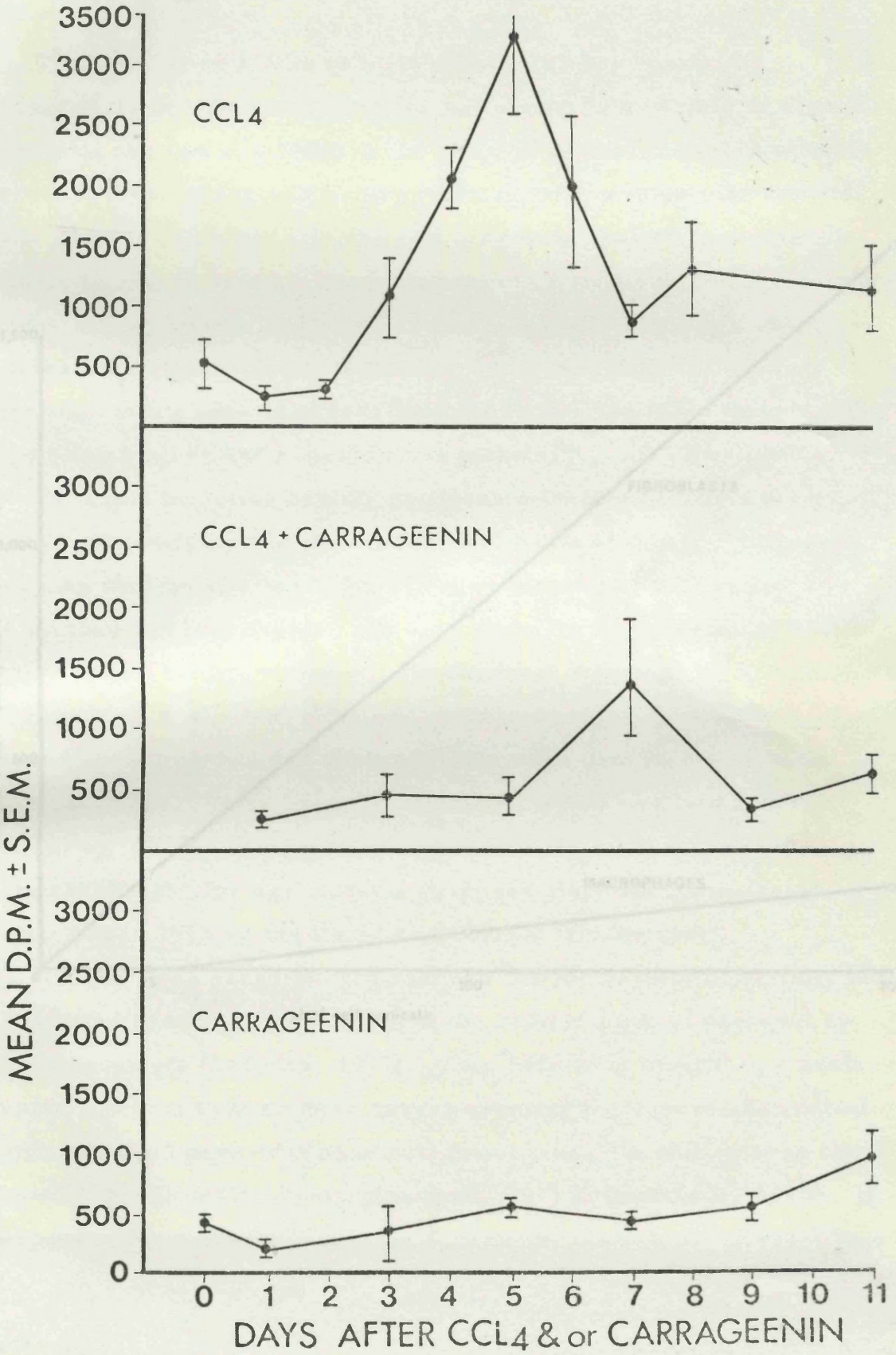
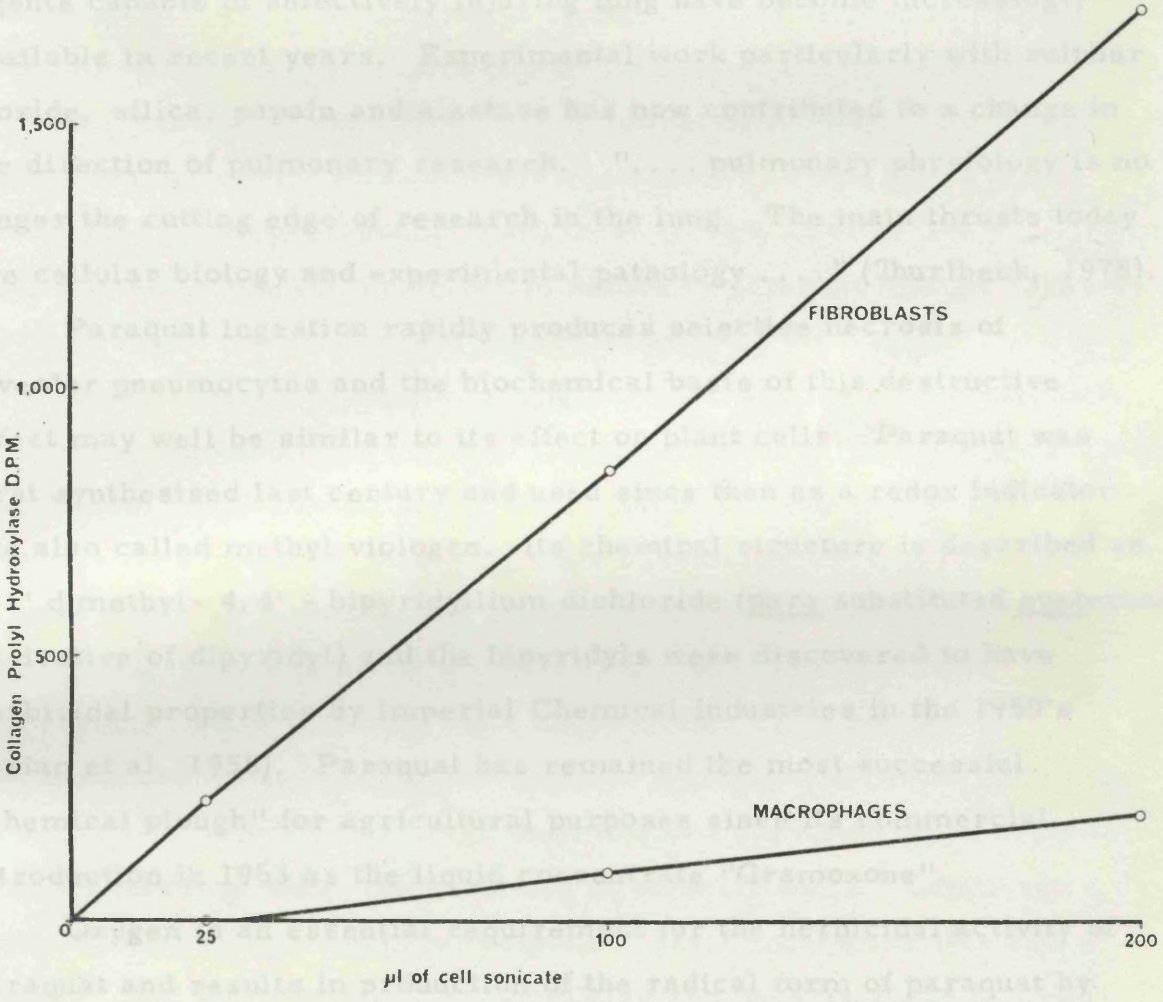


Fig 3:6 The relative levels of prolyl hydroxylase activity in macrophages and fibroblasts.



Chapter 4

EXPERIMENTAL PARAQUAT LUNG INJURY

General Introduction

The experimental use of toxic substances has constantly provided insights into pathophysiological events in a variety of organs. One recalls the use of alloxan in the study of diabetes, carbon tetrachloride in liver injury and α-bungarotoxin in neuromuscular control. Agents capable of selectively injuring lung have become increasingly available in recent years. Experimental work particularly with sulphur dioxide, silica, papain and elastase has now contributed to a change in the direction of pulmonary research. ".... pulmonary physiology is no longer the cutting edge of research in the lung. The main thrusts today are cellular biology and experimental pathology ...." (Thurlbeck, 1978).

Paraquat ingestion rapidly produces selective necrosis of alveolar pneumocytes and the biochemical basis of this destructive effect may well be similar to its effect on plant cells. Paraquat was first synthesised last century and used since then as a redox indicator dye also called methyl viologen. Its chemical structure is described as 1, 1' dimethyl- 4, 4' - bipyridyllium dichloride (para substituted quaternary derivative of dipyridyl) and the bipyridyls were discovered to have herbicidal properties by Imperial Chemical Industries in the 1950's (Brian et al, 1958). Paraquat has remained the most successful "chemical plough" for agricultural purposes since its commercial introduction in 1963 as the liquid concentrate "Gramoxone".

Oxygen is an essential requirement for the herbicidal activity of paraquat and results in production of the radical form of paraquat by plant chloroplasts (Ledwith, 1977). This radical is thought to transfer its extra electron to molecular oxygen creating the superoxide radical resulting in lipid peroxidation of cell membranes - a phenomenon capable of producing cell death (Farrington et al, 1973 ; Dormandy, 1978). It appears that the protective enzyme superoxide dismutase, present in

all cells, is incapable of reversing this effect. Inhibition of this enzyme with diethyldithiocarbamate potentiates the lethal effect of paraquat in rats (Goldstein et al, 1979). A similar mechanism has been proposed to account for mammalian toxicity by Bus et al (1976). It is interesting to note that lipid peroxidation by free radicals is now considered to be responsible for carbon tetrachloride induced liver injury (reviewed by Dianzani, 1976).

Paraquat is taken up and retained specifically in lung in contrast to a related compound diquat which has more nephrotoxic effects (Sharp et al, 1972). Within hours changes can be detected first in alveolar type I cells (membranous pneumocytes) and subsequently type II cells (granular pneumocytes). This effect is similar to oxygen and ozone toxicity except that capillary endothelium is unaffected (Sykes et al, 1977). Oxygen tension is maximal in lung and this has of course been quoted to explain the selective nature of paraquat toxicity (Fisher, 1977) but does not account for the localisation of the toxin.

The sequence of events in lung has been studied extensively both at light and electron microscopic levels in animal models (Vijeyaratham and Corrin, 1971 ; Smith et al, 1974 ; Smith and Heath, 1974 a). Observations in human poisoning show essentially the same picture (Toner et al, 1970 ; Smith and Heath, 1974 b). In the rat the alevolar type I epithelial cells swell within 4 hours and show electron microscopic features of hydropic degeneration by 18 hours. Degenerative changes of vacuolation and disruption of organelles also affect type II cells by this stage and after two days both types begin to disintegrate thus stripping the lining of alveolar walls. Capillary congestion and a few acute inflammatory cells accompany this early phase. Next intra-alveolar haemorrhage and oedema occur patchily throughout the parenchyma. Hyaline membranes are a notable feature and it has been suggested that this phase provides a model for human respiratory distress syndrome of the newborn particularly as there is a drop in pulmonary surfactant levels. This latter feature is readily attributable to destruction of type II cells rather

than failure of surfactant production. Bronchiolar damage may also contribute to a deficit in surfactant (Etherton and Gresham, 1979). Given sufficient paraquat, experimental animals or humans die during this destructive and exudative phase. Renal and hepatic failure may also occur and contribute to death which is nevertheless primarily of respiratory type.

After three days mononuclear cells become prominent in alveoli. These are a mixture of macrophages and stellate cells described by Smith and Heath (1974 a) as profibroblasts since electron microscopy shows sequential changes from a rather indeterminate form into recognisable fibroblasts. The suggestion that these latter cells "originate from blood monocytes which transform into profibroblasts and then migrate throughout the lung by means of their long pseudopodia" is a redundant speculation particularly since there is ample evidence that monocytes differentiate exclusively into macrophages (Jennings and Florey, 1970). The fibroblasts emerge first into alveoli adjacent to the bronchial tree and connective tissue septa. They have the appearance of fibroblasts in early log phage of tissue culture (Figs 4:1 and 4:2) which is probably an apt analogy for alveoli filled by inflammatory exudate. Fibroblasts continue to emerge and proliferate and reticulin fibres appear as in organisation elsewhere intil respiratory failure supervenes. This process is patchy and in some, presumably less extensively denuded, alveoli, type II cells which are the stem cells for alveolar epithelium (Adamson and Bowden, 1975) proliferate to form a cuboidal lining which seems to restrict fibroblasts to their normal compartment.

Is collagen synthesis increased in this experimental model and what is the stimulus for this alveolar fibroblast activity ? The following experimental work attempts to elucidate these questions.

Fig 4:1 A. Scanning electron microscopic view of a mouse pulmonary alveolus in the early inflammatory phase of paraquat poisoning. A fine fibrin mesh fills the alveolus. A trapped platelet is indicated (—>). X 2,415

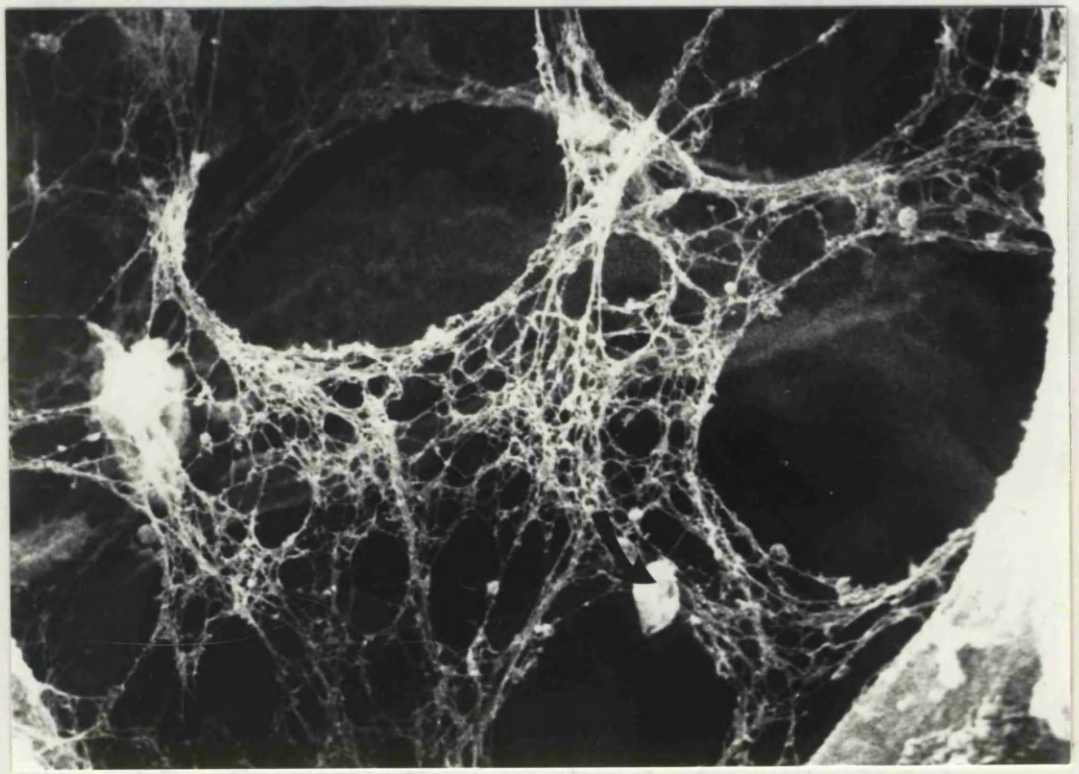


Fig 4:1 B. Normal mouse alveolus for comparison. Alveolar macrophages adherent to Type I cells are identifiable by their cytoplasmic processes (—>). X 1,605

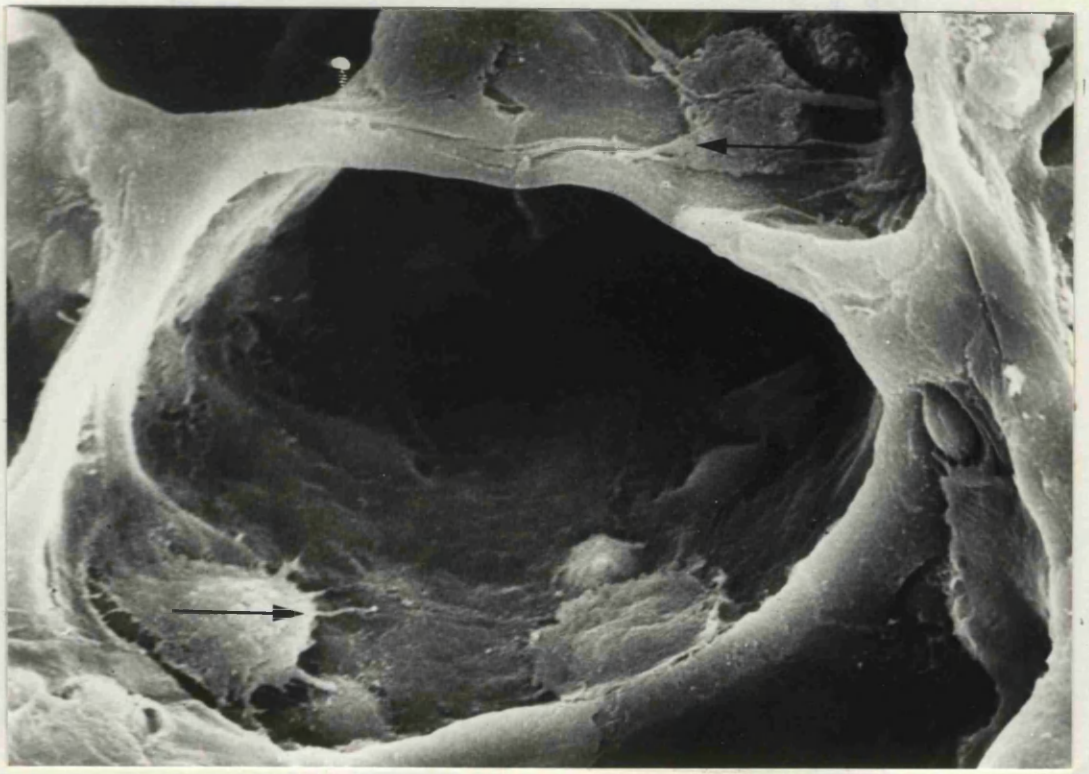


Fig 4:2 A. Light microscopic view of stellate fibroblasts (—>) in mouse pulmonary alveoli 5 days after paraquat. Fine strands of presumed collagen are stained blue. M.S.B. X 700

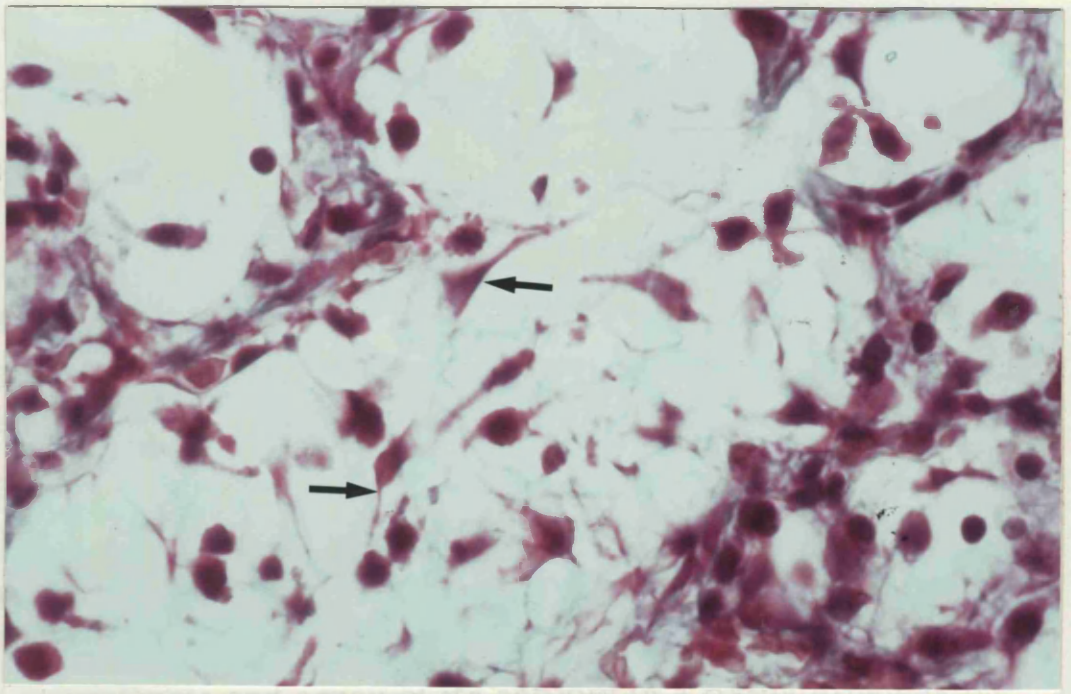
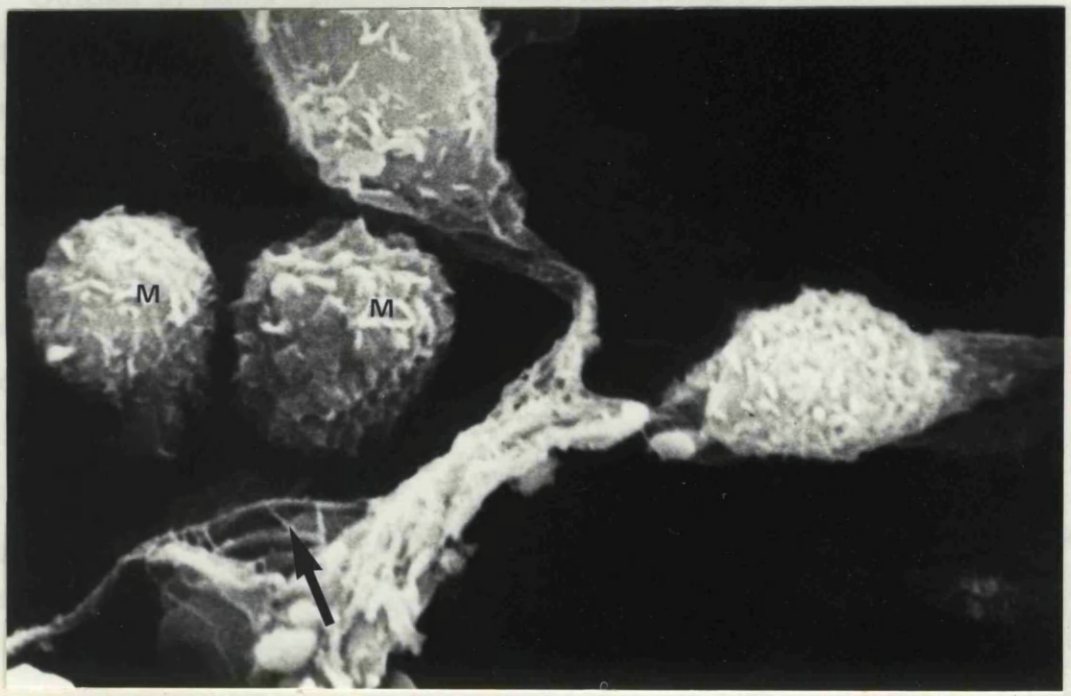


Fig 4:2 B. Corresponding scanning electron microscopic view of such an alveolus to show three elongated fibroblasts. Two adjacent macrophages showing surface ruffles are present. Long strands of presumed collagen are indicated (—>) These differ in thickness and character from the fibrin previously seen. X 4,950



Part A

COLLAGEN PROLYL HYDROXYLASE LEVELS IN EXPERIMENTAL PARAQUAT POISONING

This study represents the continuation of a theme from this laboratory concerning the application of techniques for measuring collagen synthesis to experimental animal models of fibrosis, especially in liver and skin. (Shaba et al, 1973 ; McGee et al, 1973). Paraquat lung injury offers a sequence of pathological events which is of considerable intrinsic interest as well as being a model of rapid fibrogenesis.

The enzyme collagen prolyl hydroxylase plays a pivotal role in collagen synthesis and assay of its activity in a tissue provides the most sensitive measure of local collagen synthesis (Cardinale and Udenfriend, 1974). This method has been used to assess the onset and development of fibrosis in the lungs of paraquat treated rats over a 7-day period. Other tissues and sera were also assayed to observe whether any rise in enzyme activity was peculiar to pulmonary tissue.

MATERIALS AND METHODS

Guided by pilot experiments, the following procedure was employed. Mature male-Sprague-Dawley rats (average weight 300 g) were given a single dose of paraquat dichloride in isotonic saline, 20 mg per kg body weight, by intraperitoneal injection. Control animals received a similar isotonic saline injection without paraquat. All animals were of similar weight, using litter mates as far as possible. Four test rats were killed daily for 6 days and five on the 7th day. These were weighed and samples of liver, kidney, serum and lung retained at -20^{o}C for prolyl hydroxylase assay. The right basal lobe of lung was used for this assay and was removed and weighed before freezing. The remainder of the lungs was inflated via the trachea with 10% formal saline at 15 cm pressure for 48 hours.

The left lower lobe was then sampled for histology. The right upper and middle lobes were dried to constant weight.

Because of considerable variation in the weight of the lungs at different time intervals after poisoning, the prolyl hydroxylase activity was quantitated in duplicate in 25 µl aliquots of whole right basal lobe which had been minced, sonicated, centrifuged and the 1,500 g supernatant made up to a constant final volume of 3 ml in buffer. The tritium release method of Hutton, Tappel and Udenfriend (1966) was employed following the establishment of a standard curve for control material activity (Appendix No. 3). Specimens from all test and control rats were assayed at the same time. The lung sonicates were subsequently assayed for protein (Lowry et al, 1951) and DNA content (Burton, 1956).

The same method of enzyme assay was applied to homogenates of the right main lobe of the liver and the whole of one kidney from which the capsule was stripped. Activity was related to the protein content of these tissues. The enzyme was also measured in duplicate in 0.2 ml aliquots of serum from blood obtained by cardiac puncture at the time of sacrifice.

RESULTS

The percentage changes in body weight and lung are shown in Figure 4:3. Total body weight declined steadily, losing approximately 20% by Day 5. By this time the lung dry weight had increased by 80% and the wet weight by nearly 400%. During this time also the protein content of lung supernatant increased by 600% from a mean of 4.0 mg/ml to 29 mg/ml. No change was found in DNA content.

The collagen prolyl hydroxylase levels in lung are shown in Figure 4:4. Following an initial slight fall on the first day there is a progressive rise to a maximum 5-fold increase at Day 5. This is a significant increase compared with the level at the beginning of the experiment ($P < 0.02$, Student's t test), which in the control group remained unchanged over the 7-day period.

The serum level of enzyme unexpectedly rose in a similar manner to lung, (Fig. 4:5) with a $3\frac{1}{2}$-fold increase by the 5th day ($P < 0.02$). The serum values were highly correlated with lung values ($r = 0.66$; $P < 0.001$). No increase was found in liver or kidney enzyme activity. Indeed a significant fall of 67% was noted in liver activity on the first day after paraquat with subsequent return to normal levels (Fig. 4:6).

Histology of the lungs showed the features of paraquat-induced pulmonary fibrosis as previously described. Stellate mesenchymal cells resembling fibroblasts in culture appeared within alveoli in the vicinity of small blood vessels and bronchioles by the 3rd day, which corresponds in time to the earliest detectable rise in prolyl hydroxylase activity.

DISCUSSION

Paraquat is known to produce extensive necrosis of pulmonary alveolar epithelium with consequent severe inflammatory changes. From the pilot experiments it was apparent that considerable weight changes occurred in the damaged rat lung. The increase in wet lung weight is readily attributable to the severe inflammatory changes. It was considered desirable that the prolyl hydroxylase activity was expressed in a manner independent of these changes in order to reflect total lung enzyme activity. Hence all animals were selected to be of similar size and the sonicated lung lobes for assay were made up to constant volume.

The initial fall in enzyme activity on the first day in both lung and liver is reminiscent of that which has been observed in liver following experimental acute alcohol ingestion (Patrick, unpublished), and may be a non-specific acute toxic effect. The activity began to rise above normal on the 3rd day which corresponds to the appearance of stellate mesenchymal cells among macrophages in alveolar spaces. This lends support to the electron microscopic evidence that these are 'pro-fibroblasts' (Smith and Heath, 1974 a). The highest enzyme levels were

found on the 5th day and the origin of this extra activity is uncertain. It may derive from increased numbers of fibroblasts or from increased intracellular activity, or more likely from both sources. The contribution from serum content is minor, as may be seen from comparison of activity per unit volume of lung with serum, and also from the absence of increased activity in liver and kidney. No increase in DNA content was detected in spite of an obvious histological increase in cellularity of alveoli. This is probably due to the disproportionately large DNA content of bronchial epithelium which is relatively unaffected in paraquat lung injury.

The activity by the 7th day is only twice normal despite the persistence of extensive pathological changes. A two-fold increase after seven days has also been noted in a recent preliminary communication on experimental paraquat injury (Chvapil and Hollinger, 1977), in which a comparable dose was used. These authors did not follow the time course of the changes and did not examine other tissues or serum. Autor and Schmitt (1977) failed to detect any increase in lung but were severely and correctly criticised by others at the symposium for poor methodology. Using cultured rat lung explants an increased rate of collagen in synthesis peaking after 6 days has been observed (Greenberg et al, 1978 a), thus being complementary to the present data. A rise in prolyl hydroxylase activity but not in collagen synthesis in lung has been reported (Kuttan et al, 1979) ; however these workers only looked at one time point, unaccountably choosing 9 days after paraquat by which time most animals are dead and a few recovered. The methodology is also suspect and the discussion confused.

With the tritium release method for assaying prolyl hydroxylase in human serum substantial elevations have been noted only in cases of hepatocellular carcinoma (Keiser et al, 1972). Our results suggest that significant increases should be sought in samples of fresh serum from cases of human paraquat poisoning and pulmonary oxygen toxicity. The recently developed radioimmunoassay for prolyl hydroxylase should prove superior for such estimations (Tuderman et al, 1975).

There is no proof of recovery in any human case of paraquat poisoning once intra-alveolar proliferation has begun (Matthew, 1974 ; Lancet, 1976). Prognostic assessment is hindered by inability to distinguish pulmonary oedema from intra-alveolar proliferation on x-ray and by the undesirability of lung biopsy in acutely ill patients. Serial serum assays might be of value in such assessment. Moreover the early reversal of increasing serum activity would constitute objective evidence of effective therapy.

The origin of the increased serum activity found in this study is presumably from proliferating intra-alveolar fibroblasts since there is no evidence of fibrosis in other organs. It is remarkable that this relatively large protein of 240,000 molecular weight in active form should gain access to the blood, possibly via lymphatic drainage, when its site of action is considered to be within the rough endoplasmic reticulum of the fibroblast (Al-Adnani et al, 1974 ; Cutroneo et al, 1974).

Fig. 4:3 Percentage changes over a 7-day period following a single dose of paraquat in total body weight (-●-), dry weight of right upper and middle lobes of lung (-■-) and wet weight of right lower lobe of lung (-✕-).

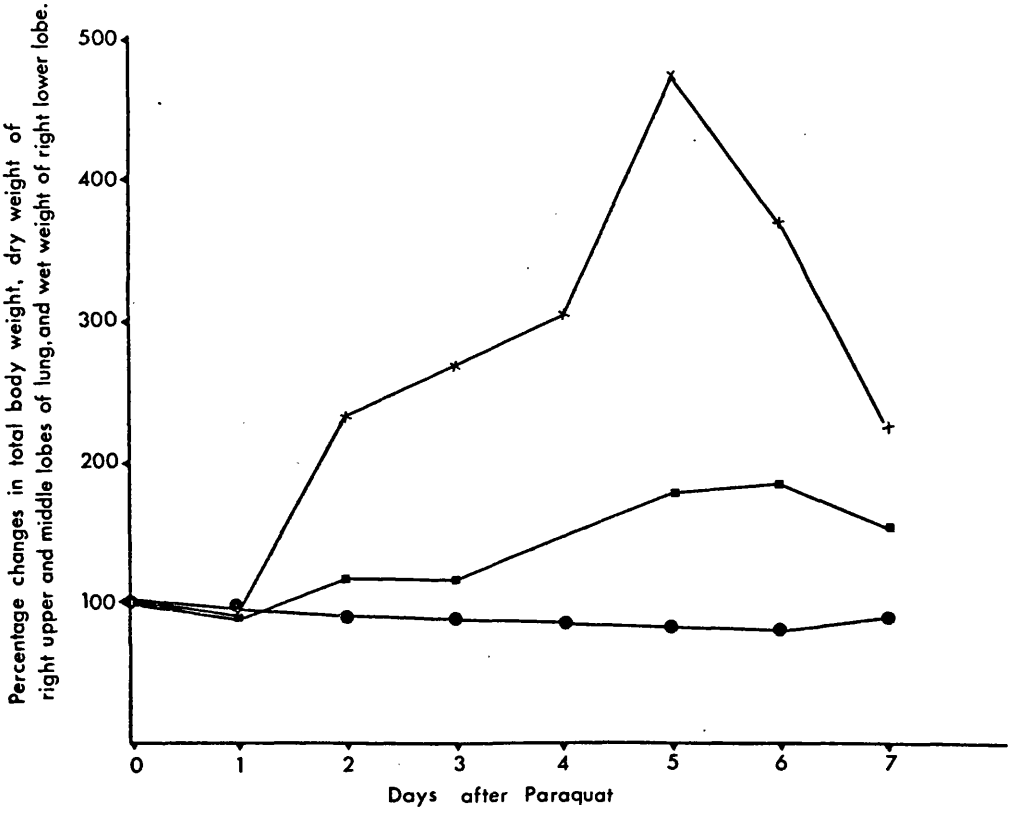


Fig. 4:4 Sequential changes in lung collagen prolyl hydroxylase activity over a 7-day period following a single dose of paraquat.

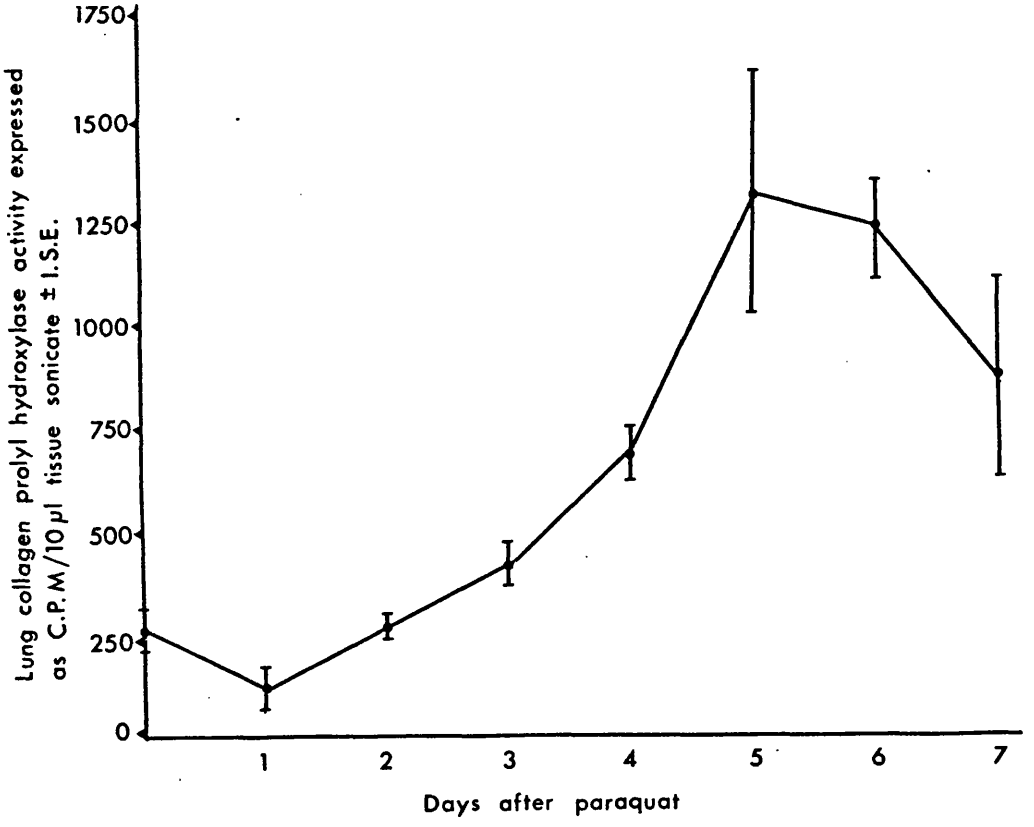


Fig. 4:5 Sequential changes in serum collagen prolyl hydroxylase activity over a 7-day period following a single dose of paraquat.

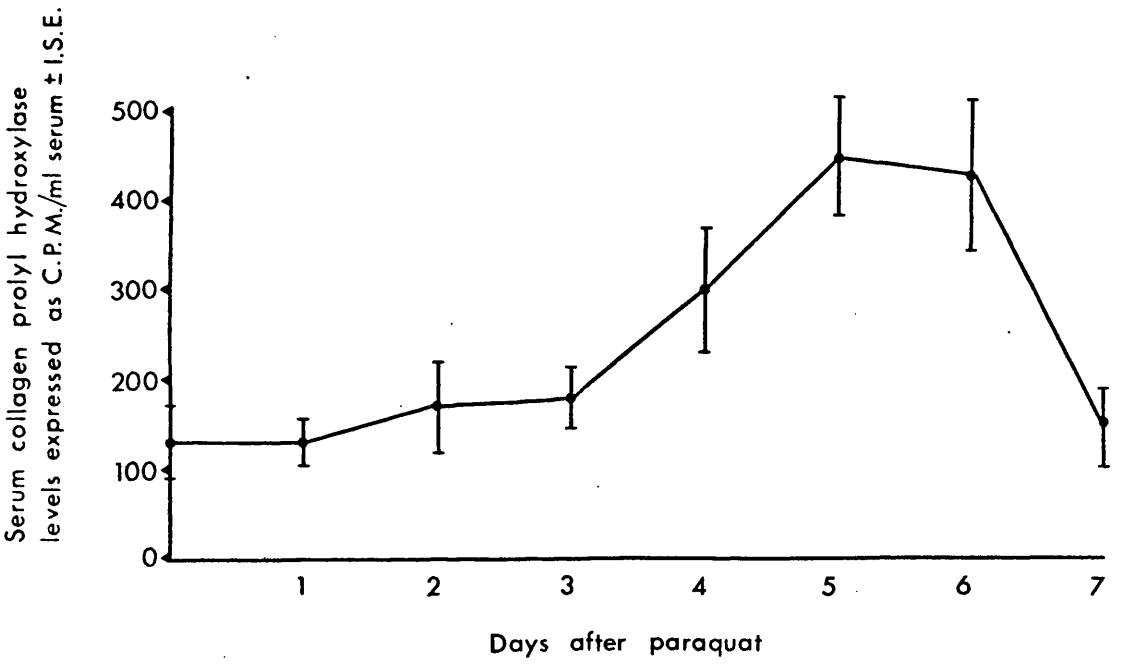
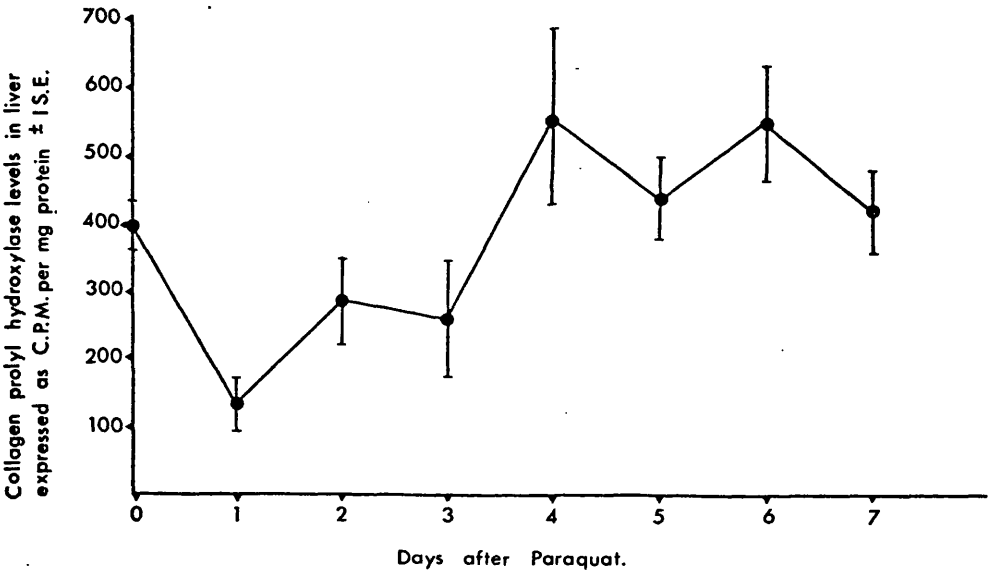


Fig 4:6 :- Sequential changes in hepatic collagen prolyl hydroxylase activity over a 7-day period.



Part B

COLLAGEN STIMULATING FACTORS IN EXPERIMENTAL PARAQUAT POISONING

The time-course of the phase of increased collagen synthesis in experimental paraquat lung injury has been found to be the same as that following liver and skin injury. Previous work from this laboratory has shown the presence of collagen stimulating factors in healing liver and skin during this phase. This effect was sought in lung injury using methodology as close as possible to that used previously.

MATERIALS AND METHODS

Preparation of lung material : Lung tissue was removed prompty and placed on ice from control Sprague-Dawley rats and from rats killed by cervical dislocation 4 or 5 days after injection of paraquat as previously described. The tissue was used fresh or in some instances after storage at -20°C. 3 g of lung from at least 2 rats was homogenised at half speed in 5 volumes of column buffer (0.05 M phosphate buffer pH 7.0 at 4°C). The homogenate was centrifuged at 12,000 g for 20 minutes at 4°C in an MSE Superspeed 50 ultracentrifuge. The supernatant (except for the surface scum of lipid) was applied to a column of G 25 Fine Sephadex (Pharmacia) which has a molecular weight exclusion of 5,000 daltons. The column height was 85 cm using a Pharmacia K 16/90 column giving a total bed volume of 150 ml. 3 ml fractions were collected at a flow rate of 30 ml per hour on an LKB fraction collector in a cold-room at 4°C. Protein content of the eluate was monitored on an LKB Uvicord flow-through spectrophotometer at 280 μ and continuously recorded on an LKB chart-recorder.

Detection of collagen stimulating activity

1 ml aliquots of fractions or buffer were added to replicate cultures of L929 mouse fibroblasts (Gibco-Biocult, Paisley) seeded 2 days previously at a density of 0.6 to 0.8 million cells per 25 cm^2 plastic culture flask (Nunc Products). The cells were grown in 5 ml of Minimum Essential Medium with the addition of 10% foetal calf serum, 2×10^{-3} M L-glutamine, 0.25×10^{-6} M ferric nitrate, 0.25×10^{-3} M sodium-Lascorbate, and 100 units/ml of penicillin plus 100 µg/ml of streptomycin. All media were obtained from Gibco-Biocult and chemicals from Sigma.

After 4 hours incubation with column fractions the cells were assayed for prolyl hydroxylase activity or collagen synthesis as detailed in Appendices 3 and 4. The effect of plating cells at varying concentrations on the intrinsic level of collagen synthesis was also examined.

RESULTS

The following experiments have each been performed at least twice with similar results.

Collagen Prolyl Hydroxylase The continuous profile of protein peaks is drawn to the same scales of % transmission and volume of column eluant in all Figures of this type in this and subsequent chapters. The protein profile of 5 major peaks is similar for both test and control lung tissue. Allowing for some variation in the chromatographic separation, there is usually greater prominence of peaks 3 and 5 in test material.

Fig 4:7 shows the effect of fractions from normal lung on replicate flasks of fibroblasts with regard to prolyl hydroxylase activity. No stimulation of activity is found compared to buffer only controls except for a small increase roughly corresponding to the second protein peak.

Fig 4:8 A and Fig 4:8 B show the pattern of activity obtained from damaged lung. Fig 4:8 A is an earlier experiment with 4 ml fractions, rather few controls though many test flasks,requiring two separate assays. An elaborate pattern of peaks above control levels of activity is obtained. The material in the first peak of protein is known to be toxic when liver or skin wounds are fractionated, and this effect is also present here. Allowing a zone of activity corresponding to the first peak of protein though largely suppressed, there is an overall pattern of 5 zones of stimulating activity corresponding roughly to the protein peaks. Fig 4:8 B with fewer flasks available shows a less complex picture with a similar pattern of peaks without the sub-peaks. Whether the sub-peaks are real or assay artefact is a matter for discussion.

The test data by the nature of the experiment obviously does not form a normal distribution. The data is amenable to statistical analysis to confirm a real increase in collagen synthesis by using the non-parametric Wilcoxon's Sum of Ranks Test. Both experiments show significant increases ($2P<0.01$). Since an increase was predicted, a one-tailed test can be justified, in which case the significance level is even greater.

Collagen Synthesis No pattern of increased collagen synthesis is seen with normal tissue fractions (Fig 4:9). Fig 4:10 A shows increased collagen synthesis most marked in the earlier fractions when damaged lung is used at once. When the remainder of the damaged lung stored frozen at -20°C overnight is used, this early activity is lost but some later activity appears (Fig 4:10 B). Comparison of test and control culture data from both experiments with paraquat lung with the Wilcoxon test shows no statistically significant differences.

The effect of plating cells at various concentrations on the intrinsic level of collagen synthesis is shown in Table 4:1 and Fig 4:11. The cells were plated by an experienced operator but the variation in cell numbers obtained from each flask plated at the same nominal

concentration is apparent. The wide spread of values for collagen synthesis is equally apparent. Nevertheless when the results are averaged a pattern emerges of a rise in level of synthesis to a maximum at 0.6 million with some falling away as cells enter late log phase.

DISCUSSION

The presence of collagen stimulating factors has been demonstrated at days 4 and 5 corresponding to the phase of increased collagen synthesis after paraquat poisoning. At least 5 zones of activity are apparent of molecular weight 5,000 daltons and below. Both prolyl hydroxylase activity and collagen chain synthesis are enhanced. These findings are consistent with those found previously with healing liver and skin and further suggest that collagen stimulating factors are a universal phenomenon basic to the chronic inflammatory process.

The complex pattern of peaks found with the more sensitive prolyl hydroxylase assay when many cultures are used (Fig 4:8 A) may be partly spurious due to the intrinsic variation of the assay. It may also point to progressive degradation of a protein yielding active material of a wide range of molecular weight. The reduction of activity after freezing overnight and thawing with the persistence of some lower molecular weight activity (Fig 4:10 A and Fig 4:10 B) also suggest degradation of material of higher molecular weight.

The methodology of this type of experiment is prolonged, time-consuming and vulnerable to error throughout its many stages. Larger numbers of control cultures were used than in previous experiments in order to permit statistical analysis. The vagaries of tissue culture are such that this requirement further reduces the chances of setting up enough cultures to be ready for use at the same time as the (fresh) material becomes available. Most difficulty was experienced in the collagen synthesis assay. The variation in plating

cells and in the assay results have been shown in Table 4:1. Fig 4:11 shows that if enough cultures are used a meaningful result can be still obtained. This result is comparable with that found by Gribble et al. (1969).

Because of activation of prolyl hydroxylase in culture can be achieved in a variety of non-specific ways without corresponding increase in collagen synthesis, the latter is the more valid assay in spite of the problems mentioned here and in Appendix No. 4. For stimulation to be obtained with the collagen synthesis assay the fibroblast cultures had to contain cell densities within the range 0.4 to 1.0 million cells per flask. The cell concentration was less critical for prolyl hydroxylase stimulation. A lot of time was wasted using frozen tissue to no effect.

The demonstration of collagen stimulating factors from column chromatography is really the demonstration of an effect rather than its measurement. This system does not lend itself to quantitation which would permit further validation and variety of experiment. Other techniques have been tried with paraquat lung and CCl_4 liver and are described in Chapter 6.

GENERAL DISCUSSION

Small animals suitable for experimental purposes tend to die during the acute destructive phase of poisoning or, with a lesser dose, to survive indefinitely with only minor lung damage - a problem encountered by other workers (Greenberg et al.,1978 A). With judicious choice of dosage within a narrow range and with older rats, the progression to the proliferative phase comparable with human poisoning is more often achieved. Many such animals tend to die at days 4, 5 and 6. The question remains, therefore, as to why it is that when alveolar destruction is complete after 2 or 3 days, death from respiratory failure is delayed until well into the proliferative phase.

well after the acute toxic effects and after paraquat levels decline. The findings in culture are nonetheless interesting, particularly when one recalls that macrophages in culture are much more susceptible to paraquat toxicity than fibroblasts (Styles, 1974). The presence of collagen stimulating factors whether similar or identical to those found at other sites of experimental injury suggests that a basic repair mechanism is responsible for control of collagen synthesis rather than a direct effect of paraquat toxicity.

Fig 4:7 The effect of gel chromatography column fractions from normal rat lung on the levels of prolyl hydroxylase activity of replicate cultures of fibroblasts.

General Points :-

The column (Pharmacia K 16/90), type of Sephadex (G25 Fine), and bed volume (150 ml) in this and all subsequent experiments of this type were kept constant to allow comparisons (except Chatper 5 on experimental hypertension).

The right-hand scale of % Transmission has been kept constant in all experiments and corresponds to the continuous profile of protein elution.

Similarly the base-line has been drawn to the same scale in each case to a constant 300 ml (regardless of the volume of individual fractions).

The left hand scale denotes the assay values obtained from the use of column tube fractions as applied in this case to replicate fibroblast cultures. Each point (open circles) indicates the assay result from a fibroblast culture to which the corresponding column fraction has previously been added. The values ranked adjacent to the left hand scale (dark squares) are those obtained from cultures to which column buffer alone has been added.

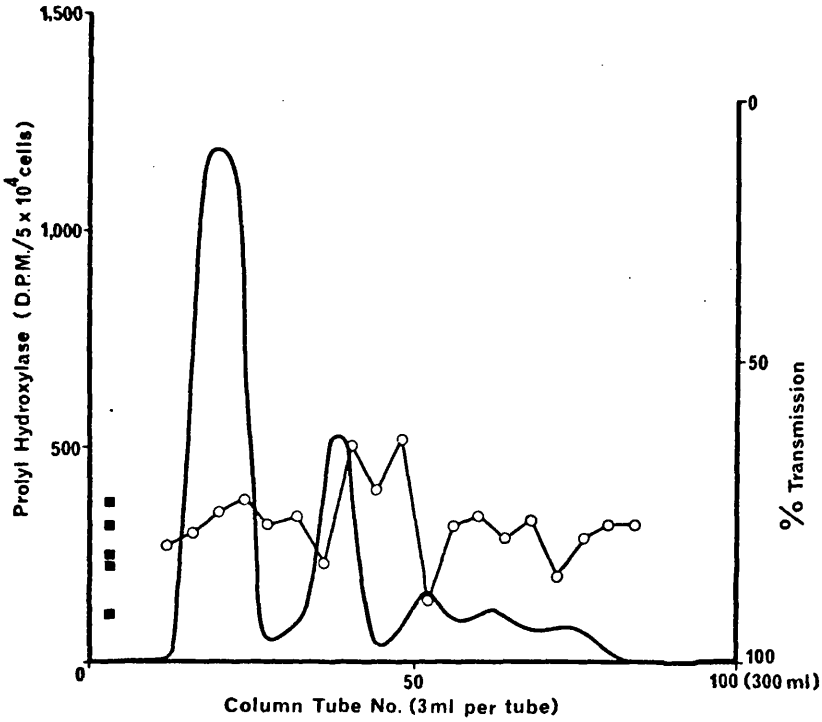

1,500
1,000
500
0
Prolyl Hydroxylase (D.P.M./5 x 10^4 cells)
0
50
100
% Transmission
50
100 (300 ml)
Column Tube No. (3 ml per tube)

Fig 4:8 A. The effect of column fractions from paraquat-damaged rat lung on prolyl hydroxylase activity of cultured fibroblasts.

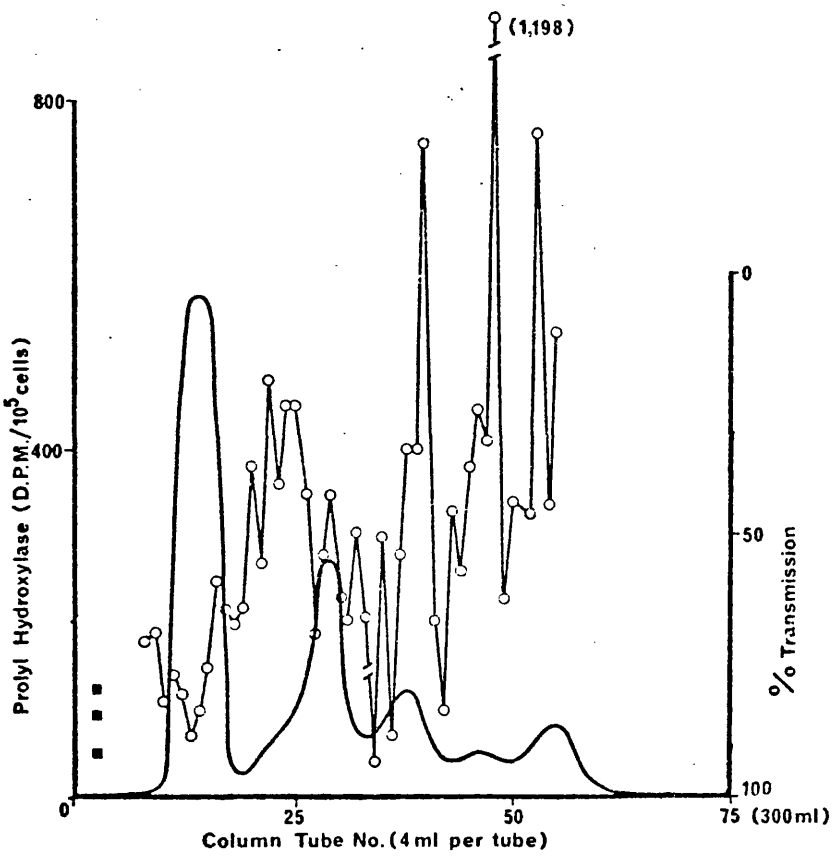


Fig 4:8 B. A further experiment similar to 4:8 A.

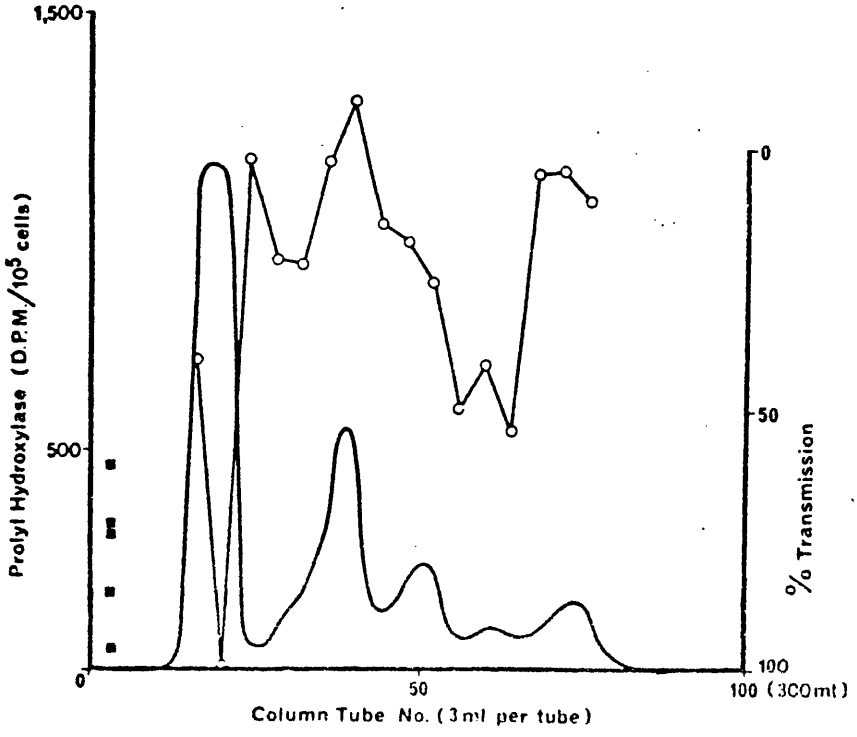
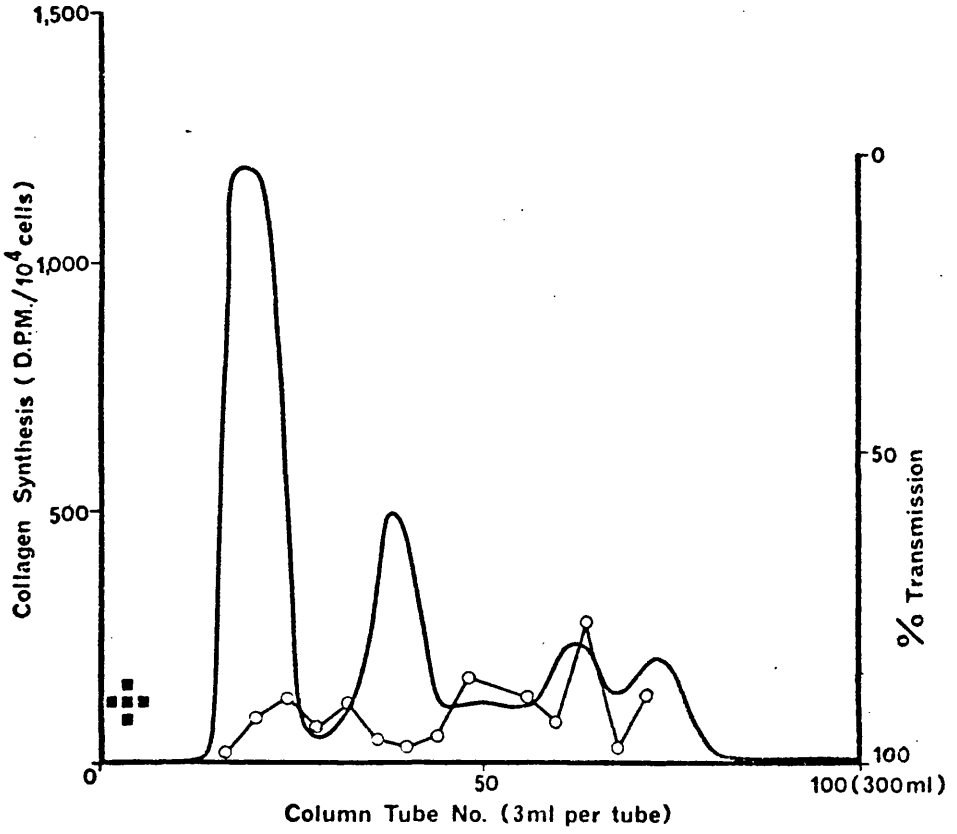


Fig 4:9 The effect of column fractions from normal rat lung on collagen chain synthesis of cultured fibroblasts.



damaged rat lung on collagen chain synthesis of cultured fibroblasts.

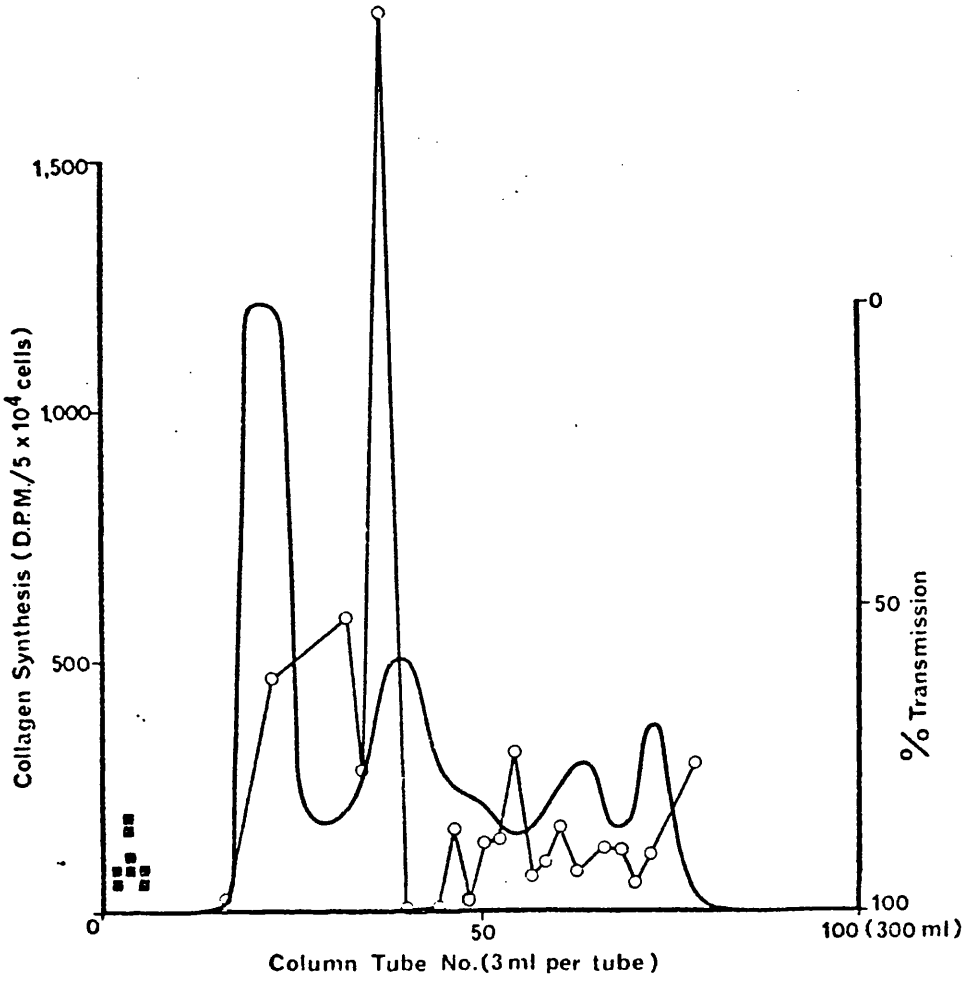


Fig 4:10 B. A further experiment similar to A but using the same lung stored frozen.

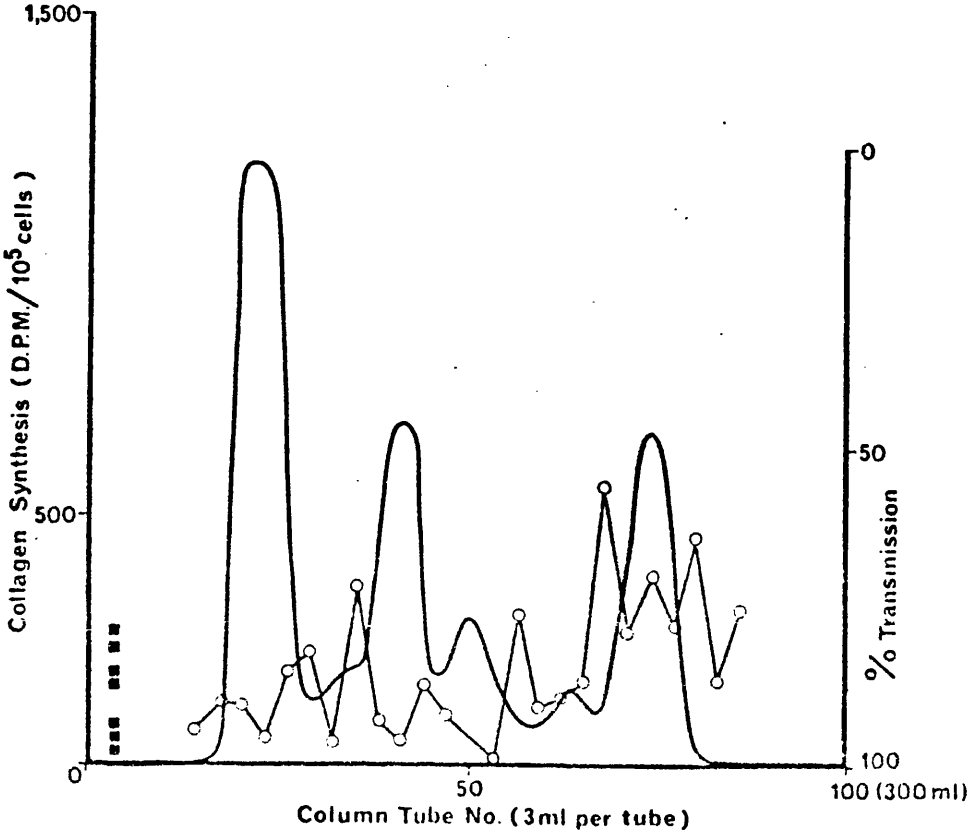


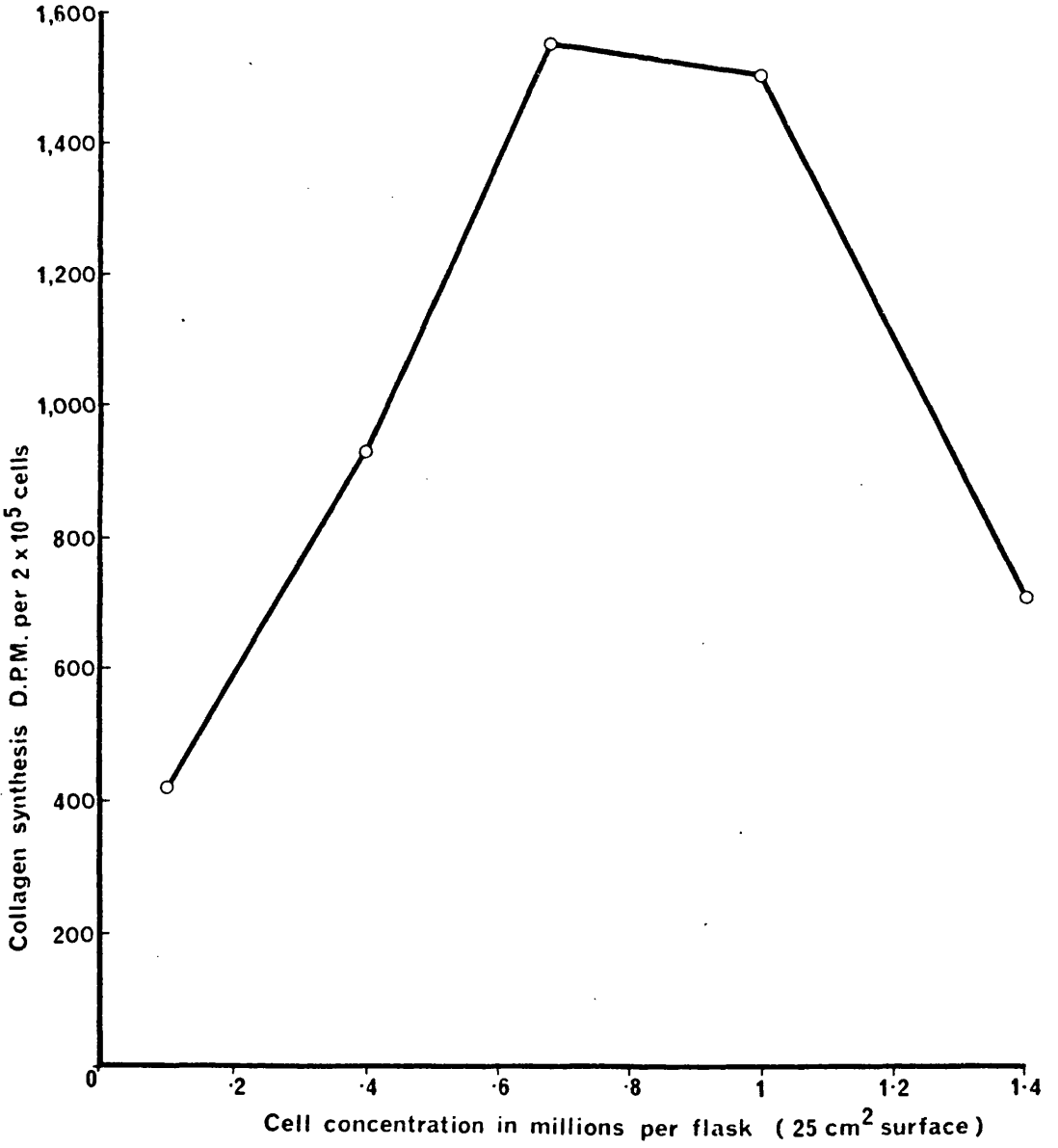
TABLE 4:1

The Effect of Cell Concentration on Collagen Synthesis

Cells Plated	Cells Counted	Mean Cell Count	DPM	Mean DPM
0.2 million	0.09	0.1	152	426
	0.11		638	
	0.12		775	
	0.14		137	
0.4 million	0.40	0.4	538	927
	0.32		1360	
	0.40		756	
	0.41		1053	
0.6 million	0.71	0.64	1319	1548
	0.59		1467	
	0.66		2071	
	0.60		1336	
0.8 million	1.17	1.0	2105	1501
	0.94		1404	
	0.98		1062	
	0.93		1433	
1.0 million	1.21	1.4	682	709
	1.23		203	
	1.75		1333	
	1.30		616	

Legend :- To show the cell counts recovered from 25 cm^2 area plastic flasks seeded 2 days previously at various nominal concentrations. The corresponding values for collagen synthesis are expressed as DPM per 2 x 10^5 cells being the actual experimental data.

Fig 4:11 The mean data from Table 4:1 displayed graphically.



Chapter 5

THE EFFECT OF EXPERIMENTAL HYPERTENSION ON ARTERIAL COLLAGEN SYNTHESIS

The composition of the arterial wall changes during the development of hypertension with corresponding changes in mechanical properties related to loss of elasticity (Feigl et al. 1963). Increased collagen deposition has long been recognised but increased collagen synthesis was first demonstrated by Ooshima et al. (1974) in the heart and arterial tree of hypertensive rats. The present work was undertaken to confirm their findings and to see if collagen stimulating factors could be isolated from the aortas of hypertensive rats. The absence of macrophages and necrosis from the hypertensive aorta formed the reason for the choice of this model of enhanced collagen synthesis.

The connective tissue functions of the fibroblast are now recognised to be performed by the smooth muscle cell in the arterial tree. Aortic smooth muscle cells in culture synthesise predominantly type I and to a lesser extent type III collagen (Layman et al. 1977 ; Burke et al. 1977). Elastin is also a major product (Boissel et al. 1976 ; Ross, 1971). The mechanical properties of the normal arterial wall can be understood as those of an elastic tube designed to cope with pulsatile flow (McDonald, 1974). Smooth muscle contraction of peripheral arteries and arterioles provide the means to control peripheral resistance and distribution of blood flow. In the larger vessels the presence of smooth muscle cells would seem haemodynamically unnecessary except to provide connective tissue maintenance at a level matched to mechanical stresses.

Materials and Methods

The same procedure as adopted by Ooshima et al. (1974) was followed to induce hypertension in male Sprague-Dawley rats. One

kidney was removed at age 6 to 8 weeks. After one week of post-operative recovery the rats began a regime of twice-weekly subcutaneous injection of deoxycorticosterone acetate (DOCA) and 1% NaCl drinking water ad libitum. The steroid was given in depot form as a suspension of 10 mg per ml in a solution of 10 mg of carboxy-methylcellulose and 2 mg of polysorbate per ml in isotonic saline. All materials were obtained from Sigma. This model is commonly referred to as "DOCA-salt hypertension". Normotensive controls were sham-operated, sham-injected, age-matched male Sprague-Dawley rats drinking ordinary tap-water. All animals received the usual pellet diet.

The animals were weighed weekly. Systolic blood pressure was measured by the tail cuff-peumatic pressure transducer method on a Physiograph Six instrument made by the now defunct E. & M. Instrument Co., Texas. The blood pressure could be measured readily to the nearest 5 mm Hg. Diastolic blood pressure cannot be measured by this method.

The aorta was dissected from cardiac to iliac ends from each animal killed by ether inhalation. Adherent fat was scraped off and the tissue placed promptly on ice. A block of tissue just above the level of the renal arteries was taken for histology. The aorta was opened and blood rinsed off with chilled isotonic saline. Each aorta was dabbed dry with filter paper. Homogenisation of aortas in a small volume proved unsatisfactory with ground glass pestle homogenisers because of the tough, rubbery consistency of the tissue. In order to achieve thorough dispersion of the tissue, the following method was employed. Each aorta was coiled and snap-frozen onto a cryostat chuck, then sectioned through at about 12 μ and all material collected.

For prolyl hydroxylase assay the shavings were added to 0.7 ml of assay buffer and vortexed. The material made a minimal alteration to the final volume which was regarded as constant. The suspension was briefly sonicated (5-10 seconds) and the 1,000 g supernatant used

for assay (see Appendix No. 3). The protein content of the supernatant was subsequently estimated by the method of Lowry et al. (1951) to allow for differences in weight and adequacy of homogenisation. Test and control aortas were assayed fresh each week together with one of identical stored aliquots of L929 fibroblast sonicate as a standard enzyme source. Mean interassay variation was 13% using the sonicate and no adjustment was made to the results from the aortas. The heart weight was also recorded.

Collagen stimulating factors were sought in the aortas after 6 weeks using the same methodology as described for lung in the previous Chapter. Aortas from 6 test or control rats were sectioned and the material pooled and vortexed in 1 ml of chilled column buffer. The 15,000 g supernatant was applied to a K9/60 column containing G25 Fine Sephadex (Pharmacia). The bed volume was 38 ml and the void volume of the system 15 ml. 1 ml fractions were collected at a flow rate of 40 ml per hour at $4^{o}C$ in a cold-room. Alternate fractions were applied to replicate cultures of L929 fibroblasts which were subsequently assayed for collagen chain synthesis as previously described. By this procedure, fractions were obtained from fresh aortas in about 4 hours.

Results

The normal systolic blood pressure of the rat is about 120 mm Hg as is seen in the normal control animals at the beginning of the experiment. When animals are less accustomed to handling, the pressure may rise somewhat and animals were not considered hypertensive until a pressure of 150 mm Hg was achieved. The pressure rose steadily over a twelve week period in only the test group, reaching 200 mm by six weeks (Fig.5:1A). A relative increase in heart weight also (Fig. 5:1 B) developed in the test group but this was of lesser magnitude. The thickness of the aortas of the hypertensive group was almost twice that of the control group by six weeks. Aortic collagen prolyl hydroxylase activity was observed to fall in the control

and test groups (Fig. 5:2). A relatively higher level was consistently maintained in the hypertensive group.

An identical pattern of peaks of protein elution from molecular sieve chromatography was obtained from extracts of both hypertensive and control rat aortas. Alternate 1 ml fractions from the test material were immediately applied to replicate fibroblast cultures. Column buffer was added to 4 control cultures. A profile of stimulating activity was obtained (Fig. 5:3 A) consisting of an initial major peak and two minor, less well defined, zones. Compared with the mean of the control cultures, the peak values represent 5, 2.5 and 2 - fold stimulation. No toxic effect on cell culture from the first peak was evident either morphologically or from the assay results. Comparing control values with all test values (Fig. 5:5) using the Wilcoxon Ranking Test for non-normally distributed data confirmed a significant difference ($2P<0.05$).

The remaining fractions were stored at $4^{o}C$ for 36 hours and then applied to fibroblast cultures. No pattern of stimulation was obtained from this material nor from fractionation of control material (Figs. 5:3 B & 5:4). A subsequent repeat experiment using 8 week hypertensive rat aortas stored for 12 days at $-70^{o}C$ failed to show convincing stimulation.

Discussion

Hypertension can most readily be induced experimentally in young rats. Collagen biosynthesis declines with age in rat tissues and this applies to the cardiovascular system as found by Ooshima et al. (1974). The present work also shows the decline in aortic prolyl hydroxylase activity over six weeks in control animals. A relatively higher level was sustained in the test group as blood pressure rose. Difficulty was encountered in efficient homogenisation of the tiny, rubbery strips of aortas, and necessitated the laborious process of frozen sectioning. The use of a high-speed micro-homogeniser would probably reduce the variability of the assay.

In a series of papers from the Roche Institute, Udenfriend and collaboratories have shown that both collagen prolyl hydroxylase activity and collagen chain synthesis increase in aorta, and also in cerebral and other arteries but not veins of DOCA-salt hypertensive and spontaneously hypertensive rats (Ooshima et al. 1974 ; Ooshima et al. 1975 ; Udenfriend et al. 1976 ; Iwatsuki et al. 1977 ; Ooshima et al. 1977 ; Iwatsuki et al. 1977). Antihypertensive drugs arrested the progress of hypertension and reduced collagen synthesis to normal. B-aminopropionitrile which inhibits lysyl oxidase, and hence extracellular collagen fibril cross-linkage, reduced blood pressure and vascular collagen in hypertensive rats. These observations, particularly the latter one, are of major significance in showing that increases in vascular connective tissue not only are sequelae of hypertension but contribute to its maintenance. This work complements the pathophysiological studies of Folkow et al. (1973) showing that increased vascular resistance is mediated by an increase in the wall to lumen ratio, i.e. the thicker vessel walls encroach on the lumen even at full smooth muscle relaxation.

This model of increased collagen synthesis was deliberately chosen for its differences from the other models studied. The absence of large-scale necrosis and particularly of macrophages would provide interesting information on the nature of collagen stimulating factors whether present or not. In retrospect this thinking was flawed. Failure to demonstrate factors could be attributable to uncertainties concerning the relatively small amount of tissue available compared with the other models, and the much slower time-course, just as readily as to true absence. Choosing somewhat arbitrarily six weeks as the time to examine aortas fortunately did give a positive result thus avoiding these conceptual inadequacies. Collagen stimulating factors were demonstrated convincingly by both pattern and statistics. The more valid collagen chain synthesis assay was employed. The failure to repeat this after

overnight storage is readily attributable to the known lability of the factors. The subsequent attempt with frozen material was also doomed, in retrospect, as stimulation has not been achieved with other types of damaged tissue after freezing in subsequent work.

The animal work and methodology of this type of experiment is onerous but the significance of these initial observations in the understanding of mechanisms in hypertension may be considerable. A more rapid model of hypertension would be more convenient to work with, and would probably provide a higher concentration of collagen stimulating activity. Just after most of the present work was completed, Foidart et al. (1978) described an abrupt rise in collagen synthesis maximal at 1 week after induction of hypertension by clipping one renal artery in rats. Using this model they showed a linear correlation between collagen content and blood pressure. They concluded that synthesis was controlled by the blood pressure level and not by circulating humoral factors from the ischaemic kidney since reserpine blocked the effect.

What is the nature of the stimulus for enhanced collagen synthesis in hypertension ? Greenberg et al. (1978) have demonstrated pressure-independent hypertrophy of veins and pulmonary arteries of spontaneously hypertensive rats. Electron microscopy suggested enhanced protein synthesis but collagen synthesis or content was not examined. A humoral mechanism was postulated, perhaps release of a stimulator of cell growth or release of a derepressor of an inhibitor of cell growth from the kidney or from the arterial side of the circulation. As previously discussed, however, collagen synthesis seems to be directly correlated with the level of pressure. Leung et al. (1976) found that rat aortic smooth muscle cells cultured on elastic membranes showed a large increase in collagen and glycosaminoglycan synthesis but not in DNA synthesis when subjected to cyclic stretching. There is no way at present of knowing how relevant this observation is to events in vivo in hypertension, but perhaps the presence of collagen stimulating factors is all the more surprising.

Another factor which is known to reflect the level of blood pressure is endothelial permeability. A direct relationship between passage of protein tracers - ferritin and horseradish peroxidase - and blood pressure has been shown in rats given catecholamines and aortic coarctation (Huttner et al. 1973). Increased pinocytotic activity of endothelial cells and focal endothelial cell damage occur in hypertension ; the extreme example being fibrinoid necrosis in malignant phase hypertension (reviewed by Koletsky, 1976). A large increase in aortic endothelial cell replication has been found in hypertensive rats (Schwartz and Benditt, 1977) and focal desquamation of endothelium is now being proposed as a crucial feature of hypertension as much as of atherosclerosis (Ross, 1976). Some fibrinogen normally penetrates the endothelial barrier (Bell et al. 1974) but this must surely differ in character from the influx of plasma through endothelial gaps and defects in hypertension.

Ross and Glomset (1973) have proposed that endothelial defects lead to exposure of subendothelial collagen with resultant platelet aggregation and focal release of material from platelets stimulating smooth muscle cell migration and proliferation. A platelet factor has been described capable of stimulating fibroblasts and smooth muscle cells quiescent in platelet-poor-plasma to proliferate (Rutherford & Ross, 1976). While this hypothesis is attractive with regard to the localised intimal lesions of atherosclerosis in which platelet material does become incorporated, it seems less relevant to hypertension in which the general hypertrophy of the media would necessitate a more permeable agent with access throughout the media. Anyway McCullagh et al. (1978) have failed to show any specific effect of platelet factor on collagen synthesis, and collagen stimulating factors such as those prepared from injured liver do not stimulate DNA synthesis.

The presence of collagen stimulating factors in experimental hypertension indicates that necrotic tissue is not essential for their production. Plasma constituents are present however in both necrotic tissue and hypertensive aortas. Again, macrophages do not appear to

be required. Endothelial cells however may play an analogous role. Chidi et al. (1979), using a standard model of embolectomy balloon arterial injury in rabbits for 30 days, showed that collagen content and intimal thickness was greatly increased only in parts of the wall covered by regenerating endothelium with minimal changes in denuded areas. They postulated an interaction between regenerating endothelium and smooth muscle. Macrophages and endothelial cells both produce plasminogen activator. The idea that the interaction of proteolytic enzymes and plasma constituents produces collagen stimulating factors is developed further in the following chapters.

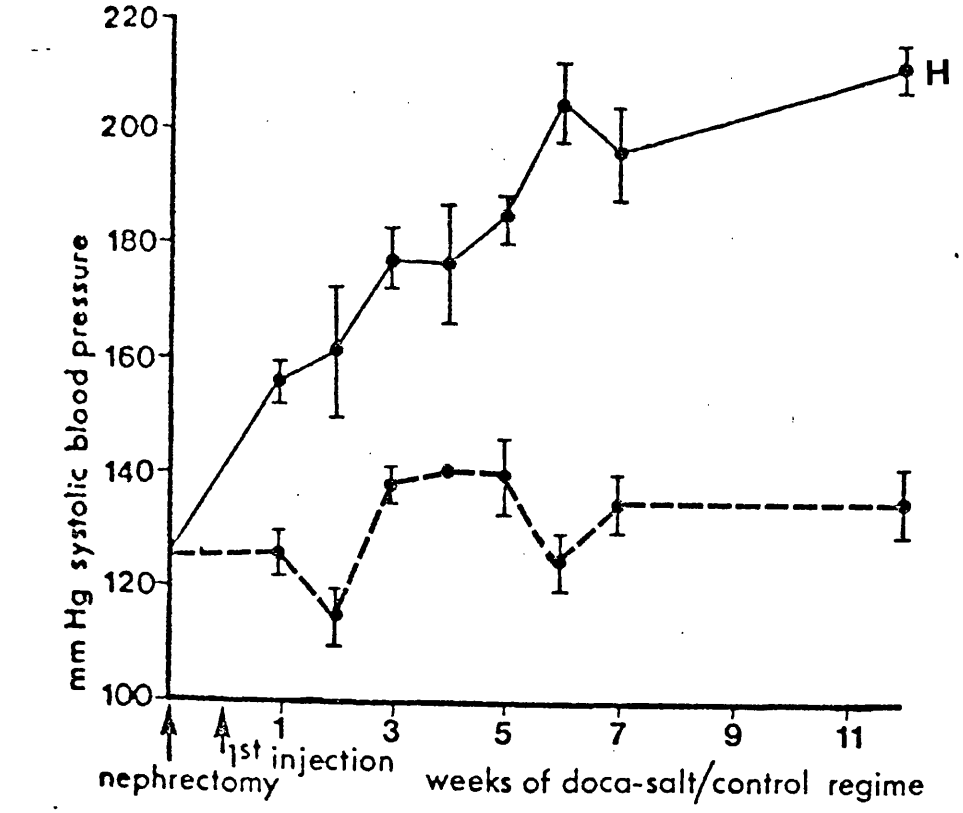


Fig 5:1 B. Heart weights of hypertensive and control rats.

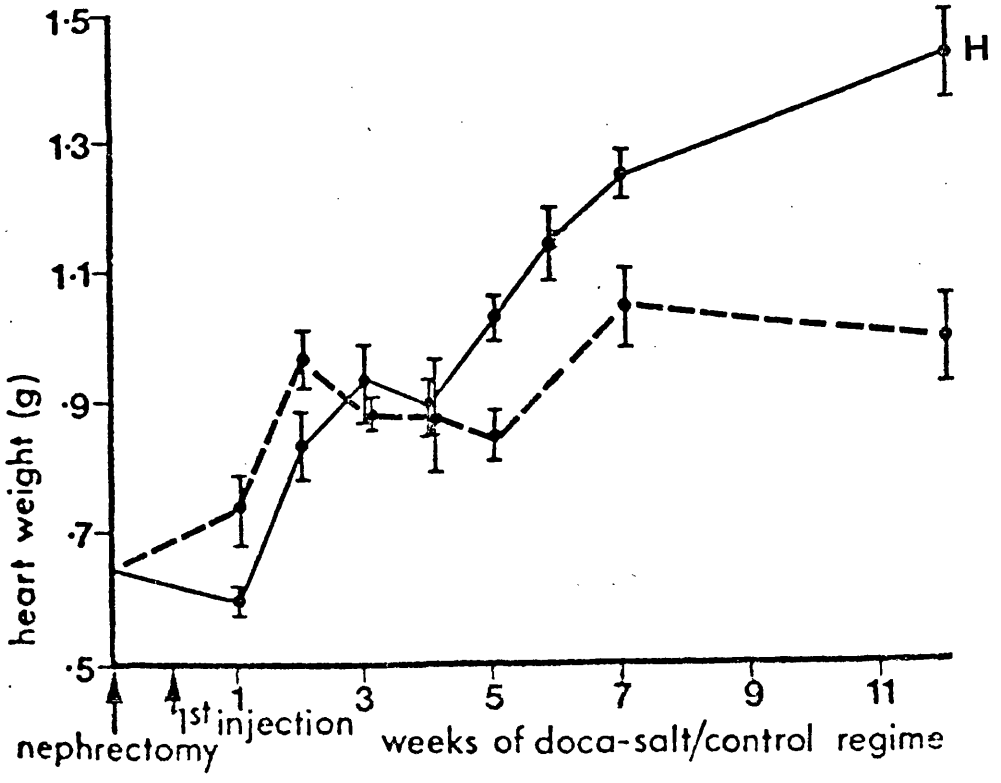


Fig 5:2 Aortic prolyl hydroxylase levels of hypertensive and normal rats.

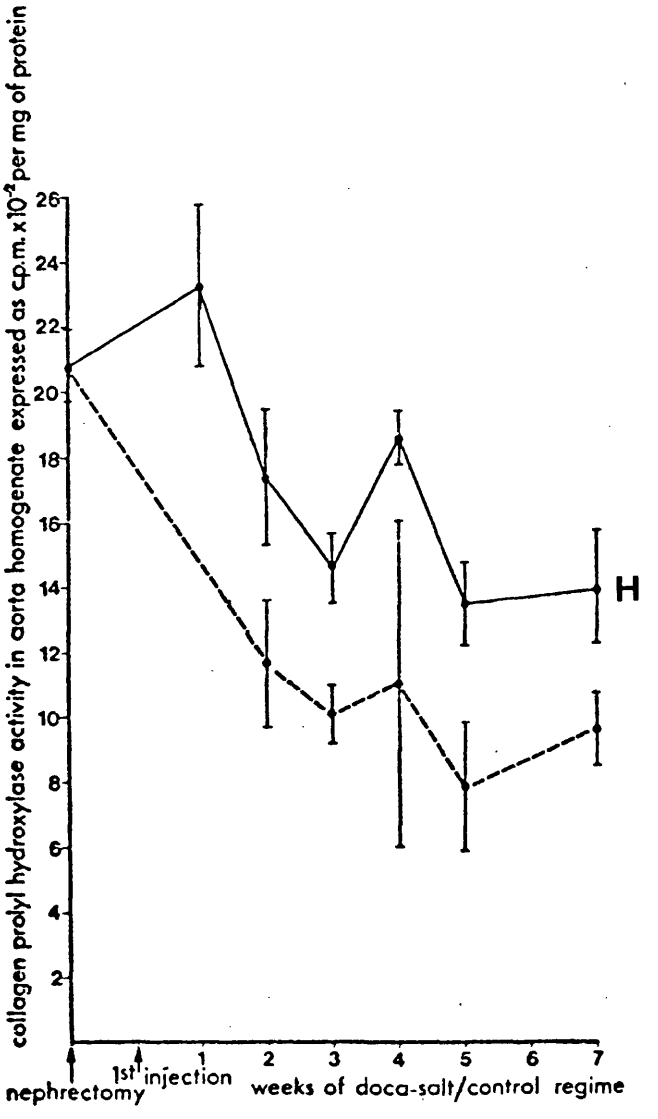


Fig 5:3 A. The effect of column fractions from hypertensive rat aortas on collagen chain synthesis in replicate fibroblast cultures.

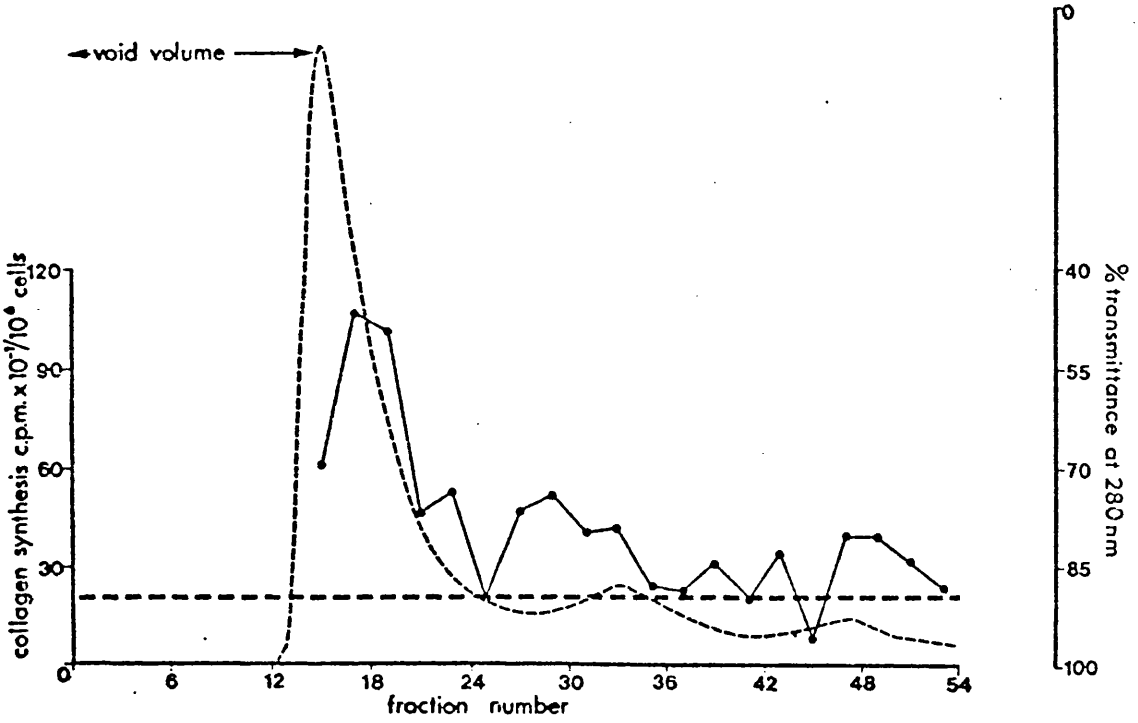


Fig 5:3 B. Further experiment after storage of fractions.

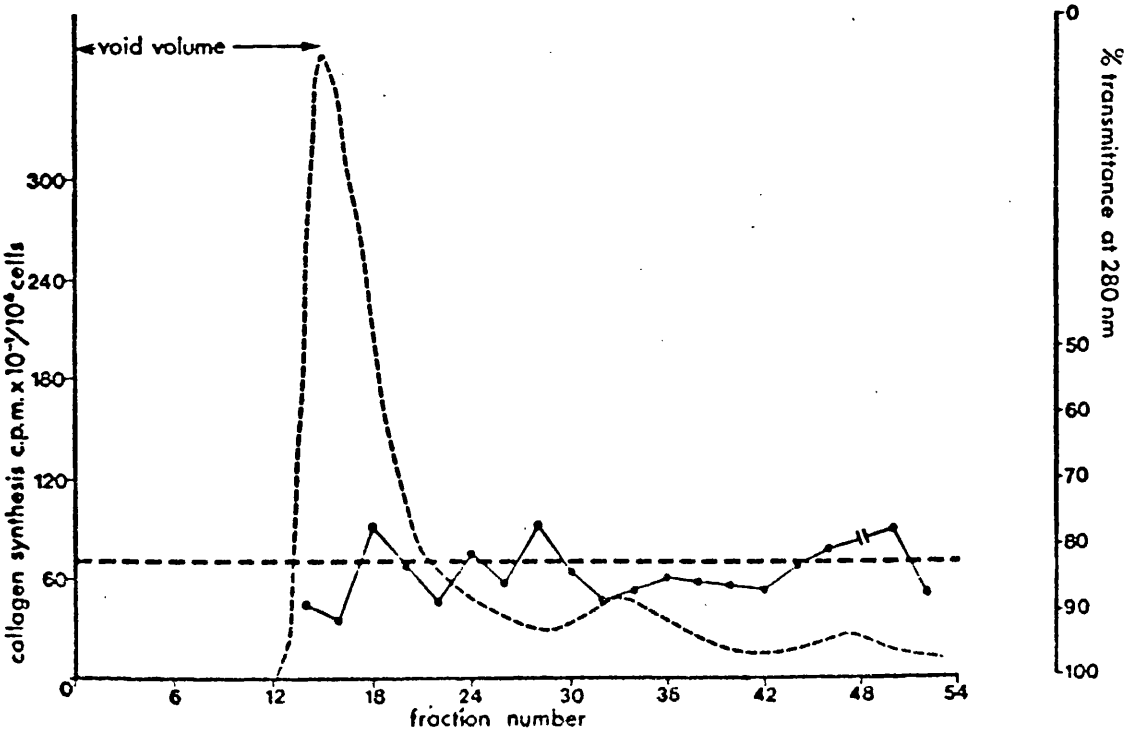


Fig 5:4. The effect of column fractions from control rat aortas on collagen chain synthesis in fibroblast cultures.

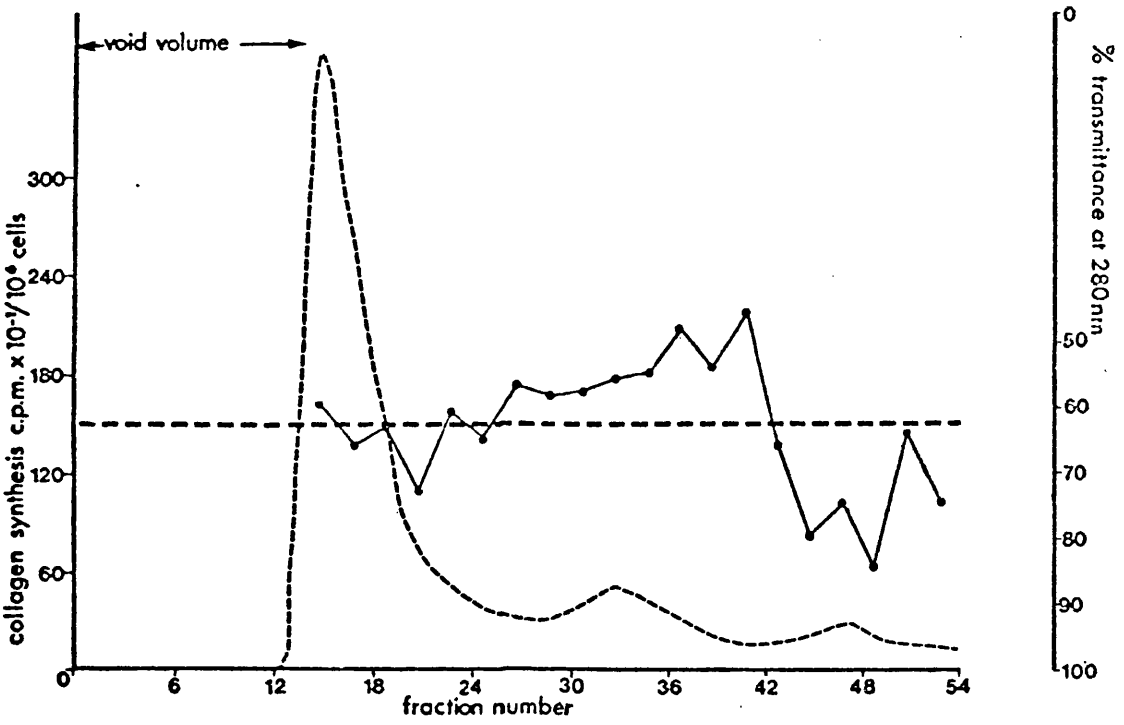
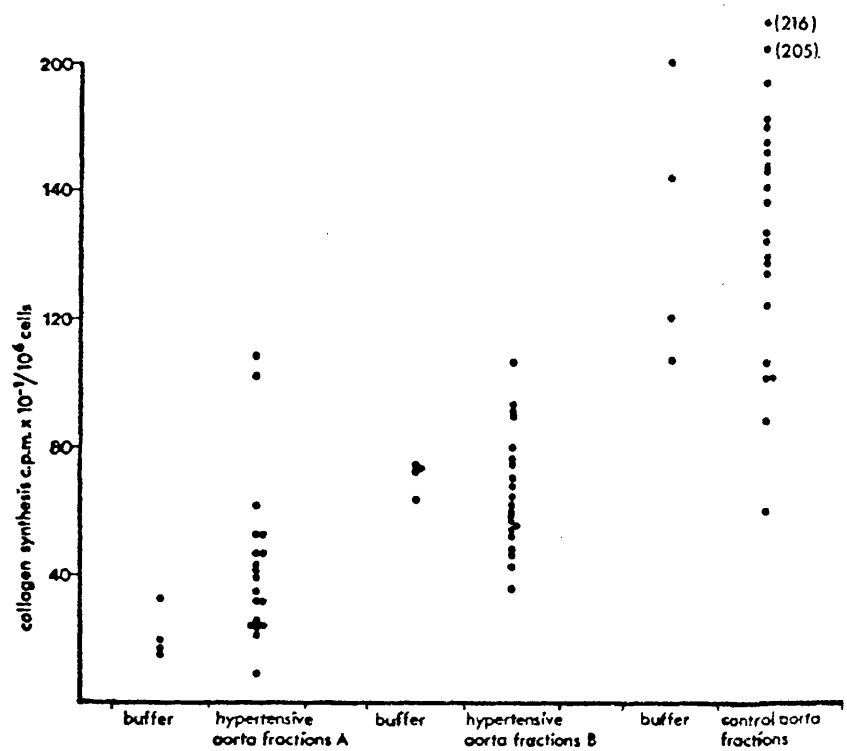


Fig 5:5. Distribution of data from the three previous column experiments.



Chapter 6

OTHER APPROACHES TO THE DEMONSTRATION OF COLLAGEN STIMULATING FACTORS

Collagen stimulating factors have been prepared for previous work by the relatively lengthy process of column chromatography overnight in a cold room, yielding many fractions. The complex pattern of stimulation subsequently obtained is not quantifiable and reproducibility is subject to variation in column performance, tissue culture plating efficiency and assay performance. Initially short columns (15 cm) of G-25 Sephadex were tried to circumvent these problems. More than one column could be run simultaneously and rapidly, allowing test and control material to be processed together. The first peak of toxic material could be discarded and the remaining active material pooled. This procedure failed simply because the less complete separation permitted trailing of toxic material into the later fractions inhibiting stimulating activity.

It was realised that what was really needed was a simple filtrate with exclusion of toxic higher molecular weight material presumed to be of at least 5,000 daltons. Amicon filtration membranes proved suitable for this purpose. The problems of using cultured fibroblasts as the test system were circumvented in later work by adopting an in vivo approach.

Methods :-

The earlier experiments were performed with 3-day carbon tetrachloride mouse liver as used in previous work on collagen stimulating factors. The filtrates were applied to tissue culture flasks. The more recent experiments have utilised 4 or 5-day paraquat rat lung and this material has been tested in vivo by intraperitoneal injection into mice.

Experiments with Carbon Tetrachloride liver

Mice poisoned with carbon tetrachloride as described previously were killed after 3 days and these livers or normal control livers homogenised at 2.5 volumes per g in chilled phosphate buffer and a 12,000 g supernatant prepared as described for paraquat lung factors. The supernatant was applied to Amicon filters of 50,000 daltons exclusion limit in the form of "Centriflo" cone filters. These cones were set in 50 ml centrifuge tubes and spun at 750 g at 4°C for about 30 to 45 minutes depending on the amount of liquid applied. This liquid was a dark red colour but the filtrate obtained was pale pink due to almost complete retention of haemoglobin. The filtrate was successively applied to Amicon "Diaflo" filters of 10,000 and 5,000 exclusion limits. The filter discs were used in an Amicon Ultra-filtration Cell Model 10-PA. In this apparatus the liquid is progressively forced through the membrane by pressure from a cartridge of Freon propellant. Application of the crude starting supernatant resulted in rapid clogging of these filters and the cone filter stage was a necessary preliminary step. The various filtrates were applied to L929 fibroblast cultures and assays for collagen synthesis performed as previously described.

Experiments with paraquat lung

Rats were poisoned with paraquat as described previously. The animals were killed after 4 or 5 days and the lungs or normal control lungs homogenised at 5 volumes per g in chilled phosphate buffer and a 12,000 g supernatant prepared as previously described. In these experiments only the "Centriflo" cone filters were used to prepare a filtrate of proteins less than 50,000 daltons molecular weight.

Rather than applying the material to tissue culture fibroblasts, the material was injected intraperitoneally into littermate male CFLP mice using animals of similar size (20-25 g) as far as possible. The problems of tissue culture were thus avoided. This was done using initially fractions from a conventional G25 Sephadex column on one occasion

and subsequently using filtrates of normal and damaged lung. Following intraperitoneal injection of filtrate or control buffer, the mice received a further injection after 2 hours of 0.1 μCi of ^{3}H-5-Proline subcutaneously in the flank. After a further 4 hours the animals were killed and liver and sometimes also lung were taken and stored at -20^{o}C for subsequent prolyl hydroxylase and collagen synthesis assays as described in Appendices 3 and 5.

Results

Experiments with Carbon Tetrachloride Liver.

Adding crude supernatant of injured or normal liver causes tissue culture fibroblasts to round up and detach. If the remaining cells are assayed, both prolyl hydroxylase and collagen chain synthesis are diminished. These toxic effects were not evident using the various filtrates.

The "Centriflo" cones permitted fairly rapid filtration but the ultrafiltration cell with "Diaflo" membranes filtered rather slowly at 4^{o}C apparently due to increased friction around the piston even when lubricated. This took many hours even at room temperature, introducing anxieties about the known lability of the factors. Filtration overnight in the fridge or cold room proved feasible but since only one apparatus was available, test and control material were not available simultaneously.

The test material was found to stimulate prolyl hydroxylase activity by nearly 6-fold compared with buffer using the filtrate less than 50,000 daltons. (Table 6:1) There was a progressive decline in the level of stimulation achieved using the next two filtrates down to 3-fold. Control liver filtrates were also found to stimulate prolyl hydroxylase, though to a lesser degree. Again there appeared to be some diminution of activity at least using the filtrate less than 5,000 daltons.

Since a rise in activity was predicted, a one-tailed Student's t test may be justified. The changes in mean levels in comparison

with adding buffer alone are significant for both test and control material. It should be noted that even if a two-tailed test is applied, nearly all these results remain statistically significant.

A remarkably similar level of stimulation (nearly 6-fold) was detected using the collagen chain synthesis assay (Table 6:2) when the 50,000 d filtrate was applied. The other filters were not used here. This result was again significant with the t test whether one or two-tailed. Normal liver in a separate experiment produced no stimulation of collagen chain synthesis in contrast to the previous result using the prolyl hydroxylase assay.

At this time, experiments began to fail to show stimulation. This was attributed to a phase of difficulty with tissue culture and enthusiasm waned. In retrospect the failures were obviously due mainly to a switch to liver stored frozen for convenience. An example of the loss of activity and possibly even a slight toxic effect is shown in the middle column of Table 6:2.

Experiments with paraquat lung

Intraperitoneal injection of collagen stimulating factors from a column resulted in increased hepatic prolyl hydroxylase activity and proline uptake producing the same type of pattern as found previously with tissue culture (Fig. 6:1). The fractions sampled do not quite extend across all protein peaks, but 4 peaks of stimulation are detected. The pattern is clearest with prolyl hydroxylase and proline uptake which show the closest correspondence. The individual values for these two assays alone are significantly correlated ($r=3.2$, $2P<0.005$). The increase in prolyl hydroxylase values alone is significant with the Wilcoxon test ($2P<0.01$). The pattern was much less clear with the collagen synthesis assay and, due to a wider spread of controls, the Wilcoxon test is not significant.

After this column experiment the earlier results from the use of filters with injured liver were reviewed and it seemed worthwhile to try this technique again and inject the filtrates intraperitoneally

into mice. Tables 6:3 to 6:6 show the results of four successive experiments. Table 6:3 shows an increase in hepatic proline uptake and collagen synthesis.

Table 6:4 shows an increase in hepatic proline uptake and collagen synthesis, but rather surprisingly, no increase in prolyl hydroxylase. Lung was also assayed in this experiment and all three assays show an increase. Table 6:5 shows the results from injection of normal rat lung filtrate using the larger 2 ml aliquot as in previous experiment. An increase in proline uptake in both liver and lung of the mice is apparent but no convincing change is seen in the two assays of collagen metabolism.

Table 6:6 shows an attempt to demonstrate a linear dose-response relationship. An increase in all three assays is apparent but the maximum effect was obtained with 0.5 ml and not 2 ml as previously. Normal lung was included in the experiment and no significant changes are seen. The dose-response curve obtained is shown in Fig. 6:2. An explanation for this less than perfect result is available. The filtrate on this occasion was noted to be rather more red in colour than before. The same set of filters was used as previously. These conical filters are stated to be re-usable but it seems probable that high molecular weight toxic material has penetrated since the dose-response curve declines rather than maintaining a plateau. The close correspondence of the three types of measurement is shown in Fig.

The results of the collagen synthesis assay of tissue, which is the assay of most consequence, is subject to many possible variations such as in animal size, isotope uptake, leakage from injection site and assay variation itself. The three experiments all show an apparent increase in collagen synthesis but, due to the spread of data, levels of significance with a t test are only achieved in the last experiment, possibly due to improving technique. Nevertheless the trend of the results is as predicted and a way of expressing this statistically was sought. Table 6:7 shows the application of the 50% Probability test

which is a special version of the Binomial test (Langley, 1970). If the mean of the buffer control cultures for each experiment is assumed to be representative of that population, then taking all the test values together, the data may be converted to binomial form by totalling those values above and those below the control mean. If no effect on collagen synthesis has been achieved then 50% of these values should be distributed above and 50% below the control mean. The significance of any deviation can be obtained from tables. From Table 6:7 it can be seen that the results of the first two experiments with damaged lung still fail to each statistical significance. The third experiment is significant ($2P<0.05$). The data in this form can be combined and it is notable that this results in increased significance ($2P<0.02$). Ideally the same test should be applied to a similar number of control results.

Discussion

With carbon tetrachloride liver filtrates the greatest stimulation of prolyl hydroxylase synthesis was obtained with the largest molecular weight filtrate and progressively less stimulation with the others. These findings suggest that the range of molecular weights of the factors extends above 5,000 daltons and indeed above 10,000 daltons. Sephadex gels other than G 25, having an exclusion limit above 5,000 daltons, do not appear to have been tried previously and the presence of higher molecular weight activity has escaped detection. The corollary of these observations is that the toxic component is of molecular weight above 50,000 daltons.

A single minor peak of activity stimulating prolyl hydroxylase but not collagen synthesis is a recognised phenomenon of normal tissue column separation. It is therefore not surprising that the filtrates of normal liver definitely stimulated prolyl hydroxylase activity, though less than CCl_4 liver. Collagen synthesis was only stimulated by CCl_4 liver and therefore gives greater contrast between test and control and indeed a truer reflection of in vivo events.

The "Centriflo" conical filters supplied the most active filtrate

and permitted rapid separation. Collagen stimulating factors may be composed of many distinct proteins or derive from disintegration of a single protein into active fragments. For many purposes the overall combined quantitative effect on collagen synthesis is what is sought. This methodology seems to fit these requirements.

The ability of paraquat lung factors in the form of column fractions or filtrates to stimulate collagen synthesis in liver and lung following intraperitoneal injection provides in vivo validation of the factors' significance. This preliminary evidence indicates that the factors are more than just a tissue culture phenomenon. Indeed it is the lack of this type of evidence which is hindering research on growth factors (Gospodarowicz and Moran, 1976). Conversely, understanding of the role of chalones in growth control is limited by chalones remaining a largely in vivo phenomenon. In this context it must be emphasised that collagen stimulating factors are stimulatory and therefore do not resemble chalones which are inhibitory.

With lung factors, proline incorporation activity did not distinguish test and control material. This is as expected when it is recalled that previous work from this laboratory showed that protein synthesis was stimulated to a similar minor degree by column fractions from both normal and carbon tetrachloride liver. Prolyl hydroxylase assay similarly failed to show changes exclusive to the test material.

It is interesting that both liver and lung appear to have been affected in mice injected with rat lung material. Presumably this means factors have gained access to the circulation. As shown in the chapter on paraquat lung injury, the only organ in the poisoned animal in which there is increased collagen synthesis is the lung. Here the effect of the stimulating factors present appears to be restricted to the damaged tissue. Whether this is due to local inactivation or to dilution of factors reaching the circulation or to some other mechanism is unclear. Certainly one can imagine that it would be inappropriate and possibly disastrous for the animal if active factors normally gained access to the circulation. It would be interesting to see the effect of

repeated injection of factors - would this cause systemic sclerosis or some novel syndrome ? - which cell types would be stimulated in normal tissue ? The cutaneous manifestations of human systemic sclerosis have been reported to have resolved with haemodialysis (Barker and Farr, 1976).

Table 6:1

Effect of various filtrates of 3 day carbon tetrachloride injured mouse liver and normal liver on prolyl hydroxylase activity of L929 fibroblast cultures.

1 ml added to each culture.

DIAFLO membrane exclusion limit	Test Material Prolyl Hydroxylase DPM/5x10^4 cells		Stim.	Control Material Prolyl Hydroxylase DPM/5x10^4 cells		Stim.
(Buffer controls)	166	$\bar{x}$		407	$\bar{x}$	
	246		x1	669		x1
"	261	187		412	574	
"	172			760		
"	91			623		
<50,000 d	1238			818		
"	711		x5.7	602		x2
"	2263	1073		1764	1119	
"	664			1377		
"	488	(t=2.7;1P<0.025)		1034	(t=2.5;1P<0.025)	
<10,000 d	973			1027		
"	1336		x4.8	1242		x2.1
"	456	892		1328	1216	
"	804	(t=4.2;1P<0.0025)		1268	(t=6.3;1P<0.0005)	
<5,000 d	616			707		
"	505		x3	1191		x1.5
"	298	557		755	884	
"	576					
"	791	(t=4.4;1P<0.0025)			(t=2.0;1P<0.05)	

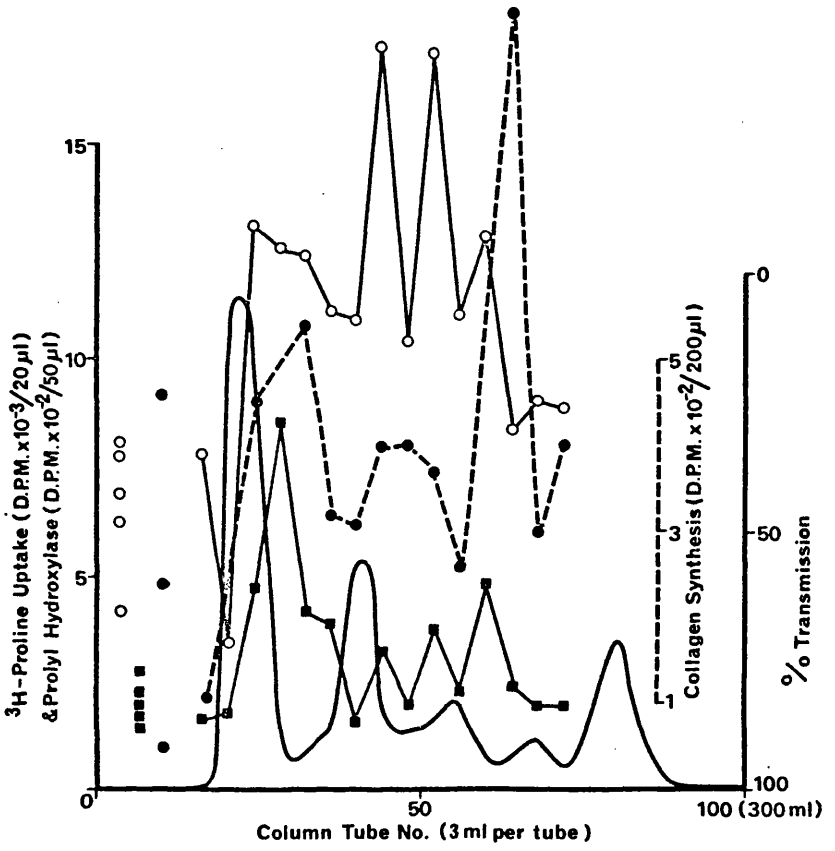
Table 6:2

Effect of various filtrates of 3 day carbon tetrachloride injured mouse liver and normal liver on collagen synthesis in L929 fibroblast cultures. 1 ml added to each culture.. 3 separate experiments.

DIAFLO membrane exclusion limit	Test Material Collagen synthesis (DPM/2x10^5 cells) Liver used fresh			Liver stored -20°C		Control Material Collagen synthesis (DPM/2x10^5 cells) Fresh normal liver	
		$\bar{x}$	Stim		$\bar{x}$		$\bar{x}$
(Buffer controls)	179			1379		125	
	196		x1	644		137	
	55	125		2199	1606	293	235
	68			2200		386	
<50,000	756			1046		270	
	1159		x5.8	234	715	393	
	243	720		925		251	323
		(t=2.6;1P<0.025)		656	(t=2.1;2P NS)*	376	(t=1.2;NS)
<10,000	-			1092		447	
				1120		106	
				1260	1101	336	351
				930	(t=1.3;NS)	515	(t=1.0;NS)

* Two-tailed test used here since a fall was not predicted (1P<0.05).

lung on prolyl hydroxylase activity, collagen chain synthesis and ^{3}H-proline uptake in livers of mice injected intraperitoneally.



—O— prolyl hydroxylase activity, top line

--●-- collagen synthesis, middle line

—■— proline uptake, bottom line

——— continuous profile of protein from column.

(buffer control results adjacent to left hand scale)

A rough correspondence is seen between the values for the three assays on each liver. The most marked increase relative to buffer control values is found with the prolyl hydroxylase assay.

Table 6:3

Results of ^{3}H-Proline incorporation and collagen synthesis assays on mouse liver following intraperitoneal injection of an extract of 4-day paraquat rat lung

Mouse No.	Substance Injected	^{3}H-Proline Incorporation (DPM/10µl homogenate)		Collagen Synthesis (DPM/0.2ml homogenate)	
1	0.5ml Buffer	3,136	$\bar{x}$	-	$\bar{x} \pm$SEM
2	0.5ml "	3,176	3,270	225	248
3	1.0ml "	3,032		256	$\pm$ 12
4	1.0ml "	3,736		264	
5	0.5ml Filtrate	5,190		818	
6	0.5ml "	5,304	5103	569	556
7	1.0ml "	4,636		195	$\pm$ 131
8	1.0ml "	5,280	(t=8.2;1P<0.01)	641	(t=1.9;NS)

(To obtain data for statistical analysis all values are grouped and averaged regardless of volume injected).

Table 6:4

Results of ^{3}H-Proline incorporation, prolyl hydroxylase activity and collagen synthesis in mouse tissue following intraperitoneal injection of 5 day paraquat rat lung extract

A) Liver :-

Mouse No.	Substance Injected	^{3}H-Proline Incorporation DPM/10 µl homogenate		Prolyl hydroxylase Activity DPM/50 µl homogenate		Collagen Synthesis DPM/0.2 ml homogenate	
1	2.0 ml	5824	$\bar{x}$	1836	$\bar{x}$	3473	$\bar{x}$ ± SEM
2	Buffer	5148	4892	1325	1703	443	1801 ± 549
3	"	4581		1742		891	
4	"	4052		2077		1662	
5	"	4853		1533		2536	
6	0.5 ml	4967		956		3556	
7	PQ lung	5708	5534	1541	1664	2944	2562 ± 403
8	Filtrate	5970		1279		2744	
9	"	6599	(t=1.3;NS)	2006		2443	(t=1.1;NS)
10	"	4425		2540		1122	
11	2.0 ml	5971		2060		4453	
12	PQ lung	4226	5510	1938	1702	1975	3113 ± 827
13	Filtrate	6876		1347		2897	
14	"	5522	(t=1.1;NS)	1544		5415	(t=1.3;NS)
15	"	4954		1620		824	

B) Lung :-

1	2.0 ml	844		3650		235	
2	Buffer	994	873	2984	3053	35	146 ± 43
3	"	924		2998		240	
4	"	789		2464		57	
5	"	815		3170		164	
6	0.5 ml	722		4547		-	
7	PQ lung	897	841	4188	3960	363	351 ± 153
8	Filtrate	825		4148		188	
9	"	991	(t=0.5;NS)	3806	(t=2.9;	774	(t=1.4;NS)
10	"	771		3109	1.P<0.01)	78	
11	2.0 ml	1186		4285		779	584 ± 2001
12	PQ lung	925	1066	4021	3963	1024	
13	Filtrate	1124		3380		427	
14	"	1062	(t=3.3;	4050	(t=3.8;	107	(t=2.3;
15	"	1031	1.P<0.01)	4078	1.P<0.01)	-	1.P<0.05)

Table 6:5

Results of ^{3}H-Proline incorporation, prolyl hydroxylase activity, and collagen synthesis in mouse tissue following intraperitoneal injection of normal rat lung extract

A) Liver :-

Mouse No.	Substance Injected	^{3}H-Proline Incorporation DPM/10 µl homogenate		Prolyl hydroxylase Activity DPM/50 µl homogenate		Collagen Synthesis DPM/0.2 ml homogenate	
1	2.0 ml	5328	$\bar{x}$	859	$\bar{x}$	1978	$\bar{x} \pm$ SEM
2	Buffer	5787	5555	1749	1167	1871	1326
3	"	6120		911		1825	± 365
4	"	4700		730		843	
5	"	5841		1586		115	
6	2.0 ml	6545		910		-	
7	Filtrate	6834	7482	-	1306	-	1460
8	"	9803		1840		2705	± 1245
9	"	6672		1239		-	
10	"	7554	(t=2.9; 1P<0.01)	1233	(t=0.4;NS)	214	(t=0.1;NS)

B) Lung :-

1	2.0 ml	1108		1632		688	
2	Buffer	1419	1109	2275	2057	51	234
3	"	943		1943		97	±116
4	"	1106		2310		167	
5	"	969		2125		167	
6	2.0 ml	1607		2306		343	
7	Filtrate	1391	1541	2021	2572	376	321
8	"	1980		3695		-	± 43
9	"	1511		2411		371	
10	"	1218	(t=2.8; 1P<0.05)	2428	(t=1.6;NS)	192	(t=0.6;NS)

TABLE 6:6

Results of ^{3}H-proline incorporation, prolyl hydroxylase activity and collagen synthesis in the livers of mice injected intraperitoneally with an extract of 5 day paraquat rat lung

Mouse No.	Substance Injected	^{3}H-Proline Incorporation DPM/10 µl homogenate		Prolyl hydroxylase Activity DPM/50 µl homogenate		Collagen Synthesis DPM/0.2 ml homogenate	
1	2 ml Buffer	2021	$\bar{x}$	728	$\bar{x}$	111	$\bar{x}$ ± SEM
2	"	3299	3001	322	479	-	381
3	"	3203		501		650	± 269
4	"	3481		366		-	
5	0.1 ml	2227		1421		485	
6	PQ lung	3854	2668	1206	1092	352	605
7	Filtrate	1964	(t=0.6;NS)	1200	(t=2.9;	358	±209
8	"	2628		541	1P<0.05)	1225	(t=0.6;NS)
9	0.5 ml	6162		1536		1043	
10	"	3554	4253	3375	1862	-	1275
11	"	3436	(t=1.7;NS)	1231	(t=2.7;	1318	±124
12	"	3860		1305	1P<0.05)	1465	(t=3.4; 1P<0.05)
13	1.0 ml	3342		1147		591	
14	"	3918	3926	741	1015	1129	814
15	"	3840	(t=2.2;	-	(t=3.3;	1092	±174
16	"	4602	1P<0.05)	1157	1P<0.05)	444	(t=1.4;NS)
17	2.0 ml	4160		1161		948	
18	"	3485	3968	1050	1238	512	814
19	"	3663	(t=2.3;	1180	(t=5.3;	982	±151
20	"	4565	1P<0.05)	1561	1P<0.01)	-	(t=1.5;NS)
21	2.0 ml	5601		1343		-	
22	Control lung	3547	3833	460	854	587	640
23	Filtrate	3614	(t=1.1;NS)	653	(t=1.7;NS)	-	±52
24	"	2571		960		692	(t=0.9;NS)

Fig 6:2. The mean values from the previous experiment (Table 6:6) displayed graphically.

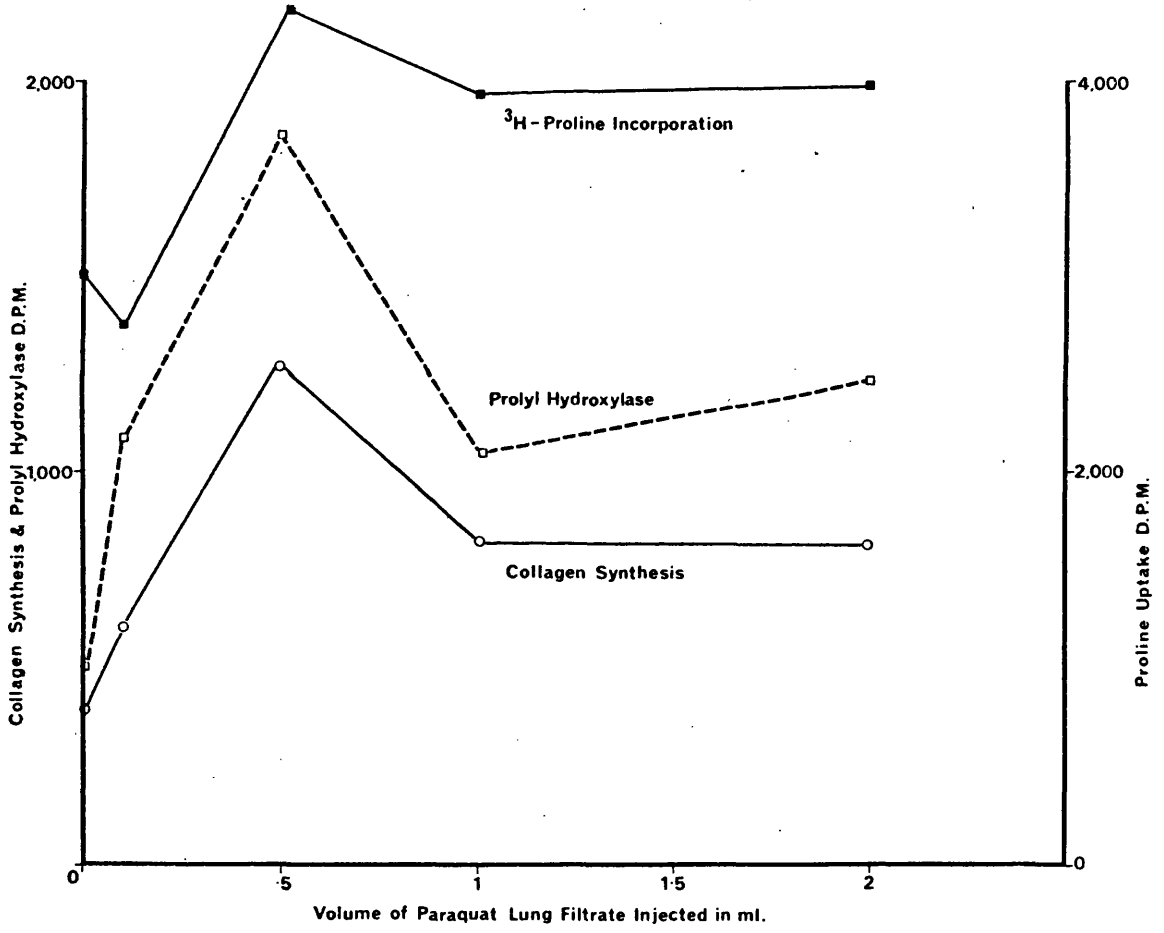


Table 6:7

Results of 50% Probability Test on Collagen Synthesis

Data converted to binomial form to permit summation

	Expt. 1	Expt. 2	Expt. 3	Combined Expt.'s
No of test results above the control mean	3	8	12	23
No of test results below the control mean	1	2	2	5
n	4	10	14	28
50% Probability test	NS	NS	$2P<0.05$	$2P<0.02$

Chapter 7

THE GENERATION OF COLLAGEN STIMULATING FACTORS IN VITRO FROM FIBRIN

The influence of macrophages on fibrogenesis in experimental liver injury and the presence of necrotic debris or plasma proteins alone at the site of enhanced fibrogenesis in all the models studied, suggested certain elements suitable for the generation of collagen stimulating factors in vitro. This section is concerned with various attempts to elicit collagen stimulating activity from macrophages and fibrin.

Fibrin deposition in inflammation has been recognised for some time to serve a glue-like function in providing temporary adhesion across tissue defects such as bleeding vessels and ulcerated or injured surfaces. It has been thought to provide an inert scaffolding for capillary and fibroblast ingrowth. Fibrin deposition is a constant feature of the inflammatory exudate at any site, and, in contrast to other constituents from plasma, it forms a persistent material.

Fibrogenesis is a phenomenon which frequently follows necrosis but it may be provoked by injury and inflammation where tissue damage is of minor proportions. This latter occurrence is exemplified by the organisation of serous exudates, often resulting in adhesions. The point is that necrotic tissue per se is not a necessary component of the fibrogenic response. That properly formed fibrin is necessary is hinted at by the poor wound healing observed in patients with hereditary deficiency of fibrin stabilising factor (Factor XIII) or fibrinogen itself. This is not a feature of the other coagulation disorders. Also, Buckman et al (1975) were able to prevent experimental post-operative adhesions in dogs by Ancrod defibrinogenation. Similarly, Ungar and Ginsburg (1955) found that the intravenous injection of trypsin reduced fibrin deposition and granulation tissue around surgical gut implanted in the livers of rats. The links between fibrin deposition and

organisation and collagen deposition are often referred to in standard texts (such as Muir's Textbook of Pathology) but the nature of the linkage is rarely discussed. Shoshan and Gross (1974) recognise that a relationship seems to exist between the fibrin network of wounds, the migration of fibroblasts and the deposition of collagen. They review the evidence that inhibition of fibrinolysis delays healing of wounds, resulting in increased scarring. They nevertheless fail to draw the conclusion that this implies prolongation of a stimulus to fibrogenesis. I would suggest that fibrinolysis, far from removing that signal, may actually generate and control it by progressive removal of its source.

Accordingly, from studying the timescale of events in various healing processes, it seemed to me that fibrin was a likely candidate to provide instructions to fibroblasts by means of its degradation products. Plasminogen is bound in inactive form to fibrin and can be activated by macrophage and endothelial enzyme secretion. The by-products of fibrin degradation are already recognised to be utilised in inflammation. As mentioned in the introductory chapter fibrinopeptides B and Y are chemotactic for leukocytes. Such products would provide a simple self-limiting mechanism for producing enhanced collagen synthesis restricted to inflammatory lesions.

Methods

The Preparation of Fibrin

The purest form of fibrinogen commercially available was used (Fibrinogen Kabi, Grade L, coagulability 90% of total protein content). The lyophilised powder was reconstituted according to the manufacturer's instruction with additional steps to convert it to be the stable cross-linked form of fibrin (Dray-Attali and Larrieu, 1977). A bottle of 1 g fibrinogen was reconstituted by addition of 100 ml of 0.15 M sterile NaCl containing 0.08 M $CaCl_2$. 500 units of thrombin (Leo) and 1 ml fresh human plasma were added via a Millipore filter to preserve sterility.

The bottle was incubated at 37°C for 6 hours before sotrage at 4°C. The resultant white fibrin clot was shaken out as a jelly onto filter paper in a sterile cabinet and finely chopped with a sterile dermatome razor blade. The lumps of fibrin were transferred to a 1 litre sterile beaker covered by sterile gauze and rinsed twice with 500 ml sterile saline. The fibrin was left soaking in 500 ml sterile isotonic saline containing 50,000 units of penicillin and 50,000 μg of streptomycin as used for tissue culture. After 48 hours the fibrin was homogenised in 100 ml of saline and antibiotics using a Silverson homogeniser until fine particles were produced. This was stored at -20°C as 5 ml aliquots of 50 mg fibrin in Steriling 20 ml plastic universal containers.

Experiments using macrophages

Macrophages were recovered by peritoneal saline washout from CFLP mice (20-25 g) injected 3 days previously with 1 ml mineral oil. About 3 to 4 million cells were obtained per animal. The fibrin (50 mg) in each Sterilin container was precipitated by centrifugation and the supernatant discarded. Approximately 20 million macrophages plus 2.5 ml of culture medium as used for fibroblasts were added to each container and gently mixed. After 24 hours incubation overnight at 37°C, the medium was centrifuged at 12,000 g. The supernatant was applied to a G25 Sephadex column and the fractions applied to fibroblast cultures as described previously.

Results

Fig. 7:1 shows a profile of peaks of activity detected with the collagen synthesis assay. 5 ml of supernatant was applied to the column. This was the first experiment of this type performed. (Addition of whole supernatant to cultured fibroblasts produced a toxic effect and no stimulation). With the Wilcoxon test there is a significant difference between the test and control culture values ($1P<0.05$) if a one-tailed test is allowable. Since an increase was predicted this seems justified.

Fig. 7:2 shows the next experiment performed, applying fresh fibroblast medium (no macrophages or fibrin used) to a column with subsequent testing of the fractions on fibroblast cultures. The protein profile is somewhat different. There is a suspicion of a minor degree of stimulating activity present but the results do not achieve statistical significance.

A further experiment with macrophages and fibrin was dogged by various mishaps resulting in only a quarter of the previous number of macrophages being incubated with half of the previous amount of fibrin. A pattern (not shown) suggesting stimulation of collagen synthesis was produced but these values were bracketed by a wide spread of control culture results.

Fig. 7:3 shows the results from application of 15 ml of supernatant from macrophages and fibrin, being 3 times the amount used in the first experiment. Fractions were taken from across the full extent of the protein profile in this experiment. A clearer pattern emerges of four peaks of stimulation of collagen synthesis. These values are significantly higher than buffer-only control values ($2P<0.05$) whether a one or two-tailed Wilcoxon test is applied. The peak values attained are over three times greater than the highest control value. Only one further repeat experiment has been carried out so far and this was vitiated by technical error at the assay stage.

Discussion

Silica was the first material to be successfully used in vitro to induce macrophages to produce factors stimulating collagen synthesis (Heppleston and Styles, 1967 ; Heppleston, 1969). This in vitro model produced results consistent with the fibrogenic reaction around silica in pneumoconiosis and in experimental animals around injected silica and around diffusion chambers containing macrophages and silica (Allison, 1978). The model offers a "clean" source of stimulating material since no other biological origin than macrophages is present.

However, the silica model is not without problems and subsequent reports, even when successful, tend not to be followed by others from the same laboratory with the exception of more recent work from Finland. Harington et al. (1973) failed to confirm Heppleston's findings and instead found inhibition of collagen synthesis. Kilroe-Smith et al. (1973) produced granulomas surrounded by collagen in guinea pigs by injection of insoluble material extracted from macrophages exposed to silica - a completely different approach from Heppleston's. Whether silica itself was present in this material is unclear, and it seems likely that the effect was primarily on macrophages at the injection site rather than fibroblasts. A more comparable model using supernatants from cultures of macrophages with silica was used by a different group to confirm Heppleston and Styles' results (Burrell and Anderson, 1973). Richards and Wusteman (1974) claimed that fibroblast collagen production was stimulated to the same extent by addition of intact macrophages whether they contained silica or not. The assays were done after 24 days culture and changes found are minor compared with controls ; no statistical analysis is offered. Nourse et al. (1975), using the supernatant from silica treated disrupted macrophages, obtained stimulation of collagen synthesis only if it was applied to freshly plated or non-confluent fibroblast.

The variety of different methods used and the conflicting results obtained make interpretation of these reports difficult and indeed they do not seem to add much to the original observation. The reports describing stimulation of collagen synthesis have one feature in common. The macrophages or the factor(s) from macrophages were incubated with medium containing serum either when added to the fibroblast cultures or at an earlier stage. It is possible that the macrophage factors interacted with serum constituents which include residual fibrinogen.

A series of papers of higher quality has come from Kulonen's group in Turku, Finland. The supernatant from homogenised untreated

macrophages inhibited collagen, DNA and RNA synthesis. The supernatant from peritoneal macrophages treated with silica stimulated collagen synthesis in granulation tissue slices. This activity was also obtained from exposure of macrophage lysosomes to silica (Aalto et al. 1976). The stimulating activity was found to reside in 2 of 30 fractions obtained by isoelectric focusing of macrophage culture medium (Jalkanen et al. 1979) but the molecular weights were not defined and small molecules less than 1, 800 daltons had been removed previously by gel filtration. Various other reports seem to add little of consequence. A recent paper (Kulonen and Potila, 1980) reports that incubation of macrophages with rheumatoid synovial fluid, connective tissue activating peptide from blood leukocytes (Castor) and an extract from carbon tetrachloride damaged liver stimulated collagen synthesis to a minor extent after prior incubation with macrophages. These reports have become increasingly complex and hard to evaluate, and the rationale of the many experimental variations is largely unexplained. The mode of action of silica is unknown but the effect seems real enough. The macrophage product(s) are undefined. Their results could be explained either by the direct action of a macrophage constituent or product, or by the action of a macrophage enzyme on a constituent present in their test systems of granulation tissue or fibroblasts. The problem with silica is that it is a rather special case involving non-biological material and one can only guess how relevant this model may be to the wider context of control of collagen synthesis in other pathological situations.

Researchers with immunological interests have investigated putative lymphocyte factors which control fibroblast collagen synthesis. Nearly all these groups have in fact used Ficoll mononuclear cell preparations from blood and, as some but not all have realised, this type of preparation contains about 20% monocytes. Johnson and Ziff (1976) found that the supernatant from phytohaemagglutinin-stimulated blood mononuclear cells produced a higher level of collagen synthesis

in fibroblasts than the supernatant from unstimulated control cells. However no control fibroblasts, to which nothing was added, are included ; it is just possible that the unstimulated cells produced a toxic effect giving a misleading impression of an apparent increase using the stimulated cells. This pitfall was avoided by Spielvogen et al. (1978) using a similar approach and they found that mononuclear cell supernatant stimulated fibroblast collagen synthesis by approximately two-fold, PHA alone by three-fold, and mononuclear cells plus PHA by four-fold. A third group used apparently similar methodology once again, but found inhibition of collagen synthesis (Jimenez et al. 1979).

It is hard to conceive of a reparative system in which an immunological reaction would itself stimulate collagen synthesis. The reaction might produce damage to cells but then the extent of fibrogenesis would match the degree of damage. The time course of the auto-immune diseases also suggests that fibrosis follows on after the active phase of immunological damage - lymphocytes are seen around the target cells and not around fibroblasts. It seems to me that these groups working with blood cells may have stumbled upon a real phenomenon that has more to do with macrophages than lymphocytes.

A simple preparation of disrupted human blood buffy coat leukocytes has been termed "connective tissue activating peptide" by Castor (1971). This material enhances hyaluronate formation but depresses collagen synthesis. Little further information of any clear significance has emerged and this material remains uncharacterised.

The experiments with macrophages and fibrin have already produced some convincing evidence for the production of collagen stimulating factors. This model may prove to be of greater relevance to the broad range of chronic inflammatory pathology leading to fibrosis than any of the previous work reviewed. The pattern obtained is very similar if not identical to that obtained from damaged tissue. Having obtained a positive result it should now be possible to explore the

effects of, for example, macrophages cultured alone, fibrin alone, medium alone and sonicated as against intact macrophages. The use of Amicon filters would probably speed up this work and allow quantitation. Such quantitative methodology would allow optimal conditions to be defined.

Fig 7:1 The effect of column fractions from the supernatant of macrophages incubated with fibrin on collagen chain synthesis in replicate fibroblast cultures.

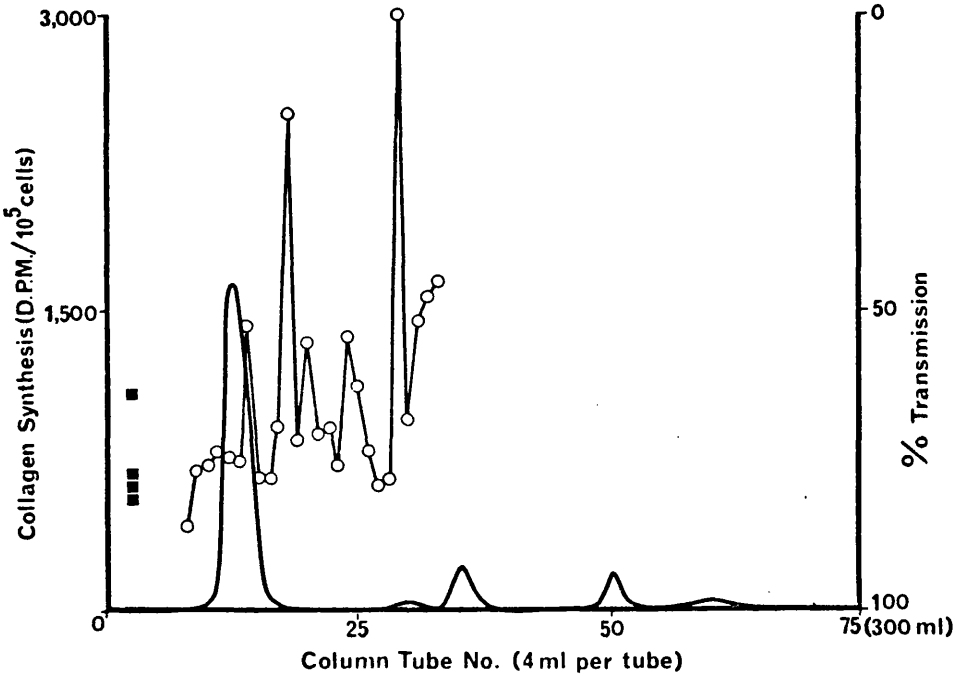


Fig 7:2 The effect of column fractions from medium alone, as a control experiment, on collagen synthesis in fibroblast cultures.

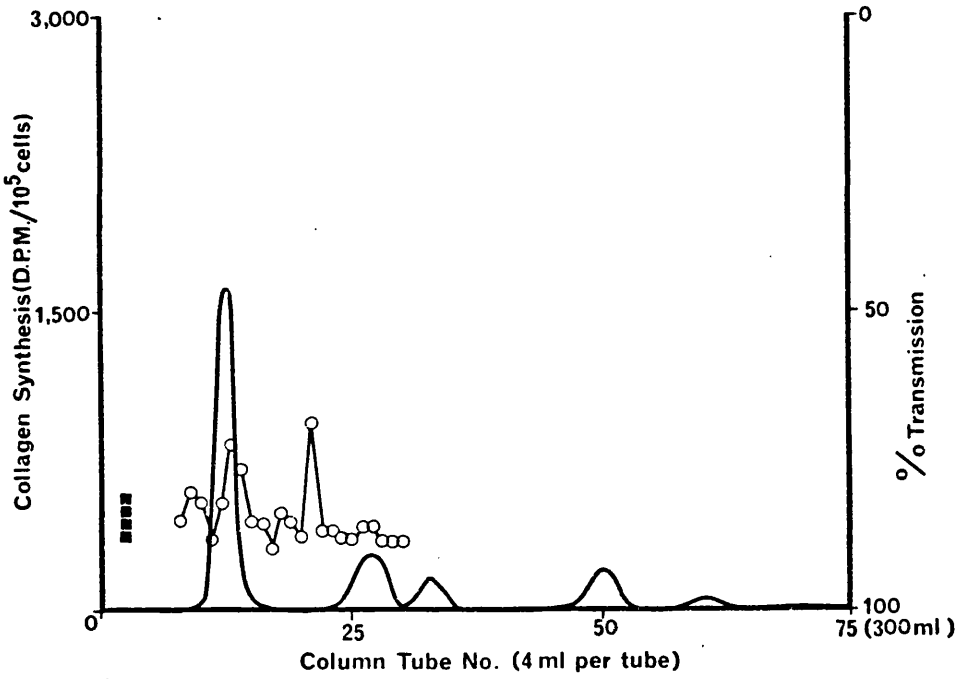
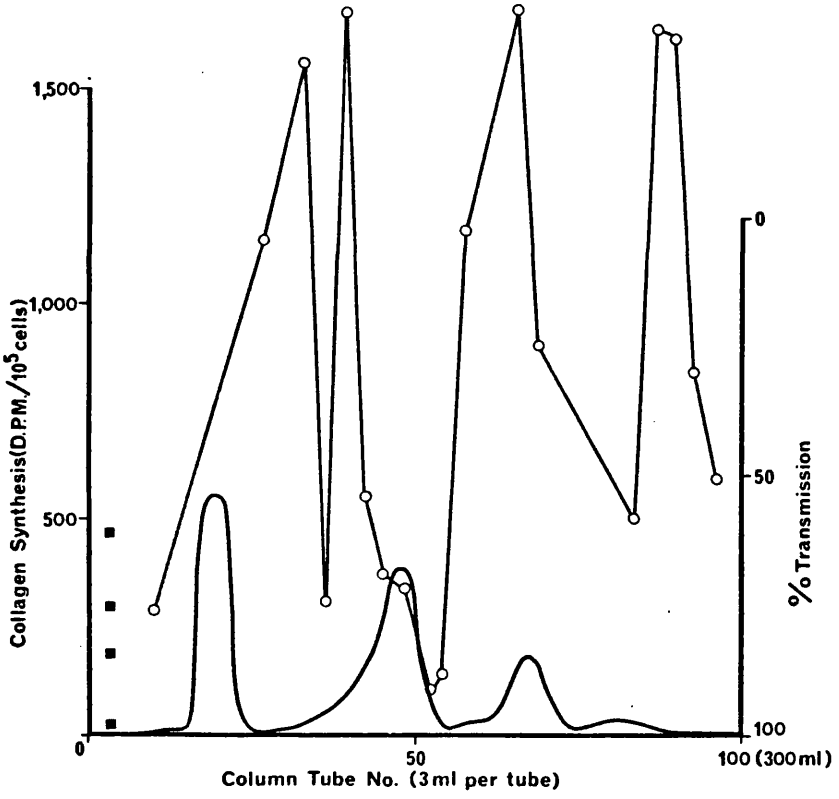


Fig 7:3 The effect of column fractions from a larger volume of supernatant (c.f. Fig 7:1) of macrophages incubated with fibrin on collagen synthesis in fibroblast cultures.



Chapter 8

Final Discussion

The major hypothesis that has emerged from the present work is that the enhanced collagen synthesis characteristic of the chronic inflammatory response is controlled by macrophage, and possibly endothelial, enzymatic degradation of necrotic tissue and fibrin into small peptides with collagen stimulating activity. The development of this hypothesis will now be traced.

The large numbers of macrophages distributed throughout most normal tissues was emphasised by the use of antimacrophage antiserum for immunohistochemistry. There is obviously no fibrosis around macrophages in these normal situations. This cannot be attributed merely to inactivity as these cells are known to be functional as in the Kupffer cell system of the liver. If macrophages influence fibrogenesis at sites of damage, then their behaviour must be altered in response to elements present only in such an environment.

In a chronic inflammatory lesion, which constituent could provide an effective control mechanism for fibrogenesis ? This constituent would require to be present and persistent during the phase of fibrogenesis and absent normally : transfer to another site should induce fibrogenesis ; the effect should be reproducible in vitro with cultured fibroblasts ; the effect should be abolished by specific antibody or other antagonist. This set of requirements begins to resemble Koch's postulates.

A number of these requirements have now been met for collagen stimulating factors but what is being considered here is the overall control mechanism which gives rise to these factors. In the work on CCl_4 liver injury it was found that interference with macrophage function in turn interfered with fibrogenesis. Further than this, the time-course of this macrophage activity in this, as in other situations, is evidently dictated by the time taken to dispose of debris. Therefore it is proposed that the stimulus to fibrogenesis is created from the degradation of necrotic debris and persistent constituents of the inflammatory exudate.

It is evident that this line of thought has occurred to others. "In acute injury, the principal proliferating cells are the sinusoidal macrophages or Kupffer cells which display excess engulfed PAS-positive material - both by light and electron microscopy this resembles components found in hepatocytes. This suggests that particulate liver cell breakdown products may be the stimulus for cell proliferation of mesenchymal cells" (Rubin and Popper, 1967). The stimulus for proliferation may differ from that for collagen synthesis but its control and even its source may be similar.

It was previously argued that in many inflammatory situations leading to fibrosis, fibrin, being the most persistent constituent of the inflammatory exudate, might substitute for necrotic debris as the substrate for collagen stimulating factor production by enzymatic degradation. The source of such enzyme activity would generally be macrophages but in certain situations such as in experimental hypertension and possibly in the capillary buds of granulation tissue, endothelial cells may also oblige. This proposal differs from previous work on silica, for example, in which the implicit assumption is that macrophages respond by secretion of a substance which directly stimulates collagen synthesis.

This type of notion has a respectable antecedent. "At the same time growth-activating polypeptides may be manufactured by the leukocytic ferments from cell debris and coagulated fibrin lying on the surface of the wound ". (Carrel, 1930).

It is possible that fibroblasts might be stimulated by peptides of rather non-specific type derived from breakdown of both fibrin and cell debris - rather in the way in which chemotaxis of leukocytes is stimulated by a wide variety of small peptides. Perhaps those experiments of Nature, the congenital deficiencies of fibrinogen and fibrin stabilizing factor which affect wound healing, provide the best evidence available so far, that fibrin plays a crucial role.

Accordingly, there is a convergence of evidence pointing to the central role of fibrin degradation in the control of collagen synthesis in chronic inflammation. How might this hypothesis be tested ? Purification and chemical characterisation of collagen stimulating factor from tissue seems so laborious as to be impracticable. The evidence from the use of filtratesof stimulating factors indicates that active material exists of molecular weight between 10,000 and 50,000 daltons. This is likely to be antigenic and might cross-react with anti-fibrin antiserum. The effect might be abolished by prior incubation with such an antiserum. Another approach would be to incubate fibrin with macrophages in diffusion chambers implanted in animals. Fibrosis might develop around the implants as previously demonstrated for silica.

To control the process of fibrogenesis is the aim of many who wish to suppress the effects of a wide variety of disease processes. Attempts to achieve this have generally been aimed at interference with the collagen biosynthetic pathway. From this thesis, two further approaches are apparent - control of the activity of macrophages, and control of fibrin degradation.

Appendix No. 1

Immunoperoxidase method for light microscopy :-
(adapted from Mason et al, 1969, and Mason and Taylor, 1975)

1) Tissues are fixed in formol saline and processed to paraffin sections in the conventional way.

2) Sections taken to water.

3) Endogenous peroxidase is blocked by immersion of sections in methanol (400 ml) containing concentrated H Cl (0.8 ml) and hydrogen peroxide (100 volumes, 2 ml) for 30 minutes.

4) Rinse in water then in tris-saline (0.05M tris buffer pH 7.6 (100 ml) plus 0.85% sodium chloride 900 ml).

5) Normal swine serum (N.S.S.) diluted 1:5 with 0.05M tris buffer, pH 7.6 is applied to sections for 15 minutes. The swine serum is not rinsed off.

6) After pouring off excess N.S.S., the specific antibody at optimum dilution in tris buffer (or corresponding absorbed serum or normal rabbit serum as appropriate) is applied for 30 minutes. One drop of N.S.S. is previously admixed with each ml of diluted antibody.

7) Wash in tris-saline for 15 minutes.

8) Swine anti-rabbit serum (DAKO-Immunoglobulins, Copenhagen) 1:40 plus 1 drop per ml of N.S.S. is applied for 30 minutes.

9) Wash in tris-saline for 15 minutes.

10) Peroxidase antiperoxidase conjugate (DAKO) 1:100 plus 1 drop per ml of N.S.S. is applied for 15 minutes.

11) Wash in tris-saline for 15 minutes.

12) Sections are immersed in 0.03% 3, 3' diaminobenzidine tetrahydrochloride (Sigma) in tris buffer plus 1 drop of 100 volumes hydrogen peroxide for about 10 minutes with intermittent checking of intensity of staining under the microscope.

13) Rinse in tris-saline and then water.

14) Counterstain lightly with 1% methyl green or haematoxylin as appropriate.

15) Sections are dehydrated, cleaned and mounted in H.S.R.

Appendix No. 2

Immunoperoxidase method for electron microscopy of mouse liver (adapted from Wilson-Kimball and Nakane, 1978).

Mice were killed by cervical dislocation and the abdomen opened. The same fixative was used as for light microscopy. The liver was immediately injected with several ml 0.12 molar phosphate buffered formalin at $4^{o}C$ causing an area of swelling and blanching. Tissue was then excised adjacent to the needle track and small blocks, not more than 1 cubic mm, trimmed and fixed overnight in the same fixative at $4^{o}C$.

The following day these blocks were transferred to 0.05 molar sodium phosphate buffer at pH 7.2 to which were added 10% sucrose and 4×10^{-5} molar digitonin (Sigma). Following a further 24 hours in the solution at $4^{o}C$ the tissues were passed through buffered sucrose solution 15%, 20% and 20% + 10% glycerol, 4 hours in each. They were then transferred to small aluminium foil boats and frozen quickly by placing these over crushed carbon dioxide snow. They were transferred without thawing to a cryostat where 12 - 15 micron sections were cut.

Sections were transferred without delay to sucrose 10% in phosphate buffer (PBS) at pH 7.2 and at $4^{o}C$. They were then passed through the following solutions :

1. PBS two changes each.15 minutes.
2. Endogeneous peroxidase suppression solution as used for the light microscope preparations. 30 minutes.
3. PBS. 15 minutes.
4. Normal swine serum 1 part PBS 4 parts. 15 minutes.
5. Antimacrophage serum diluted 40 times with PBS and with normal swine serum 1 drop per ml. 30 minutes.

6. PBS . 15 minutes.

7. Swine antirabbit serum diluted 40 times with PBS. 30 minutes.

8. PBS. 15 minutes.

9. PAP complex dilutes 100 times in PBS. 15 minutes.

10. PBS. 15 minutes.

11. Diaminobenzidine 3 mg in 100 ml 0.05 m tris HCl buffer pH 7.6 + 2 drops of fresh hydrogen peroxide. 30 minutes.

12. PBS overnight.

13. Osmium tetroxide 2% in phosphate buffer without sucrose. 1 hour.

14. Phosphate buffered with saline 3 changes each of 10 minutes.

15. Increasing grades of ethanol from 25% to 100% each for 10 minutes and with constant agitation and followed by 3 changes of 100% ethanol again for 10 minutes and with constant agitation.

16. Propylene oxide 2 changes each of 10 minutes.

17. Equal parts propylene oxide and Emix for 30 minutes at 37^{o}C.

18. Pure Emix resin 2 changes each of 40 minutes at 37^{o}C.

19. The tissues are finally blocked out in fresh resin in gelatine capsules and retained at 16^{o}C overnight.

Appendix No. 3

Method for assay of collagen prolyl hydroxylase activity of cells and tissue
(Hutton et al, 1966)

The enzyme collagen prolyl hydroxylase exhibits a remarkable degree of substrate specificity which permits measurement of its activity amongst the multifarious constituents of homogenised cells or tissues. Hydroxylation involves the direct displacement of the hydrogen atom on carbon 4 of proline. A suitable substrate of unhydroxylated collagen containing 3, 4 tritiated proline will release these hydrogen atoms in the form of tritiated water in proportion to enzyme activity. This tritiated water is then separated from residual substrate by vacuum distillation and measured by scintillation counting. A substrate blank is included to assess tritium dissociated on storage and the value subtracted from all other results.

1) With L929 fibroblast tissue cultures, the flasks are emptied of medium, and the cells rinsed twice with approximately 1 ml of saline. The cells are scraped off with a plastic "policeman" into an accurate 1 ml of saline and counted, then transferred to tubes and centrifuged at 700 g for 5 minutes.

2) The cells are resuspended at the rate of 1 million cells per ml in 0.25 M sucrose at 4^{o}C containing 10^{-5} M ethylenediamine tetraacetic acid, 10^{-3} M dithiothreitol, 0.05 M tris-HCl pH 7.2, and 0.1% Triton X-100. The cells were disrupted by sonication for 10 seconds and the tubes kept on ice.

3) Tissue for assay is weighed and homogenised in the same chilled buffer in proportions previously determined.

4) An aliquot of each sample, usually 50 µl, is added to 0.2 ml of water 0.7 ml of Mix and 50 µl of substrate, making a final reaction volume of 1 ml.

5) The tubes are incubated at 30°C for 30 minutes. The enzyme is rapidly denatured at 37°C possibly by proteases in the homogenate. The Mix is made up in the following proportions : 0.1 ml of 0.5 M tris-HCl pH 7.2 ; 0.1 ml of 10^{-2} ferrous ammonium sulphate ; 0.1 ml of 50 mM sodium ascorbate ; 0.2 ml of 1% denatured bovine serum albumin ; 0.02 ml of catalase ; 0.07 ml of water ; 0.1 ml of α-keto-glutaric acid ; 0.01 ml of dithiothreitol. (All ingredients from Sigma).

6) The reaction is stopped by adding 0.1 ml of 50% trichloroacetic acid. The material may be stored frozen at -20°C for later completion.

7) The tube contents are transferred to distillation tubes. These are placed in water just below boiling point and the water content transferred by vacuum distillation to tubes sitting in a freezing mixture of carbon dioxide snow and alcohol.

8) The distillates are melted and 0.8 ml of each tube content is transferred to 10 ml of Aqualuma (L.K.B.) for scintillation counting.

Additional points :

Substrate is composed of underhydroxylated collagen chains prepared in batches extracted from 10 dozen 7 day old chick embryos in the presence of 3,4 ^{3}H proline and also α, α'-dipyridyl. This is an iron chelator which blocks the intrinsic prolylhydroxylase activity while permitting collagen chain synthesis. Iron is an essential cofactor as is α-ketoglutarate. The method is as described by Hutton et al (1966).

In order to determine the optimum amount of substrate for use in subsequent assays, standard curve experiments are performed and an example is illustrated. The aim is to finally use an aliquot of substrate which contains about 4,000 to 5,000 total releasable CPM. For a particular tissue, the aliquot of normal tissue must release about 200 to 400 CPM for accuracy of counting and to permit determination of up to 6 fold increases within the linear range of the assay whilst still allowing detection of possible low activities. A typical linearity graph is shown for L 929 cells. Such preliminary experiments require to be done for every new batch of substrate and also for every batch of animal tissue since the intrinsic enzyme activity declines with age.

Precision of the Assay :-

The within-assay variation was estimated by assay of 25 replicates using pooled L 929 fibroblast sonicates. An aliquot of 25 µl of cells at 5 million per ml was chosen as this gave approximately 1,500 CPM which is about the middle of the linear portion of the enzyme activity curve. The precision of the assay was found to be 8.8% (coefficient of variation = Standard Deviation ÷ Mean X 100).

Fig A:1 An example of the testing of a new batch of substrate for the prolyl hydroxylase assay. Increasing aliquots of enzyme as cell sonicate are added to constant substrate to demonstrate the linear range of release of counts.

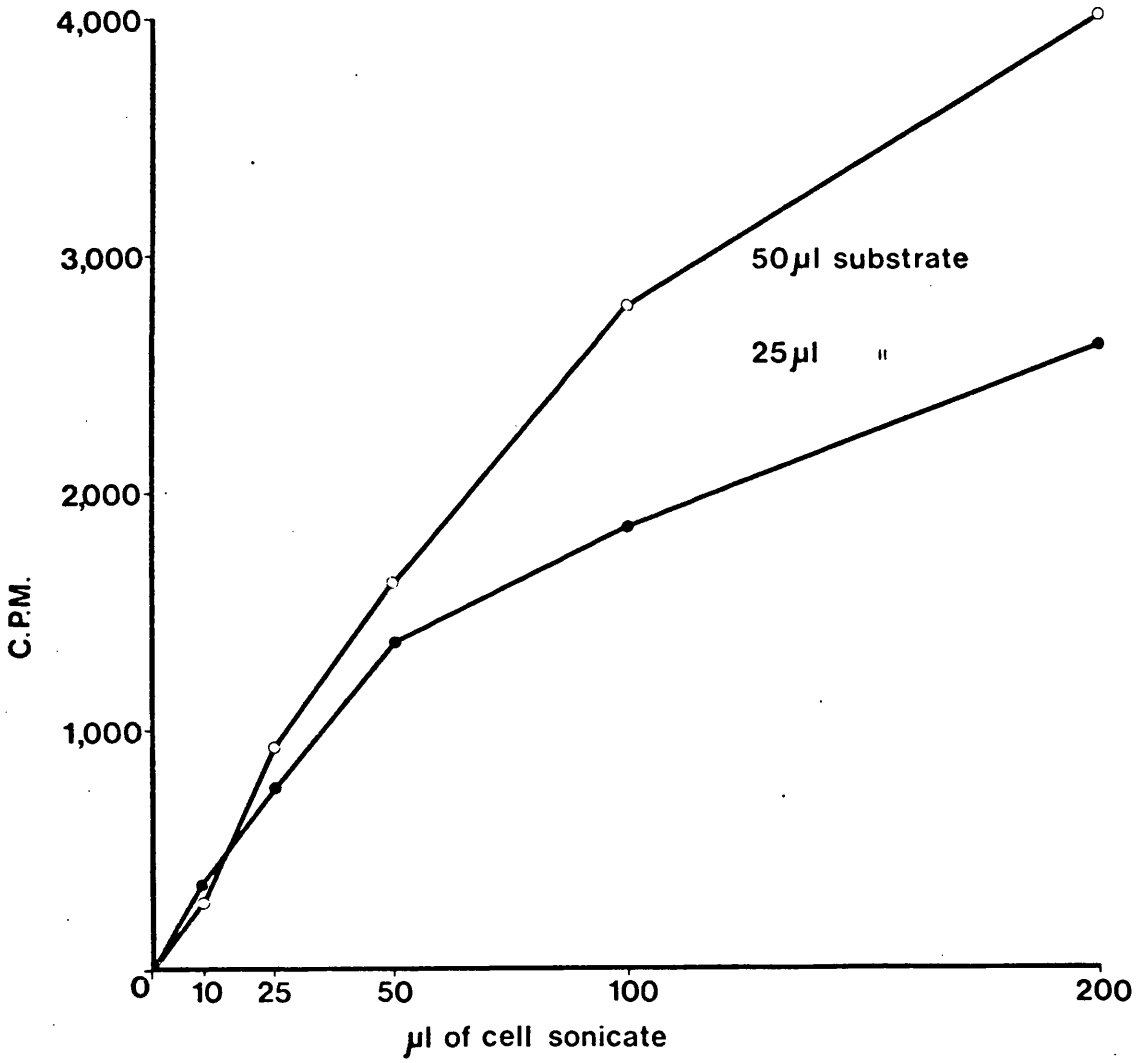
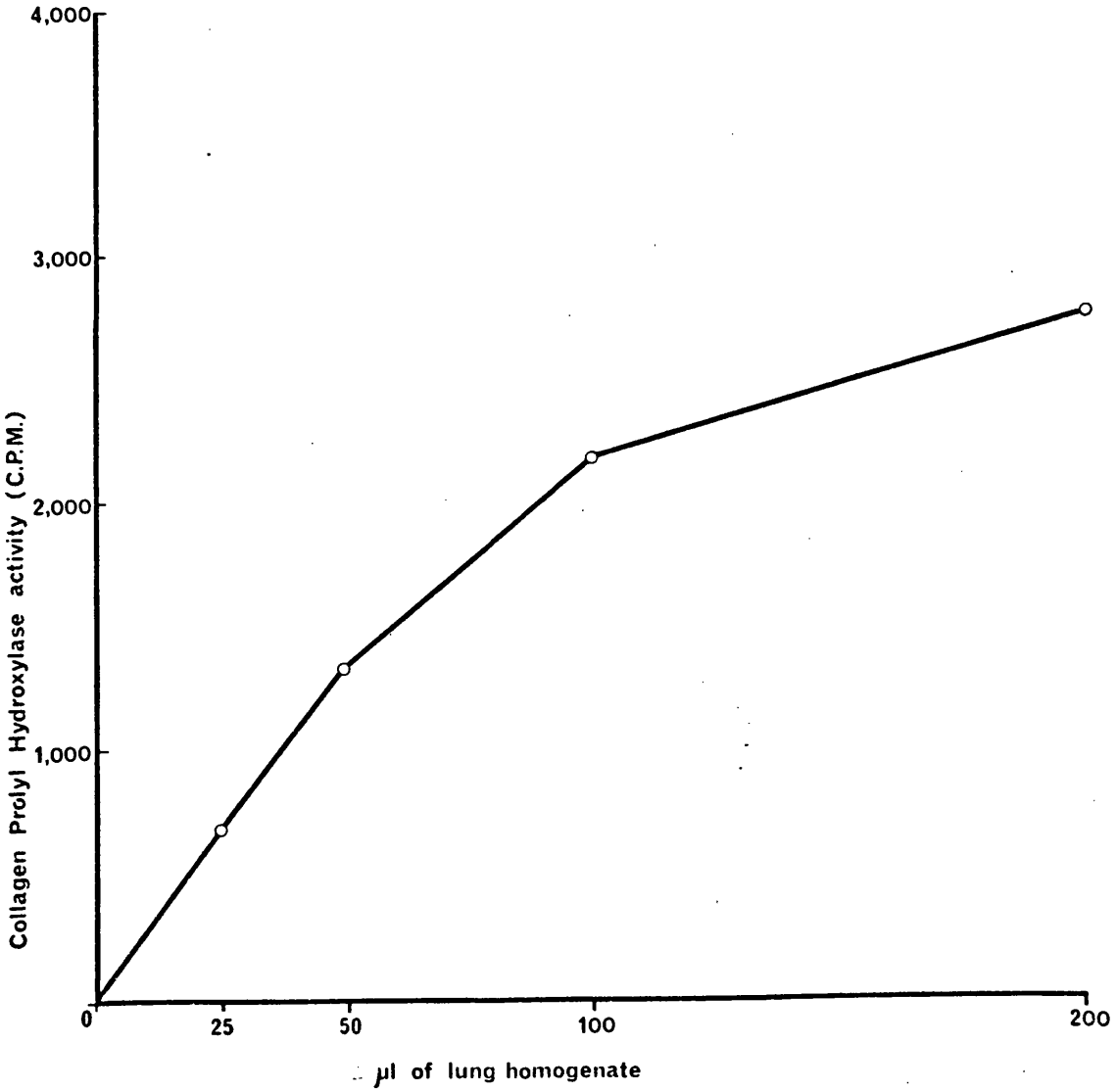


Fig A:2 An example of the preliminary testing of a sample from a batch of tissue to establish the optimum aliquot of tissue homogenate for prolyl hydroxylase assay. On the basis of this assay, 25 µl of lung homogenate and double the aliquot of substrate would subsequently be used.



Appendix No. 4

Method for collagen synthesis assay for L929 fibroblasts (adapted from Peterkofsky and Diegelmann, 1971).

The rationale for this method is that purified bacterial collagenase will release radioactive proline labelled collagen peptides exclusively from a mixture of cell or tissue proteins. A satisfactory preparation of collagenase must not release labelled tryptophan peptides from proteinaceous material as it is known that this amino acid is not present in collagen. The method is accepted as the best one there is for estimating collagen synthesis in vivo and in vitro. It is nevertheless intrinsically far less sensitive and accurate than that for collagen prolyl hydroxylase and involves considerable technical skill for the many stages.

1) Following exposure of fibroblast cultures to experimental material usually for 3 hours, each flask is pulsed with 10 µl (10 µCi) of L-(5-^{3}H) Proline (Amersham). (Labelled Tryptophan may be used alternatively or in addition). The hydrogen atom on carbon 5 of proline is not displaced by hydroxylation.

2) Incubate flasks for a further hour at 37.4^{o}C.

3) The medium is discarded and the flasks each rinsed twice with approximately 1 ml isotonic saline and drained totally.

4) An accurate 1 ml of saline is added to each flask and the cells scraped off with a plastic "policeman".

5) The cells are transferred to 5 ml disposable plastic tubes and counted.

6) The cells are pelleted at 700 g for 5 minutes.

7) The supernatant is discarded and the cells resuspended in 0.5 M Tris-HCl pH 7.6 at a concentration of 1 million cells per ml. The assay can be halted and the material stored at -20°C for later completion.

8) The cells are disrupted by sonication for 10 seconds.

9) Aliquots of 10 µl of sonicates can be added to 10 ml Aqualuma scintillant (L.K.B.) and counted to assess total proline uptake. Aliquots of 100 µl may also be deducted and stored frozen for subsequent collagen prolyl hydroxylase assay.

10) Each tube is incubated with 20 µl of ribonuclease (Sigma) (1 mg per ml in tris buffer) for 10 minutes at 37°C. This slightly improves the yield of total counts by release of collagen chains still bound to ribosomes.

11) Protein is precipitated by adding an equal volume of 20% trichloracetic acid (T.C.A.) and standing on ice for 5 minutes or at 4°C for 10 minutes to allow an opalescent precipitate to form.

12) The protein is pelleted at 1,000 g for 5 minutes.

13) The supernatant is discarded thus removing free isotope.

14) The material is resuspended in 10% T.C.A., 0.5 ml per tube and precipitated and washed a further 2 times with 0.5 ml 5% T.C.A.

15) The final precipitate is redissolved in 0.2 M NaOH using the original tube volumes. This material is used as the substrate containing labelled collagen for digestion by collagenase. The tubes are incubated for 5 minutes at 37°C and spun at 1,000 g and this time the supernatant is kept for assay.

16) The assay is performed by adding two aliquots of 0.2 ml substrate from each tube to a fresh pair of tubes. To each is added 0.05 ml water, 0.15 ml of mix, and 0.1 ml of 0.16 M HCl to neutralise the NaOH. 10 µl of collagenase is added to alternate tubes. The total reaction volume is 1 ml. Thus the cell material from each flask is incubated with and without collagenase to allow eventual subtraction of non-specific counts from collagenase-released counts.

17) The mix is composed of the following proportions :- 0.1 ml of 1.2 M Hepes (N-2-hydroxyethylpiperazine) ; 0.05 ml of 0.25 M N.E.M. (N-ethylmaleimide, inhibits non-specific proteases) ; and 0.005 ml of 0.05 M Ca Cl_2.

18) The tubes are incubated at 37°C for 30 minutes on a shaking waterbath.

19) 0.1 ml of 0.5% albumin in water is added to each tube in order to help bring down the precipirate of protein formed at the next stage.

20) The reaction is stopped by adding an equal volume (0.6 ml) of 10% T.C.A. : 0.5% tannic acid. The tubes are stood on ice for 10 minutes to allow the precipitate to form.

21) The tubes are centrifuged at 1,000 g for 10 minutes to form a firm pellet.

22) The supernatant is transferred to 10 ml of Lumagel scintillant (L.K.B.) for counting.

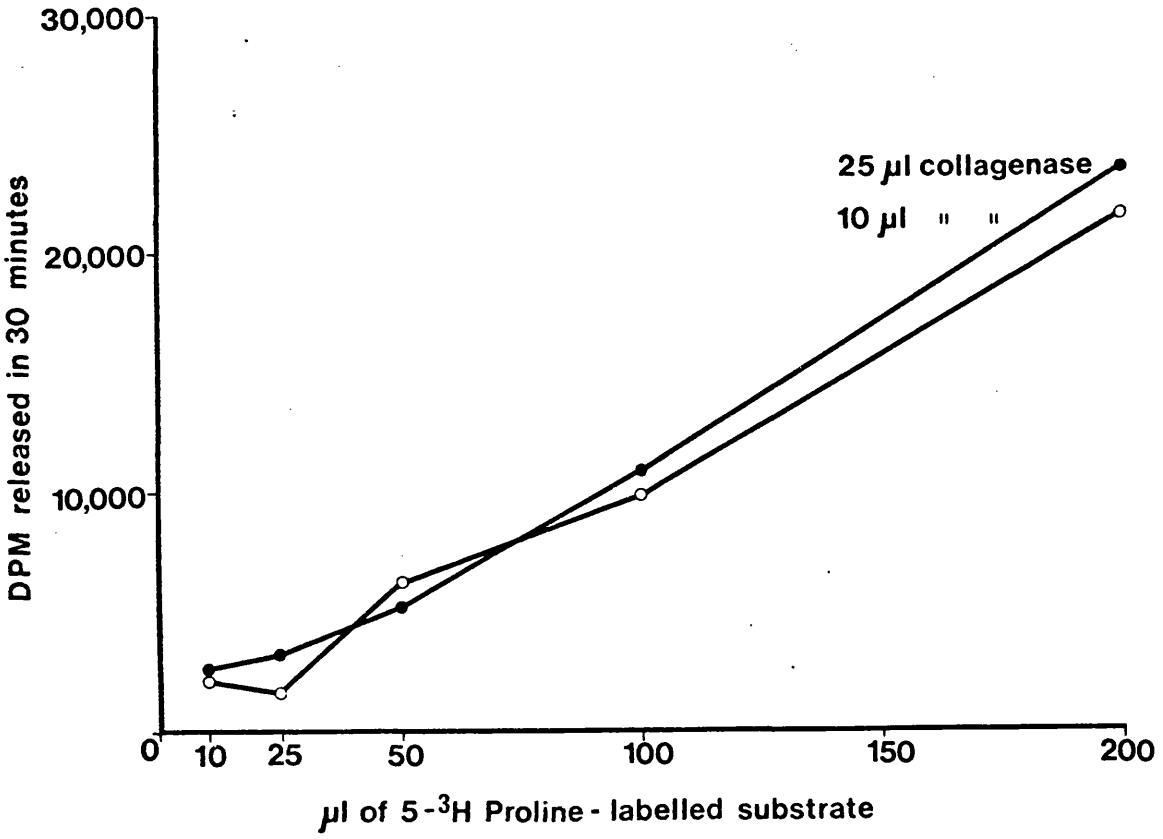
Additional points :-

Clostridial collagenase (Sigma Type 3) contains unwanted non-specific protease activity and requires further purification. This is achieved by gel filtration (molecular sieve chromatography) on Sephadex G200 (Pharmacia). The fractions are tested both for protease activity as previously described, and also non-specific protease activity (in spite of N. E. M.). Fractions, usually most of the first peak of the protein elution profile, containing exclusively collagenolytic activity are pooled and concentrated if necessary. From a standard linearity graph (fig.), a saturating amount of collagenase is determined suitable for assay purposes, that is, able to release at least 10,000 CPM in 30 minutes.

The final stage of the method involves precipitation by trichloracetic and tannic acids and creates an awkward combination of quenching problems. Variable colour and chemical quenching plus unacceptable chemiluminescence are produced with many scintillants rendering conversion to DPM impossible. While CPM is adequate for day to day experimental work, the expression of results as DPM in published data is preferable as these values are independent of the varying efficiencies of counts or scintillants. Fortunately Lumagel (LKB), one of the new generation of scintillants, proved resistant to the acids added. The automatic quench correction and chemiluminescence monitoring facilities of the LKB Rack-Beta scintillation counter used latterly for this work permitted satisfactory quench curves to be obtained for both this assay and the prolyl hydroxylase assay. An example is shown (fig.) of part of the print-out of a quench curve obtained by the spline-curve fitting function of the counter computing quench data resulting from the LKB "Hat-trick" method of progressive quenching. This latter method uses a simple device to continuously add carbon tetrachloride as quenching agent to a standard pellet of isotope of known DPM in the presence of all other ingredients used experimentally. This is ideal for solving the quenching problems described.

Sometimes the collagen synthesis assay gives "negative" values on subtraction of the counts with and without addition of collagenase. These often, but by no means exclusively, occur where low values might be expected. The assay is itself much more erratic than that for prolyl hydroxylase and inconsistencies in the final precipitation step are a further source of technical error. Too many assumptions would have to be made to include these values even as "zero" results and it seems more acceptable to exclude them altogether even when this vitiates an experiment.

Fig A:3 An example of the testing of a new batch of collagenase to establish a linear range suitable for the collagen synthesis assay. Here 10 µl appears more than adequate.



```
MODE 1 SELECTED
ISOTOPE IPM ->172137
METHOD 1,2,3 ->2
START RATIO  ->3.2
AUTO PLOT AND STORE (R) ->R
NOW LOAD SAMPLE (R) ->R

      CPM1   EFF1%  RATIO

   86006.0   49.97  3.145
   85095.0   49.44  3.083
   83651.0   48.60  3.002
   82164.0   47.73  2.906
   80145.0   46.56  2.713
   77870.0   45.24  2.622
   75139.0   43.65  2.506
   73259.0   42.56  2.325
   71392.0   41.48  2.213
   69486.0   40.37  2.124

BUSY CALCULATING
ISOTOPE 1, WINDOW 1
```

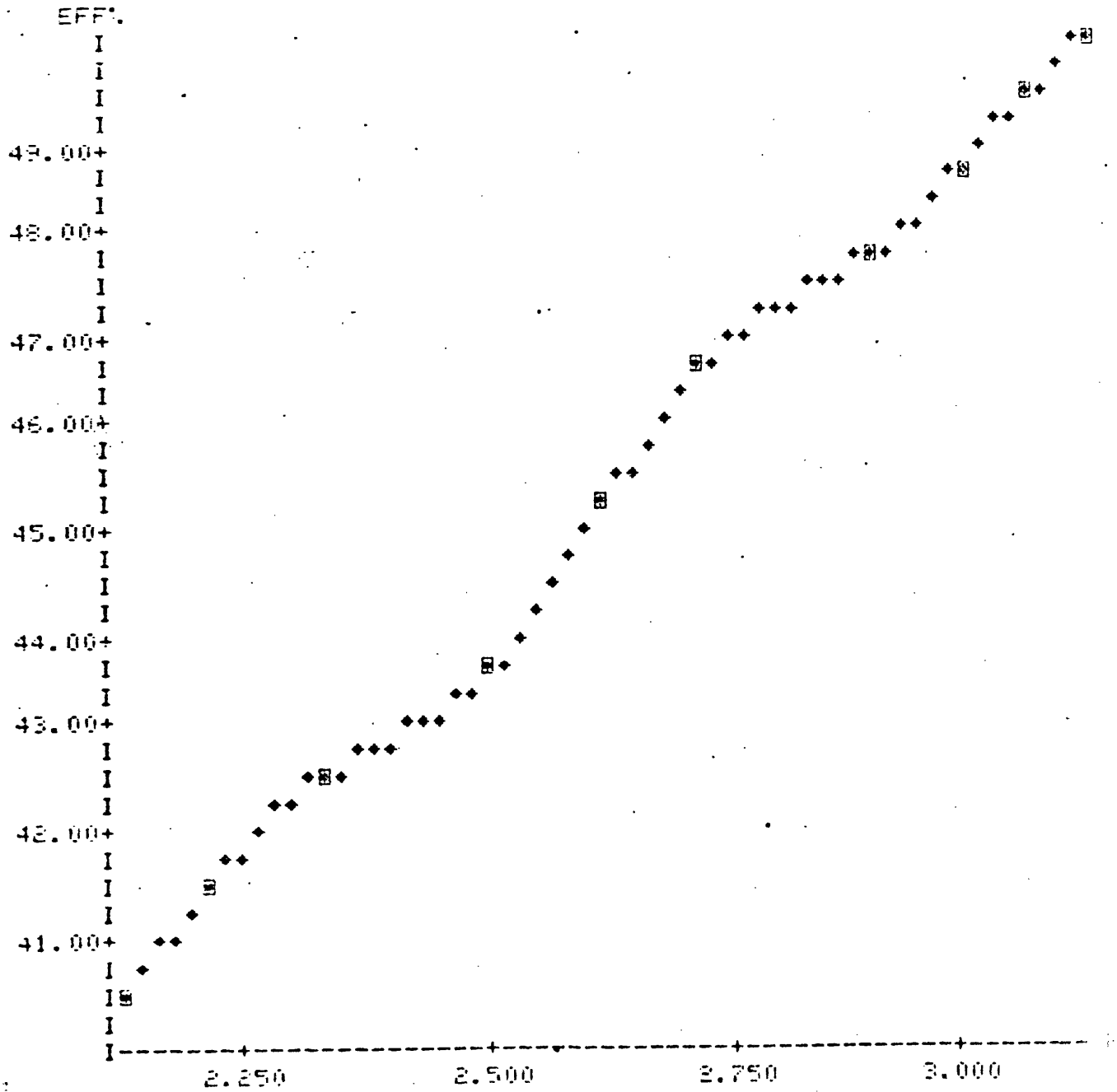


Fig A:4 Part of the printout from the LKB Rack-Beta automatic quench curve calibration.

Appendix No. 5

Method for collagen synthesis assay as applied to experimental animal tissue.

This assay is a modification of the previous method used for L 929 fibroblasts.

1) The material - liver or lung for example - will have come from mice or rats previously pulsed with L - (5-^{3}H)-proline and may be stored at -20^{o}C. The presence of this label does not interfere with assay for collagen prolyl hydroxylase which may also be performed on the same tissue.

2) Approximately 1 g of each sample is weighed out and 3 volumes of 0.05 M tris-HCl buffer pH 7.6 is added.

3) The tissue is homogenised using a Polytron micro-homogeniser at half speed until no particulate material remains. The homogeniser is rinsed with water and dried with paper tissue between samples.

4) Each sample is completely transferred into 0.5 cm diameter Visking dialysis tubing previously softened in warm water. The sample must be carefully knotted tightly into the minimum tubing volume under pressure to avoid water uptake during dialysis.

5) The dialysis sacs are numbered and placed in 2 litre flasks filled with water, at about 5 sacs per flask.

6) The flasks are set on magnetic stirrers in a cold-room at 4^{o}C for 3 days with at least one change of water. This dialyses out free isotope.

7) The samples are transferred to 5 ml plastic tubes. 10 µl of each is added to 10 ml Aqualuma to assess total proline incorporation (as compared with uptake).

8) Each sample is vortexed and split into pairs of aliquots for the collagenase digestion procedure as previously described (Appendix No. 4).

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