THE PROTEIN BALANCE OF NORMAL, LYMPHOEDEMATOUS AND INJURED TISSUES AND THE ACTION OF A BENZOPYRONE, COUMARIN

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SUMMARY

The protein balance of the blood-tissue-lymph system was investigated under normal conditions and during various forms of oedema, in an attempt to obtain a clearer picture of the changes which occur during inflammation. The ability of the system to resolve the oedema and to return to normal was studied, together with the effects of the benzopyrone, coumarin.

Various injuries increase vascular permeability and result in local oedema in the tissues. The increased transport of macromolecules from the blood to the tissues has been widely investigated, particularly as the result of moderate histamine injury. However, the possibility of backflux from the tissues to the blood has not previously been considered. A compartmental model of all available pathways for protein within a blood-tissue-lymph system (using Guyton's capsules) was developed to allow all the rates of flux to be calculated. The effect of histamine injury, and hence acute inflammation (local oedema), was also studied. Protein flux against its concentration gradient was detected under normal conditions and the rate was increased by histamine injury. Much of this flux was across close and open junctions, although it seems likely that vesicles also contributed. More protein returned to the blood directly from the tissues than passed to the

lymphatics under both normal and histamine-injured conditions. As expected histamine also caused significant increases in the rates of protein flux from the blood to the tissues and from the tissues to the lymphatics. In both normal and histamine-injured conditions the net protein flux between the blood and the tissues was directed from the blood.

Local oedema was produced by injecting various substances into the subcutaneous tissues. The rates at which these oedemas were resolved were calculated and compared. The resolution of high-protein oedema was biphasic. The first phase was more rapid and probably dependent on optimal lymphatic function. The second phase was slower and continued until the tissues returned to normal. It was probably dependent on proteolysis and began when the more central lymphatic collectors became filled, causing local lymph flow to be progressively reduced. The benzopyrone, coumarin, increases the proteolysis of accumulated abnormal protein. It also significantly reduces high-protein oedemas. One purpose of this study was to link these effects of the drug directly by showing that the products of proteolysis, such as amino acids, are removed from the site of oedema more rapidly than the proteins themselves. This was proven by the fact that oedema caused by the injection of amino acids was resolved more rapidly than that caused by

plasma. The effect of coumarin on the resolution of these oedemas was investigated. It had no effect in the non-protein oedemas, nor did it affect the resolution of protein-induced oedema in the first four hours. It did increase the rate of resolution in the second (or proteolysis) phase of plasma removal. This further confirms that coumarin enhances proteolysis and reduces high-protein oedema.

During the course of this study, it became necessary to obtain more detailed information regarding an often-used experimental procedure for producing lymphostasis. Of particular interest was the time course of the development of lymphoedema. This information had not been obtained previously. Therefore, a complete study of this experimental procedure was undertaken. Lymphostasis led to four separate, quite distinct phases in the development of lymphoedema. An initial rapid rate of increase in limb volume (approximately 1.5 percent/hour) followed by a plateau and a slight reduction in the swelling. Then followed a longer period at a slower rate of swelling (0.32-0.38 percent/hour) until a maximum of a 46-48 percent increase in limb volume was reached. The limb volume then returned to normal (0.34-0.45 percent/hour). The lengths of the various stages appear to be dependent on the tightness of the ligatures. Lymphostasis reduced the rate of resolution of

high-protein oedema by approximately 40 percent (in the first four hours) indicating that the lymphatics do play a very vital part in those initial stages of oedema resolution. The macrophages are very important in the resolution of high-protein oedema only after four hours but their destruction did not reduce the effectiveness of coumarin. It appears that coumarin is able to enhance proteolysis by other cells.

Willoughby and Di Rosa (1970) hypothesized that altered proteins in the tissues were a cause of chronic inflammation. It has also been suggested that lymphoedema is a form of inflammation. Therefore, it is possible that the accumulation of plasma proteins in the tissues as a result of lymphostasis could be responsible for all the pathological changes found. Repeated doses of plasma were injected into the subcutaneous tissues of immunologically-tolerant rats for up to 64 days and, effectively, "lymphoedema was produced without lymphostasis". Changes typical of chronic inflammation were found in the skin, and to a much greater extent, in the fascia. Coumarin significantly reduced the extent of this inflammatory reaction in the skin but not in the fascia. The accumulation of excess proteins in the tissues did cause chronic inflammation. Thus chronic lymphoedema can be regarded as a form of chronic inflammation. Evidence also indicates that, in the

presence of excess plasma proteins, the benzopyrone, coumarin, is capable of stimulating cells other than macrophages in the skin.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University except where referred to in Section 2. To the best of my knowledge and belief this thesis contains no material previously published except where due reference is made in the text.

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SECTION 1

GENERAL INTRODUCTION

The normal functioning of the blood-tissue-lymph system is complex and dependent on a fine balance in the relationship between the various components of the system. A disturbance or injury to any one of these components will result in imbalance and in local oedema of the tissues. The ability of the system to rectify the problem depends on the response of each component to the accumulation of protein and fluid in the tissues and, of course, on the extent of the injury. An integrated response to mild imbalance can allow the tissues to return to normal. If these homeostatic mechanisms are not sufficient, other forms of intervention, such as therapy, are required. One very effective drug therapy is provided by the benzopyrones, which are capable of enhancing the removal of oedema by a combination of their many varied actions.

1.1 Structure, Function and Permeability of the Blood-Tissue-Lymph System:

Electron microscopic studies have shown the structure of blood capillaries to differ between different

organs and even between different areas of the same organ. These capillaries can be classified into three broad types (Majno, 1965); continuous capillaries with continuous endothelium and basement membranes, fenestrated capillaries with discontinuous endothelium and continuous basement membranes, and sinusoid capillaries with open endothelial junctions and a discontinuous or absent basement membrane. The exact structural details vary even within the same group.

1.1.1 Continuous Capillaries:

The intercellular junctions of continuous capillaries are of varying complexity and occur between the lateral aspects of adjacent endothelial cells. There can be more than one foci of narrowing within a junction (Cotran, 1967). "Close" junctions narrow to about 6 nm for approximately 30 nm from the lumen (Casley-Smith et al., 1975) whilst "tight" junctions narrow to about 1 nm and are impermeable to all molecules, except perhaps the very smallest.

Extensive physiological and electron microscopic studies of the permeability of normal capillary endothelium indicate that water and low molecular weight solutes pass through "close" junctions by diffusion and ultrafiltration (Landis and Pappenheimer, 1963; Karnovsky

and Cotran, 1966; Karnovsky, 1967). The net force controlling water flow is the result of internal tissue hydrostatic pressures and colloidal osmotic pressures (Starling, 1896, 1909; Landis and Pappenheimer, 1963; Yoffey and Courtice, 1970; Zweifach, 1970). Other small molecules are moved mainly by diffusion with convection and molecular sieving becoming more important as molecular diameter increases (Landis and Pappenheimer, 1963; Curry, 1974).

An endothelial cell possesses an extensive system of intracytoplasmic vesicles. In normal endothelium they are uniform in size and are more numerous towards the cell surfaces. They have frequently been shown to transport material, quite slowly, across blood endothelium (Carter, Joyner and Renkin, 1974; Perry and Garlick, 1975), apparently by a random process powered by Brownian motion (Casley-Smith, 1963; Shea and Karnovsky, 1966; Casley-Smith et al., 1975). Vesicular transport could account for the slow leakage of proteins out of the continuous capillaries but not for the much more rapid passage of smaller molecules (Casley-Smith et al., 1975; Casley-Smith, 1976c).

Large vacuoles are occasionally seen within the endothelial cells of normal capillaries (Fawcett, 1962; Mohamed, 1975) and they transport material across the

cells (Casley-Smith and Carter, 1979). Calculations have shown that the basement membrane has a negligible effect on the passage of small molecules (Casley-Smith et al., 1975). The passage of molecules the size of albumin is impeded, temporally dependent on the rate of fluid flow (Casley-Smith, 1976a), which is low for continuous capillaries.

1.1.2 Fenestrated Capillaries:

These differ structurally from continuous capillaries by possessing holes (fenestrae), approximately 50 nm in diameter, through thin areas of the endothelium. Fenestrae are more numerous on the "venous" limbs than on the "arterial" limbs, and whilst many are sealed with a diaphragm, variable proportions are not (Casley-Smith, 1971, 1976c; Casley-Smith, O'Donoghue and Crocker, 1975). Fenestrae are very permeable to proteins (Casley-Smith, 1970, 1976c). It appears that the extravascular tissues, not the endothelium (fenestrae), control the permeability of fenestrated capillaries to small molecules, including water. The other structural components of the fenestrated capillaries probably have permeabilities as described for continuous capillaries.

Large molecules have been shown to pass out of the fenestrae on the arterial limbs and in at those on the

venous limbs (Casley-Smith, 1970). As the fenestrae on the arterial limbs are freely permeable to proteins and fluid, a much greater outflow will occur into the perivascular space, than is possible for continuous capillaries. This will result in the banking up of the larger molecules against the basement membrane, increasing their concentration in the perivascular space to above that of the plasma. A large effective colloidal osmotic pressure can be maintained across pores the size of fenestrae (Casley-Smith and Bolton, 1973a) and this would further increase fluid outflow. It has been hypothesized (Casley-Smith, 1971, 1973, 1975) that this rapid outflow drags proteins with it, against their concentration gradient and in greater concentrations than can diffuse back into the lumen against the fluid flow (protein diffuses relatively slowly). One aim of this project was to investigate the validity of this hypothesized mechanism. The bank up of proteins against the lumenal side of the basement membrane implies a sharp fall in protein concentration across it. Tissue channels have been shown to end at fenestrae (Casley-Smith et al., 1975; Casley-Smith and Vincent, 1978), and it has been suggested that protein concentration gradually rises along them, from the arterial to the venous limbs (Casley-Smith, 1976a), to be just below that in the perivascular space of the venous limbs. It is thought the mechanism hypothesized above also operates here but in the opposite

direction. The protein concentration gradient causes fluid inflow which drags proteins with it.

1.1.3 Interstitial Tissue:

Extensive investigations indicate that the interstitial tissue consists of a random network of channels and that their numbers and dimensions probably vary with the type of tissue and the prevailing conditions (Casley-Smith and Vincent, 1978). These channels have been observed to offer easy routes for proteins and they probably also offer the easiest routes for the filtration and diffusion of small molecules (Casley-Smith, 1976c).

Tissue proteolysis, due to cellular activity, influences the protein transport between the blood and the lymphatics. Macrophages are found throughout the interstitial tissue and are capable of phagocytosing almost any kind of foreign particle. Their numbers increase dramatically during lymphoedema and other forms of inflammation (Kalima, 1970, 1971; Casley-Smith et al., 1977a, 1978; Clodius and Altorfer, 1977; Olszewski, 1977; Casley-Smith, Földi-Börcsök and Földi, 1978). It is well known that they migrate into oedematous areas and it is generally accepted that tissue catabolism of proteins takes place in macrophages (reviewed by Földi and Casley-Smith, 1978). In addition, other cells produce

proteolytic enzymes (Houck and Sharma, 1969) which probably assist in lowering the tissue protein concentration.

The main functions of the lymphatic system are the uptake of large molecules, particles and excess fluid by the small peripheral initial lymphatics, and their transportation by the collecting lymphatics from the periphery to the central lymphatics, and eventually to the venous system.

1.1.4 Initial Lymphatics:

The initial lymphatics are very similar in structure to the venous capillaries but their endothelial cells are connected to the interstitial tissue by many fine, probably non-contractile, fibrils (reviewed by Casley-Smith, 1973, 1977a). These pull the initial lymphatics open during oedema. The initial lymphatics in quiescent regions have similar permeabilities to venous capillaries but, in addition, the interendothelial junctions have a number of open regions, the extent of which depends on the activity of the region. The functioning of the initial lymphatics can be divided into three main phases; a filling phase; an intermediate resting phase and an emptying phase.

The filling phase commences when the total tissue pressure falls, during tissue relaxation (e.g. when an adjacent muscle contracts). During oedema or motion, pulling on the fibrils tends to dilate the vessels, to cause the cells to come apart from each other and to force the junctions open. If there is no oedema or movement, the junctions are opened by the fluid inflow which is caused by a net inwards pressure (see below). This is possible because the junctions have very few of the adhesion devices seen in the junctions of blood capillaries and the cells have a very tenuous basement membrane. Thus the very easily opened junctions act as inlet flap valves, opening during the filling phase and allowing water and proteins to enter the initial lymphatic.

The forces causing the fluid inflow are in dispute (Casley-Smith, 1977a). Several suggestions have been made (reviewed by Földi and Casley-Smith, 1978) and refuted. Normally, the hydrostatic pressure difference in most interstitial tissue is directed away from the initial lymphatic. This would most likely cause fluid to leave the vessels, not to enter them. Active pumping of fluid across the endothelial cells in the vesicles is extremely unlikely, as these are passively moved by Brownian motion. Direct pressure measurements show that the collecting lymphatics do not exert a suction on, and

through, the initial lymphatics. It has been suggested that the fluid inflow is caused by the effective colloidal osmotic pressure of the lymph (Casley-Smith, 1977a) and that it drags proteins with it. This hypothesis depends on a similar mechanism to that suggested for fenestrae (page 13). There is considerable evidence that the lymph in the initial lymphatics has a much higher protein concentration than the fluid in the tissue channels (Casley-Smith and Sims, 1976; Casley-Smith, 1977c, d; Földi and Casley-Smith, 1978). This probably causes fluid, and hence proteins, to enter the initial lymphatics via the open junctions and to dilute the lymph. Muscular contractions or respiratory movements will periodically increase the total tissue pressure and compress the tissues. Then the open regions of the junctions will close, becoming permeable to small molecules only.

When the net pressure across the junctions is zero the intermediate phase occurs; the vessels remain filled, with closed junctions waiting for the next tissue compression to begin the emptying phase. The compression of the vessels will force fluid back into the tissues, concentrating the lymph and simultaneously forcing some of the lymph into the collecting lymphatics (Casley-Smith, 1977a, c).

1.1.5 Collecting Lymphatics:

The number of open junctions decreases towards the more central collecting lymphatics, until the junctions are all close or tight. The thickness of the vessel walls increases. Hence permeability is reduced. The smooth muscle in the walls of the collecting lymphatics contracts intermittently. Combined with varying external tissue pressures and with the aid of the intra-lymphatic valves, the lymph is propelled towards the blood, with each segment contracting according to its load (Casley-Smith, 1977a). In the remote collectors, the smaller intralymphatic forces cause water to be reabsorbed through the vessel walls, hence rediluting the lymph (Casley-Smith, 1977c).

1.2 Oedema:

The inflammatory response to a wide variety of stimuli and injuries is similar. The different aspects of this response may occur together or quite separately, and their relative prominence depends on the type and extent of the injurious stimulus. An initial short-lived arteriolar constriction is usually followed by a sustained dilatation of all small blood vessels in the injured area. Initially blood flow through these vessels is rapid (Aschheim and Zweifach, 1962). The permeability, usually of the post-capillary venules, may be increased.

Oedema is an important part of this inflammatory reaction. Its clinical effects include swelling, pain, loss of function, increased risk of infection and susceptibility to minor traumas due to the poor blood supply, and in some cases, to the high tissue protein concentration. Physiological effects include: increased numbers and dimensions of tissue channels (Casley-Smith, Földi-Börcsök and Földi, 1979; Casley-Smith and Vincent, 1980), increased permeability of the interstitial tissue to all types of molecules (reviewed by Casley-Smith, 1982c), and various effects on the blood vessels. Metabolism and gaseous exchange in the tissues are also affected. The initial lymphatics become dilated, and do not collapse. The fibres act as guy-ropes and many more endothelial junctions are opened (Casley-Smith, 1982b, d). Usually lymph flow increases. If oedema becomes severe the initial lymphatic fibres may separate from the interstitial tissue (Virágh, Papp and Rusznyák, 1971; Leak and Kato, 1972), the initial lymphatics will collapse and lymphostasis will complicate the situation.

Resolution of acute inflammation (i.e. the complete restoration to normal of both structure and function of the damaged area) depends on the nature of the injurious stimulus, the type of tissue injured, and the degree of destruction caused by the damaging agent. This usually occurs after mild infection, physical or chemical

injury of limited duration, and requires the removal of all abnormal material from the tissues of the inflamed area. Some of the fluid and small molecules will pass back into the blood. The possibilty of removal of some protein via this pathway will be investigated in this project. The bulk of the fluid and some of the small molecules and proteins will be removed by the lymphatics. It is hoped that this project will enable the relative effectiveness of these pathways to be quantified. Macrophage numbers increase in inflamed areas after 4 hours (Paz and Spector, 1962; reviewed by Hurley, 1972) and they actively phagocytose red cells, fibrin, cellular debris and foreign particles, including bacteria. They are assisted by fibrinolytic and autolytic enzymes.

1.2.1 Low-Protein Oedema:

Low-protein high-flow oedema is caused by a disturbance in the Starling equilibrium (Földi, 1969; Rusznyák, Földi and Szabó, 1967; Yoffey and Courtice, 1970). Raised blood hydrostatic pressure, lowered plasma colloidal osmotic pressure, or lowered tissue pressure (often caused by increased tissue compliance) can cause excess fluid to leave the capillaries. Since vascular wall permeability is normal, this fluid will have a very low protein concentration. The fluid accumulates in the tissues and oedema develops. The tissue hydrostatic

pressure rises, tissue proteins are diluted, and lymph flow increases (Taylor et al., 1973). These tend to remove the excess. If lymphatic capacity is overwhelmed, any further fluid accumulation could initiate clinical oedema. Raised central venous pressure can cause lymph drainage to be impaired and low-protein low-flow oedema may result.

1.2.2 High-Protein High-Flow Oedema:

Some traumas or diseases increase the permeability of the vascular endothelium, either directly or indirectly via mediators, allowing excessive amounts of protein and fluid to enter the tissues. The lymphatics will function at their maximum and macrophage accumulation and proteolysis will increase to remove this excess. If these combined actions are not sufficient the osmotic action of the accumulated protein will cause even more fluid to enter the tissues and the chronic phase will develop.

The effects of histamine injury are of particular interest because it is often released from damaged tissues and mediates the inflammatory response. Mild doses increase the permeabilty of small veins and venules to protein and fluid, although larger doses may also affect the capillaries (reviewed by: Hurley, 1972; Casley-Smith and Window, 1976; Renkin, 1977; Gabbiani and Majno, 1978;

Casley-Smith, 1980, 1982b, c). The structural changes in the vascular endothelial cells include: a reduction in the numbers and mean diameter of the vesicles, a reduction in mean cell width, and an increased proportion of open junctions. These open to a maximum of about 50 nm, 10 minutes after injury and return to normal by 30 minutes after mild injury and 90 minutes after moderate injury. Their width is approximately equal to that of the fenestrae indicating that the same hypothesized protein flow mechanism may operate. Protein may pass back into the blood vessels against its concentration gradient. Therefore, moderate histamine injury will be used here to investigate this mechanism. Histamine injury also increases the number of free and attached vacuoles which persist after the junctions have closed again (Casley-Smith and Window, 1976). These vacuoles transport material across capillary walls (Alksne, 1959; Casley-Smith and Carter, 1979) and may contribute to the increased permeability to macromolecules.

1.2.3 Chronic Inflammation:

Chronic inflammation is generally considered to be any inflammatory reaction of long duration. It may be the result of an acute response which fails to subside or may develop slowly and progressively over days and weeks without an initial acute phase. The macroscopic and

histological features vary widely with different stimuli but there are certain common features which distinguish it from the acute phase. The most characteristic of these is the accumulation of a variety of cells in the extravascular tissues. Polymorphs, eosinophils, macrophages and their derivatives, function actively to dispose of dead tissue and destroy invading organisms. They may also cause cellular damage themselves. Lymphocytes and plasma cells are active in the immune response and antibody manufacture. Fibroblasts manufacture collagen and are responsible for the increasing fibrosis observed in chronic inflammation. In addition, increased numbers and morphological alterations of the capillaries and initial lymphatics are usually observed. In many types of chronic inflammation the healing and inflammatory processes are intermingled to form a mass composed of inflammatory cells, areas of dead tissue and newly formed scar tissue (Hurley, 1972).

Willoughby and Di Rosa (1970) have hypothesized that the accumulation of plasma proteins in the tissues could mediate the chronic inflammatory response. This seems likely as high-protein oedemas are usually more damaging than low-protein oedemas and they usually persist for a longer period after the initial causative stimulus has subsided. The rates of resolution of low and high-protein oedemas will be compared in this project.

The proteins would attract the accumulation of cells, provide a base for increased fibrosis and create a stagnant environment. The supply of oxygen and nutrients to the tissue cells would be depleted, causing more cellular damage and death. Eventually an ideal environment for infection would result. This hypothesis will also be investigated thoroughly as part of this project.

1.2.4 High-Protein Low-Flow Oedema; Lymphoedema:

Deficiencies in the lymphatic system itself allow protein and fluid to accumulate in the tissues. The collecting lymphatics may be blocked by fibrosis caused by infection or irradiation, the lymph nodes may have been excised and the vessels interrupted; the pumping mechanisms may malfunction; or there may be a congenital lack of lymphatics. The smaller molecules can escape back into the blood, but most of the larger molecules will remain in the tissues and retain the oedema fluid by their osmotic action. The pathogenesis of lymphoedema varies slightly with different causes and regions.

The Acute Phase:

Distal to an obstruction the vessels dilate and the lymph pressure rises. With time, these become so

extreme that the intralymphatic valves become incompetent and the dilatation and increased pressure spread peripherally. Eventually the initial lymphatics also dilate and their interendothelial junctions also become incompetent. Lymph escapes back into the tissues and the tissue fluid and lymph equilibrate. Possibly tissue proteolysis prevents the protein concentration in the interstitial tissue from increasing until it equals the plasma protein concentration. Some protein may return to the blood vessels via random vesicular transport across the endothelium. If there are any open blood vascular junctions, the osmotic action of the plasma proteins may cause some fluid flow into the blood vessels, dragging proteins with it as hypothesized above (page 13).

The Latent Period:

In both clinical and experimental lymphoedema the acute phase is almost always followed by a latent period of variable length. During this period the previously affected area appears to be normal, although the initial cause has not been removed. There is some evidence to suggest that the tissues have not actually returned to normal. Small amounts of oedema fluid are probably still present and fibrosis may be occurring (Piller and Clodius, 1976; Olszewski, 1977; Clodius and Piller, 1978). The cause of this latent period is unknown. Possibly

increased proteolysis by the macrophages, which migrate into the area, reduces the effects of the lymphostasis for this variable period. Alternatively, collateral lymphatics may arise. When this capacity is also overhelmed (sometimes by a very mild trauma such as a scratch) or when increasing fibrosis causes further complications, the chronic phase begins.

The Chronic Phase:

In addition to the changes described for acute lymphoedema, the tissues now contain large numbers of macrophages, fibroblasts, and much excess fibrous tissue. This latter continues to increase with time and the tissues become very hard (Piller and CLodius, 1976). The blood capillaries may also be affected (Casley-Smith et al., 1977a; Clodius and Altorfer, 1977); possibly increasing macromolecular permeability (Földi and Casley-Smith, 1978). Recently it has been suggested (Willoughby and Di Rosa, 1970) that stagnant proteins in the tissues may act as the primary stimulus for chronic inflammation. This may explain why chronic lymphoedema is so similar to chronic inflammation. This hypothesis and its implications will be investigated here. It has also been suggested that debris from disintegrating macrophages may cause some of the excess fibrosis seen in lymphoedema (Földi and Casley-Smith, 1978).

1.3 Therapy:

1.3.1 Low-Protein Oedema:

There are certain general forms of therapy for all types of oedema which are designed to improve lymph flow. These include gentle massage and elevation of the affected area (Földi, 1969; Casley-Smith, 1977b; Clodius, 1977). In addition, pressure bandages to reduce vascular outflow and increase lymphatic drainage; and cold to cause vascular constriction are used. Diuretics are very valuable in low-protein oedemas.

1.3.2 High-Protein Oedema:

All of the above forms of therapy, except diuretics, can be used to treat high-protein oedema with variable success. In addition, various drugs such as anti-biotics, and cortisone are used in specific types of high-protein oedema.

1.3.3 The Benzopyrones:

This group of drugs is effective in all types of high-protein oedema as they alone are capable of removing the excess proteins. They are very effective in both animals and man (Gabór, 1972; Piller, 1975a, b, 1976c, d,

f, 1977a, c; Casley-Smith, 1976a; Piller and Clodius, 1976; Dunn et al., 1977; Clodius and Piller, 1978). By reducing the amount of protein in the tissues they also enhance the removal of fluid (Casley-Smith and Bolton, 1973b; Casley-Smith, Földi-Börcsök and Földi, 1974, 1978; Piller and Casley-Smith, 1975; Piller, 1976a, c, 1977a; Casley-Smith et al., 1977a, 1978). There is considerable evidence to suggest that these drugs do not reduce the amount of protein escaping from the capillaries, rather the reverse is true. They actually increase vascular permeability by releasing histamine. Although they are capable of increasing lymph transport under normal conditions; this does not happen during oedema when lymphatic function is already at its maximum, or in lymphoedema when lymphatic function is impaired. Therefore, their effectiveness in high-protein oedema is not due to any direct increase in lymphatic transport. Simple phagocytosis of the excess proteins does not appear to be the major step in the drugs' action. It does appear that the benzopyrones reduce the pathogenesis of high-protein oedemas by increasing cellular proteolysis. The cells involved appear to be the macrophages, although other cells have not positively been excluded from consideration. The drugs stimulate macrophages directly, and may increase macrophage accumulation in the oedematous areas.

Can oedema be reduced by merely splitting a few large molecules into many small ones, which must increase the local osmotic pressure? The macromolecules can not leave the tissues except via the lymphatics or tissue proteolysis, because their concentration gradients are directed from the blood to the tissues. When they are split into many tiny fragments their concentration gradient is directed towards the blood. These fragments are able to leave the tissues by diffusion, suffering very little molecular sieving and passing very easily through close junctions. This much more rapid removal of the results of proteolysis has not been tested directly and will be investigated here. This removal of proteins from the tissues releases the oedema fluid and allows it to re-enter the blood vessels. The reduction of the oedema will also allow the lymphatic system to return to normal functioning if this is possible. In addition, tissue oxygenation will improve, susceptibility to infection will be reduced and, if Willoughby and Di Rosa are correct, the stimulus for chronic inflammation will be removed and its results including fibrosis will also be reduced. This latter will also be investigated in this project together with the effects of the benzopyrone, coumarin. The benzopyrones have been found to reduce the fibrosis of chronic lymphoedema in the long term (Clodius, Deak and Piller, 1976; Piller and Clodius, 1976; Clodius and Piller, 1978).

SECTION 2

PROTEIN FLUX IN A BLOOD-TISSUE-LYMPH SYSTEM

2.1 Introduction:

The passage of water and solutes from small blood vessels has been widely investigated under normal and injured conditions. Normally, water and low molecular weight solutes pass through the close endothelial junctions by diffusion and ultrafiltration (Renkin, 1977; Crone and Christensen, 1979). Macromolecules are transported by small vesicles (Renkin, 1977) apparently by a random process powered by Brownian motion (Casley-Smith, 1963; Shea and Karnovsky, 1966; Casley-Smith, O'Donoghue and Crocker, 1975). Renkin, Carter and Joyner, (1974) ascribed about half the normal macromolecular transport to the vesicles but they indicated that results from other workers implied contributions varying between 0 - 100 percent, depending on the site and conditions. The balance of macromolecular transport is probably via close endothelial junctions as very few vacuoles are present in normal vascular endothelium (Casley-Smith and Window, 1976).

Vascular permeability increases during

inflammation, leading to the formation of a protein-rich exudate and to local oedema in the tissues (Hurley, 1972). The increased transport from the blood to the tissues has been widely investigated. In particular, moderate histamine and burn injuries cause significant increases in the proportion of open junctions in the venules, veins and capillaries (Majno and Palade, 1961; Cotran, 1967; Hurley, 1972; Dunn, 1973; Casley-Smith and Window, 1976), and these are freely permeable to large macromolecules. The number and sizes of small vesicles is little changed by histamine and burn injuries (Casley-Smith and Window, 1976), implying that they do not contribute to the increased permeability. However, great numbers of large vacuoles appear in the vascular endothelium (Casley-Smith and Window, 1976), and these have been shown to transport fluid and macromolecules (Casley-Smith and Carter, 1979).

Previous studies took no account of backwards passage from the tissues to the blood and are therefore incomplete. It is possible that effective colloidal osmotic pressures can be maintained across open endothelial junctions (Casley-Smith and Bolton, 1973a), forcing fluid to flow from the tissues to the blood (i.e. into the more concentrated solution). It has been proposed that this fluid carries proteins with it against their concentration gradient (Casley-Smith, 1970, 1971,

1973, 1975, 1982a). If vesicles and vacuoles move randomly, it seems likely that they will also transport material from the tissues back into the blood (DeFouw and Berendsen, 1979).

This experiment was designed to investigate protein flux in a blood-tissue-lymph system, under normal and histamine-injured conditions, in order to provide clearer and more complete information on the changes that occur during inflammation. In order to do this it was necessary to inject into, and sample from, the interstitial fluid. Usually the fluid spaces are so small (Casley-Smith and Vincent, 1978) that this would be impossible in practice. Guyton's (1963, 1965a, 1965b) capsules were used to provide a workable volume of interstitial fluid. Intracapsular pressure measurements were made to indicate when inflammation from surgery subsided and hence, when the protein flux experiment could commence.

2.2 Materials and Methods:

The experimental procedures and a very simplistic analysis of the results were carried out as part of the requirements for the Degree of Bachelor of Science with Honours. The materials and methods are only summarized here but are included in full as Appendix 7.1.

RISA (^{125}I) was injected into the blood by heart puncture. Radioactive chromic chloride (^{51}Cr) bound to albumin was injected into the fluid of the Guyton's capsules with either physiological saline or a histamine acid phosphate solution. Samples of both blood and the capsule fluid were taken at 5, 20, 45, 80, 320 and 1440 minutes and their ^{51}Cr and ^{125}I activities were counted on a Packard Tri Carb Scintillation Counter for one minute.

2.3 Compartmental Analysis:

A compartmental model was developed to describe the physiological system as illustrated in Figure 2.1. The aim of the analysis was to calculate all the rates of protein flux shown in Figure 2.1. This model is a modification of similar systems described by Jacquez (1972) and Rubinow (1976), and was designed to suit the particular system applicable here. The fundamental compartmental equations which describe the system are:

$$V_C \frac{dx_C}{dt} = -V_C (R_{BC} + R_{LC}) x_C + V_B R_{CB} x_B \qquad (1)$$

$$V_B \frac{dx_B}{dt} = -V_B (R_{EB} + R_{CB}) x_B + V_C R_{BC} x_C \qquad (2)$$

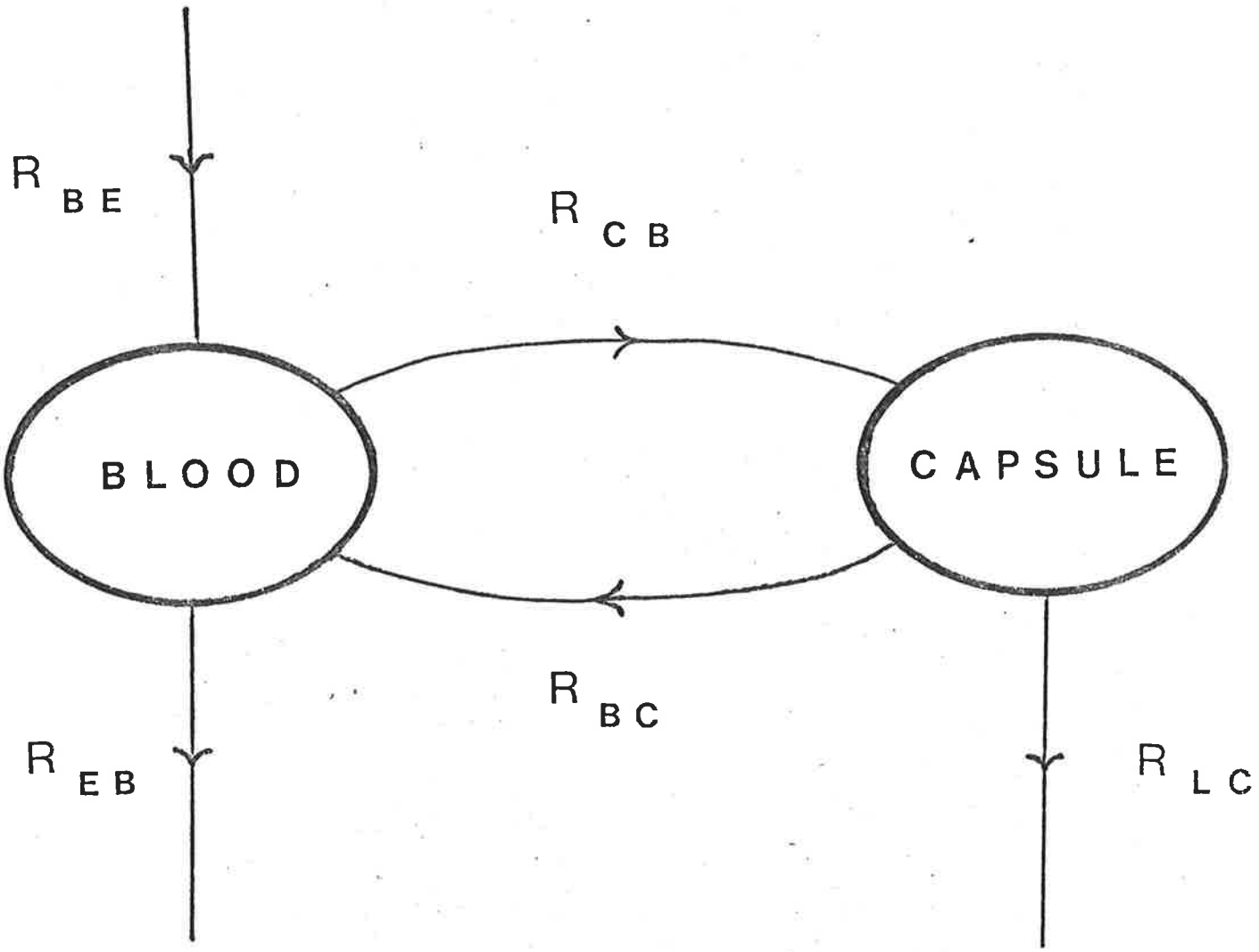


Figure 2.1:

A compartmental representation of the physiological system investigated. R_{ij} is the rate of material flux from compartment j to compartment i per unit volume in compartment j.

The subscripts indicate:

B - Blood;

C - Capsule;

L - Lymphatics + Proteolysis;

E - Exterior Tissue Compartments.

where V_C and V_B are the volumes of the capsule and blood plasma compartments respectively; x_i is the concentration of a particular tracer in compartment i; and R_{ij} is the rate of flux of material from compartment j to compartment i per unit volume in compartment j. The other subscripts are explained in Figure 2.1.

The general solution for these equations is:

$$x_C(t) = A_{C1} e^{k_1 t} + A_{C2} e^{k_2 t} \quad (3)$$

$$x_B(t) = A_{B1} e^{k_1 t} + A_{B2} e^{k_2 t} \quad (4)$$

A semilog plot of $x_i(t)$ against time (Figure 2.2) becomes a straight line for large t, as only one exponential term survives. This is the one with the largest k_j and is usually designated k_1. The constants of the general solution are obtained from the experimental data by the method of exponential peeling. The exponential $A_{i1} e^{k_1 t}$ is fitted to the count data ($x_i(t)$) for the large values of time; in this case for 80 - 1440 minutes. This yields k_1 and A_{i1}. From equations (3) and (4):

$$A_{i2} e^{k_2 t} = x_i(t) - A_{i1} e^{k_1 t} \quad (5)$$

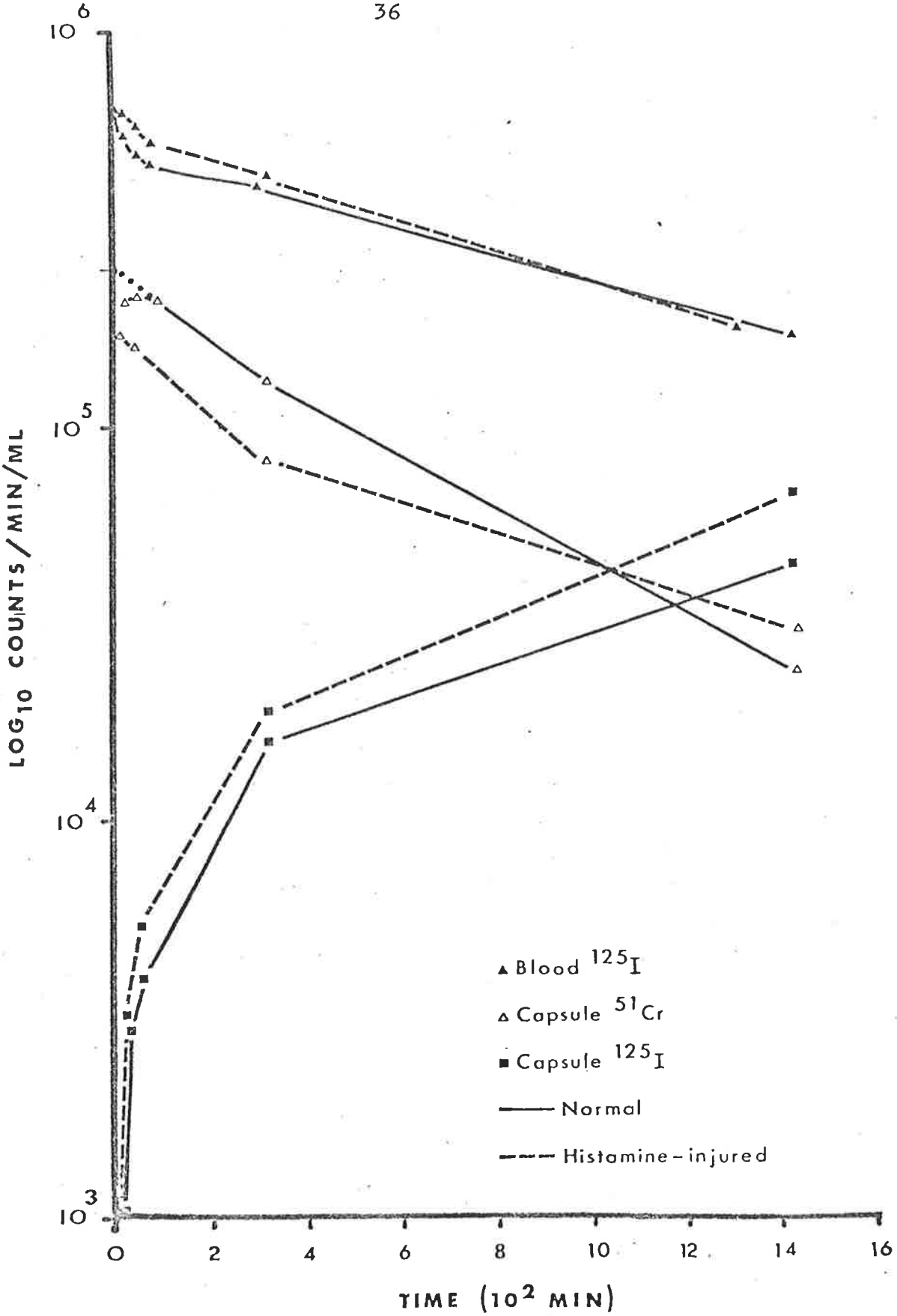


Figure 2.2:

The concentration of radioactive tracer in the compartments for one individual animal: $x_i(t)$.

The function:

$$x^*_i(t) = x_i(t) - A_{i1} e^{k_1 t} \qquad (6)$$

is formed for the smaller values of time. The data points obtained from this function are fitted to the exponential $A_{i2} e^{k_2 t}$ yielding k_2 and A_{i2}. In a steady state system similar to that in Figure 2.1, all the rates of flux can be calculated, even if only one compartment receives radioactive tracer and even if only that compartment is sampled. In this case the system was solved for the capsule. The following equations to calculate the rates are adapted directly from Jacquez (1972) and Rubinow (1976):

$$R_{BC} + R_{LC} = - \frac{(A_{C1} k_1 + A_{C2} k_2)}{A_{C1} + A_{C2}} \qquad (7)$$

$$R_{EB} + R_{CB} = - (R_{BC} + R_{LC}) - (k_1 + k_2) \qquad (8)$$

$$R_{BC} = \frac{(R_{BC} + R_{LC})(R_{EB} + R_{CB}) - k_1 k_2}{R_{CB}} \qquad (9)$$

In a steady state system conservation of matter in the capsule implies:

$$V_B R_{CB} = V_C (R_{BC} + R_{LC}) \qquad (10)$$

R_{CB} can be calculated using equation (7). V_B is the

volume of the blood plasma (approximately 6 mls for rats of this weight, according to Donaldson, 1915). V_C is the volume of the capsule in mls and can be calculated according to:

$$V_C = \frac{p_C}{x_C\ (0)} \tag{11}$$

where p_C is the absolute amount of tracer injected into the capsule initially and:

$$x_C\ (0) = A_{C1} + A_{C2} \tag{12}$$

from equation (3).

Substituting for R_{CB} in (8) yields R_{EB} and in (9) yields R_{BC}. Substituting for R_{BC} in (10) or (7) yields R_{LC}. The conservation equation for the blood plasma:

$$R_{BE} + V_C\ R_{BC} = V_B\ (R_{EB} + R_{CB}) \tag{13}$$

allows the calculation of R_{BE}. Subtracting (13) from (10) yields;

$$R_{BE} = V_B\ R_{EB} + V_C\ R_{LC} \tag{14}$$

These rates of flux (R_{ij}) can be converted to mg of albumin/minute by multiplying by the absolute amount of

albumin in compartment j. Concentrations of 0.03024 gm albumin/ml for plasma and 0.014698 gm/ml for capsular fluid have been calculated from percent protein data for rats (Libermann et al., 1972) and albumin/globulin ratios for rats (Szabó, Magyer and Posch, 1976). The volume (V) of compartment j can be obtained as described above.

For the control animals, we can assume a steady state exists and can use all the above relationships on the capsule ^{51}Cr counts to get the rates of protein flux illustrated in Figure 2.1. Table 2.1 illustrates the stepwise calculations for the normal condition from 0 - 1440 minutes.

However, for histamine injury a steady state can not be assumed. Therefore, equations (7 - 9) can be used, but not those requiring conservation. In equation (9) R_{CB} is needed before R_{BC} can be calculated. An estimate of R_{CB} was obtained by directly calculating an average rate of arrival of ^{125}I in the capsules from the blood over the 1440 minutes, for each rat. From equations (5) and (6) R_{LC} and R_{EB}, respectively, can be calculated. Without the conservation equations, R_{BE} can not be obtained, but it seems likely that a steady state would still be maintained in the exterior part of the system implying that equation (14) would be applicable. The stepwise calculations are shown in Table 2.2.

Table 2.1: Stepwise Protein Flux Calculations - Normal Condition (0 - 1440 minutes)

A_{C1}	k_1	A_{C2}	k_2	$A_{C1}+A_{C2}$	V_C	$R_{BC}+R_{LC}$	V_B R_{CB}
113392	-.00055	42948	-.00294	156342	4.53	.00121	.00546
330392	-.00123	172570	-.00282	502962	1.41	.00178	.0025
182234	-.00172	57640	-.0062	239874	2.95	.0028	.00825
209985	-.0029	50287	-.01	260272	2.72	.00427	.0116
152280	-.0019	54464	-.0075	206744	3.43	.00338	.0116
200010	-.003	99989	-.01	299999	2.36	.00533	.01258
227668	-.0024	43464	-.0085	271132	2.61	.0034	.0089
194878	-.00112	237967	-.00326	432754	1.64	.0023	.00377
154305	-.00181	47468	-.0074	201773	3.51	.00313	.01097
208705	-.00197	101197	-.0065	309902	2.29	.00345	.0079
193233	-.00267	38945	-.0039	232178	3.05	.00157	.01

R_{CB}	$R_{EB}+R_{CB}$	$R_{CB}R_{BC}$	R_{BC}	R_{EB}	R_{BE}	R_{LC}
.0014	.00228	.00000114	.000814	.00088	.005182	.00391
.00042	.00227	.0000006	.00135	.00185	.01174	.000428
.001375	.005123	.00000367	.002666	.003748	.019681	.000132
.00194	.00863	.00000785	.004046	.00669	.06279	.000224
.00193	.00602	.0000061	.003159	.00409	.0253	.000221
.0021	.00767	.0000109	.00518	.00557	.0338	.000149
.0015	.0074	.00000476	.00317	.0059	.03613	.00023
.00063	.00208	.00000113	.001798	.000282	.01145	.000499
.00183	.00609	.00000562	.00307	.004255	.02573	.000053
.00132	.005018	.00000452	.003424	.003698	.02227	.000026
.002	.005002	.000002567	.001284	.003002	.026097	.000289

Table 2.2: Stepwise Protein Flux Calculations - Histamine-Injury (0 - 1440 minutes)

$R_{BC}+R_{LC}$	$R_{EB}+R_{CB}$	$R_{CB}R_{BC}$	R_{CB}	A_{C1}	k_1	A_{C2}	k_2
.00635	.02725	.0001367	.006771	246819	-.002772	19892	-.020964
.005731	.011519	.0000544	.006672	200684	-.00086	92247	-.01215
.001654	.00397	.00000288	.006293	142993	-.00085	29934	-.0105
.006436	.038052	.00020533	.007919	192067	-.00165	56301	-.0165
.003996	.009605	.00002132	.008265	177857	-.00115	43764	-.01425
.008469	.016415	.00012129	.0085	212644	-.0008	19534	-.0140
.005266	.009961	.0000388	.00761	89073	-.001	112492	-.01878
.005211	.025514	.00009148	.007551	140108	-.00149	46823	-.01535
.002426	.00543	.00000029	.00804	122291	-.000931	40525	-.012142

$A_{C1}+A_{C2}$	V_C	$R_{BC}+R_{LC}$	$R_{EB}+R_{CB}$	$R_{CB}R_{BC}$	R_{BC}	R_{LC}	R_{EB}	R_{BE}
266711	2.66	.0041291	.019607	.00002284	.003373	.0007558	.012836	.07903
292931	2.42	.0044153	.008595	.0000275	.004122	.0002937	.001923	.01225
172927	4.10	.0025204	.008829	.00001333	.002118	.0004023	.0025366	.016869
248368	2.85	.0050163	.013134	.00003866	.004882	.0001347	.0052147	.031672
221621	3.2	.0037369	.011663	.000027196	.003291	.0004465	.0033981	.021817
232178	3.05	.0029819	.011818	.00002404	.002828	.0001537	.0033181	.024596
201565	3.51	.0109229	.008857	.00007797	.010245	.0006778	.0012471	.009862
186931	3.79	.0049617	.011878	.000036065	.004776	.000855	.004327	.026665
162816	4.35	.0037214	.009352	.0000235	.002936	.0007858	.001348	.011506

As the open junctions caused by histamine injury return to normal after approximately 30 minutes, further calculations were performed on the raw data in two stages; 0 - 45 mins and 45 - 1440 mins. Single exponential curves gave good fits (correlation coefficients $>$ 0.95) to the count data in these time intervals. R_{CB} was calculated from the arrival of ^{125}I in the capsule. $(R_{BC}+R_{LC})$ was calculated from the rate of removal of ^{51}Cr from the capsule. After an initial rapid increase to the maximum, R_{LC} would not change at 45 minutes and would probably remain constant over the period of the experiment. Therefore, the R_{LC} calculated over 0 - 1440 minutes was subtracted from $(R_{BC} + R_{LC})$ to yield R_{BC}. Table 2.3 shows the results of these calculations for each individual animal.

These rates of flux (R_{ij}) for each animal were then converted to mg albumin/minute. It was not possible to do this for R_{BE} without knowledge of the volume of the exterior tissue compartments or their albumin concentration. The mean for each rate of flux was then calculated and the normal and histamine-injured rates were compared using the Student's t-test (Table 2.4). The mean for R_{BE} is included in Table 2.4 but the units are mls/minute.

Table 2.3: Stepwise Protein Flux Calculations

Histamine-Injury

(0 - 45 minutes)			(45 -1440 minutes)		
R_{CB}	$R_{BC}+R_{LC}$	R_{BC}	R_{CB}	$R_{BC}+R_{LC}$	R_{BC}
.00995	.003668	.002913	.001879	.002784	.002393
.01361	.005462	.003515	.001654	.000860	.000512
.06615	.002213	.001810	.000767	.000892	.000760
.02247	.004223	.004088	.001770	.001702	.001478
.07476	.003162	.002716	.001311	.001192	.000971
.03133	.001648	.001494	.002775	.000816	.000667
.04576	.009234	.008556	.002239	.001155	.000925
.04117	.004242	.004056	.003099	.001544	.001304
.08540	.003169	.002384	.000982	.000999	.000758

Table 2.4: Mean Rates of Albumin Flux (mg/minute); except E → B (mls/minute)

	$B_a \rightarrow C$	C → B	C → L	B → E	E → B
NORMAL					
(0 - 1440 minutes)	0.2816 (0.0285) 10_b	0.1086 (0.0164) 11	0.0094 (0.0020) 11	0.6532 (0.1133) 11	0.0255 (0.0047 11
HISTAMINE					
(0 - 1440 minutes)	1.3607 (0.0463) 9 $***_c$	0.2081 (0.0428) 9 *	0.0215 (0.0050) 9 *	0.7278 (0.2152) 9 n.s.	0.0260 (0.0071) 9 n.s.
(0 - 45 minutes)	7.8606 (1.6366 9 ***	0.1835 (0.0393) 8 *			
(45 - 1440 minutes)	0.3317 (0.0469) 9 n.s.	0.0516 (0.0074) 9 ***			

a. The headings for the columns are B, blood; C, capsule; L, lymphatics + proteolysis; E, exterior. The arrow indicates the direction of the flux.

b. The figures for each column are mean, (standard error) and the number of observations.

c. The significance of the difference from normal using Student's t-test: n.s. = not significant; * = $0.05 > p > 0.01$; *** = $p < 0.001$.

It was decided that the capsules of the non-infected animals had reached an equilibrium with the surrounding tissue after approximately 57 days (Appendix 7.1). It seems reasonable to conclude that they enclosed a true, normal tissue space filled with interstitial fluid; although it may differ in some constituents and be relatively isolated (Casley-Smith, 1982a). Therefore, the capsule compartment is referred to as a tissue compartment in the following discussion.

2.4 The Calculated Rates of Protein Flux:

The results for normal and histamine injury are summarized in Table 2.4. Histamine caused very significant increases in the rates of protein flux to and from the capsule. The rate of flux from the blood to the tissues from 0 - 1440 minutes was increased approximately five times and the backflow from the capsule to the blood was doubled. The rate of flux from the capsule to the lymphatics and proteolysis was increased 2.3 times. Normally, the rate of flux from the capsule to the blood was 11.6 times that from the capsule to the lymphatics and proteolysis. With histamine injury this ratio was 9.7. Histamine injury increased the rate of protein flux from the blood to the tissues in the first 45 minutes approximately 28 times, but had no significant effect from 45 - 1440 minutes. It doubled the backflow from the

capsule to the blood in the first 45 minutes but halved it from 45 - 1440 minutes. The rates of exchange between the blood and the exterior were unchanged by histamine injury.

2.5 Protein Flux in the Blood-Tissue-Lymph System:

Histamine injury caused a significant increase in the rate of protein flux from the blood to the tissues, which is consistent with repeated observations (Carter, Joyner and Renkin, 1974; Joyner et al., 1974; Renkin, Carter and Joyner, 1974; Renkin, 1977). This increased permeability would be due to the increased proportion of open junctions, and to the greatly increased numbers of large vacuoles in the endothelium of the capillaries, venules and small veins (Casley-Smith and Window, 1976; Casley-Smith and Carter, 1979).

Histamine injury also caused a significant increase in the rate of protein flux from the tissues to the lymphatics and proteolysis, which agrees with many studies on inflammation (e.g. Taylor et al., 1973; Joyner et al., 1974).

This experiment has also shown the existence of protein flux from the tissues back into the blood against the concentration gradient, under both normal and histamine injured conditions. The former has often been

reported (reviewed Casley-Smith, 1982a); the latter has never been investigated. Histamine significantly increased this rate of flux, and in both normal and histamine injured conditions approximately ten times as much protein passed to the blood as passed to the lymphatics and proteolysis. It is possible that the proportion of open junctions in capsular capillaries is higher than normal. Newly formed blood vessels in granulation tissue are more permeable than mature vessels. This is particularly true at the tips where abnormally loose junctions are found (Schoefl, 1963 and Cliff, 1963 as reviewed by Hurley, 1972).

2.6 Protein Flux Across the Vascular Endothelium:

The rates of protein flux between the blood and the tissues were divided into the relevant pathways according to the available information (Table 2.5). Under normal conditions vacuoles play a negligible part in the protein transport across the vascular endothelium, whilst vesicles and close junctions may each transport anywhere between 0 and 100 percent (Renkin, Carter and Joyner, 1974; Renkin, 1977). These extremes and a 50 percent contribution by each were calculated to give a complete range of all the possible values.

If their movement is random, vesicles would

probably transport albumin between the blood and tissues in proportion to the concentration difference between the two compartments;

$$\frac{\text{Rate(Blood to Tissues)}}{\text{Rate(Tissues to Blood)}} = \frac{\text{Blood albumin conc}}{\text{Tissue albumin conc}} = \frac{.03024}{.14698} \cong \frac{2}{1}$$

Thus an estimate of vesicular transport from the tissues to the blood was obtained. The remainder of the flux in that direction must have been through junctions.

The temporally open junctions return to normal (closed again) approximately 30 minutes after moderate histamine injury. Vesicle transport probably continues throughout histamine injury at the same rate as under normal conditions (Casley-Smith and Window, 1976). However, vesicular transport could be reduced in the first 90 minutes, as the mean diameter and number of vesicles are reduced by histamine injury (Casley-Smith and Window, 1976). Reduced cell width could compensate for this in the first 30 minutes. Therefore, the rates of flux calculated for vesicles could be slight overestimates, implying that those calculated for junctions would be slight underestimates. Having calculated the normal values for both these pathways (Table 2.5), the remainder of the flux in both directions after 45 minutes must be due to vacuoles. The mean rate of flux from the blood to

Table 2.5: Mean Rates of Albumin Flux (mg/minute) Between Blood (B) and Tissues (T) Via Different Paths; when the Contribution to Normal B⟶T Flux is:

		100 % Vesicles	50 % Vesicles 50 % Junctions	100 % Junctions
NORMAL CONDITIONS: No significant contribution by vacuoles				
B⟶T	Vesicles	0.282 (0.028)10 [a]	0.141 (0.014)10	
	Junctions		0.141 (0.014)10	0.282 (0.028)10
T⟶B	Vesicles	0.109 (0.054)11	0.070 (0.007)10	
	Junctions		0.038 (0.006)10	0.109 (0.054)11
HISTAMINE-INJURED CONDITIONS (0 - 45 minutes): Contribution by vesicles as normal; and vacuoles as histamine injury 45 - 1440 minutes				
	Junctions			
B⟶T		7.529 (0.820) 9 ***[b]	7.670 (0.798) 9 ***	7.811 (0.759) 9 ***
T⟶B		0.075 (0.006) 8 ***	0.113 (0.002) 8 ***	0.184 (0.024) 8 *

a. Numbers are mean, (standard error), number of observations.

b. The significance of the difference from normal using Student's t-test: * = .05 > p > 0.01; *** = p < 0.001.

the tissues via vacuoles was calculated to be 0.050 mg albumin/minute (with a standard error of 0.004 on nine observations). The transport from the tissues back into the blood by vacuoles can also be calculated as described for vesicles above (assuming the random motion of vacuoles). No backflux due to vacuoles was detected.

After 45 minutes the total flux from the blood to the tissues returned to normal. Any increased vacuolar transport was not large enough to significantly increase this total above normal. The flux from the tissues back to the blood was reduced to half normal after 45 minutes, indicating that vesicular and/or junctional transport must have been reduced. If, as explained above, vesicular transport is actually reduced by histamine injury, then this further reduction could be attributed to the junctions. There is evidence to suggest that junctions may close tighter after histamine injury than they were normally. The junctions are unresponsive to a second dose of histamine for some hours (Casley-Smith and Window, 1976).

An increased number of vacuoles is evident as early as one minute after histamine injury. This number is still approximately constant at ninety minutes (Casley-Smith and Window, 1976) and probably remains so for many hours (Hurley, 1972). Therefore, the flux via

the vacuoles from 0 - 45 minutes would be approximately the same as that calculated for 45 - 1440 minutes. Vesicle transport would be normal (or less than normal), implying that the remainder of the flux in both directions was through junctions, particularly the now-opened ones (Table 2.5).

Histamine injury caused very significant increases in the rates of protein flux through the open junctions, in both directions, in the first 45 minutes regardless of what proportion of the total flux passed through junctions under normal conditions.

2.7 Protein Flux Against its Concentration Gradient:

Of special interest is the fact that in all the possible situations covered in Table 2.5, increased protein flux through open junctions against the concentration gradient was detected. It is very unlikely that vesicles could be responsible for 100 percent of the protein flux from the blood across normal endothelium. Similarly, it is very unlikely that open junctions could transport 100 percent. It is probable that the 50:50 contributions are the best indications of what is actually occurring; as suggested by Renkin, Carter and Joyner (1974). The extremes were included here to give a complete range of the possible values. In all cases,

histamine significantly increased the rate of protein flux from the tissues back to the blood through the open junctions.

The existence of this backflux and its increase when there are many more open junctions, seems to validate an hypothesis proposed by Casley-Smith (1970, 1971, 1973, 1975, 1982a). This was suggested to explain the passage of proteins from the tissues into the blood capillaries via the fenestrae in the venous limbs, and also into the initial lymphatics via the open endothelial junctions. In these cases, the proteins appear to move against a hydrostatic pressure gradient as well as against their concentration gradient. Briefly the hypothesis states (Casley-Smith, 1975) that "when two protein solutions of different concentrations are separated by a membrane which is quite permeable to the proteins, there is still a relatively large effective osmotic pressure forcing fluid into the more concentrated of the solutions". Effective colloidal osmotic pressures have been shown to be maintained across pores even up to ten μm wide (Casley-Smith and Bolton, 1973a). It is proposed that this fluid flow carries proteins with it from the dilute to the more concentrated solution. Casley-Smith (1981) has proved that the macromolecules and fluid can move into the more concentrated solution.

2.8 The Direction of Net Protein Flux:

The direction of the net protein flux will depend on the particular conditions, especially the net hydrostatic pressure and the width of the pores (Casley-Smith, 1976a). The proposal that the net flux is in the direction of the more concentrated solution at venous fenestrae and open lymphatic junctions has been the cause of controversy: with some in favour (Perl, 1975; Casley-Smith, 1982a) and others opposed (Michel, 1974). In this experiment the net protein flux between the blood and the tissues was directed from the blood, in both normal and histamine injured conditions. The net flux across the junctions was also directed towards the tissues under both normal and histamine-injured conditions. This could be due to the increased hydrostatic pressure in the microvascular system (especially on its venous side) during inflammation and the greater number of open junctions. It is also possible that the some of the capsular capillaries were still developing and that this type of tissue has greater permeability (increased proportion of open junctions) to protein than normal.

SECTION 3

OEDEMA AND BENZOPYRONE THERAPY

3.1 With Normal Lymphatics and Proteolysis:

3.1.1 Introduction:

A group of drugs, the benzopyrones, is very effective in reducing most forms of high-protein oedema (Casley-Smith, 1976b; Dunn et al., 1977; Clodius and Piller, 1978; Földi and Casley-Smith, 1978; Piller, 1980), including lymphoedema and thermal oedemas. They increase the proteolysis of the accumulated abnormal protein (Casley-Smith, 1976b; Piller, 1977a, b, c), apparently by stimulating the macrophages (Dunn et al., 1977; Piller, 1977c, 1978). Other cells such as fibroblasts (Houck and Sharma, 1969) and neutrophils may also be involved, although this has not been proven for the benzopyrones. A very strong positive correlation has been shown to exist between benzopyrone induced proteolysis and the oedema-reducing effect of the drug (Piller, 1976c). A direct relationship between these two factors has not been proven. It is necessary to show that the products of proteolysis are removed from the site of oedema more rapidly than the proteins; that the splitting of proteins

increases the rate at which the oedema is reduced. In this study, the rate of disappearance (resolution) of oedema caused by the injection of plasma proteins was compared with that of an equivalent concentration of amino acids.

In oedema the excess plasma proteins can be removed from the tissues by the lymphatics, or by proteolysis, because their concentration gradient is directed from the blood to the tissues. Once proteolysis splits them into fragments, mainly amino acids and di-peptides (Ehrenreich and Cohn, 1967; Lospalluto, Fehr and Ziff, 1971), their concentration gradients are directed from the tissues to the blood. Their small sizes allow them to diffuse rapidly, they suffer little molecular sieving in the tissues, and they should be able to pass easily through the narrow, close junctions (Karnovsky, 1970; Renkin, Carter and Joyner, 1974; Casley-Smith et al., 1975) of the blood endothelium. Hence they should be able to pass into the blood very quickly, reducing the tissue osmotic pressure and enabling the oedema fluid to flow. This study was designed to see if this was so.

Non-metabolizable PVP, of similar molecular weight and concentration to the plasma proteins, was injected as a control to determine the effect of proteolysis in the

resolution of oedema. Since the amino acid and PVP solutions were made with physiological saline it was used as an additional control. The effects of a benzopyrone, coumarin, on the resolution of the oedemas produced by the injection of plasma, amino acids, PVP and saline were investigated to obtain more information about the action of the drug.

3.1.2 Oedema Production and Coumarin Treatment:

For the experiment, male hooded rats of average weight 360±40 gm were randomly divided into four groups. These received artificial oedemas from the injection of plasma or amino acids. PVP and physiological saline were used as controls. Each of these groups was divided into two subgroups, one of which was treated with coumarin.

Homologous rat plasma was used after the complement system had been inactivated by treatment with 0.025 M ammonium hydroxide at 37 °C for 60 minutes. The ph was then brought back to 7.4 by dialysis against at least a 1000 times volume of physiological saline (0.85 percent) at 4 °C. Siliconized equipment was used to ensure that the Hageman Factor was not activated. Since this plasma has a concentration of six percent protein (Donaldson, 1915), the amino acids and PVP solutions were made to that concentration with physiological saline. PVP

(Pharmacia) of average molecular weight 40,000 was used. Subcutaneous injections of 2.0 mls. of one of these solutions were made into the anterio-lateral aspect of the right hind leg, over a period of one minute.

When required, coumarin (Schaper & Brümmer, West Germany) was injected intra-peritoneally at 25 mg/kg in a 2 percent solution of A. R. ethyl alcohol in physiological saline. The other groups recieved equivalent injections of the alcohol in physiological saline. It was administered daily, commencing three days before the actual experiment. A dose was also given immediately prior to the leg injections.

3.1.3 Measurement and Analysis:

The right hind legs were closely shaved at least 24 hours before being injected. The limb volumes were measured by plethysmography immediately before the injection, then at 1, 2.75, 4, 6.5, 12, 24 and 30.5 hours. At each time, the average of three measurements was recorded.

An exponential decay curve $V_t = V_0 e^{-kt}$ was fitted to the volume data for each rat. V_0 is the initial volume plus the 2.0 mls. injected. A correlation coefficient $>$ 0.75 was considered to indicate a good fit

of the data to the theoretical curve. This yielded the rate (k) at which the leg volume returned to its initial value (termed here, the rate of resolution of the oedema). The mean rates of resolution for each group were compared using the Students t-test.

3.1.4 The Rates of Resolution of Oedema:

The mean volume changes for each treatment are shown in Figure 3.1.

The exponential decay curve gave a good fit to the data for all groups, except those which received plasma with or without coumarin (correlation coefficients ranging from 0.36 to 0.75; with only three being greater than 0.75). On closer examination of the raw data (Figure 3.1) these treatments seemed to yield a biphasic response: an initial rapid rate of resolution from zero to four hours followed by a slower phase until the return to normal. In these cases a separate curve was fitted to each phase. The mean rates of resolution appear in Table 3.1.

Coumarin had no significant effect on the rates of removal of saline, PVP, amino acids or on the first four hours of plasma removal. Therefore, the mean rate of resolution for each treatment without coumarin has been combined with the mean rate for that treatment with

Figure 3.1:

The mean volume of injectant remaining in the legs as measured by plethysmography for animals with normal lymphatics and proteolysis.

The curves in part A illustrate the normal responses to oedema whilst those in part B illustrate the coumarin treated responses.

The symbols indicate the type of injectant:

⊙ - Plasma;

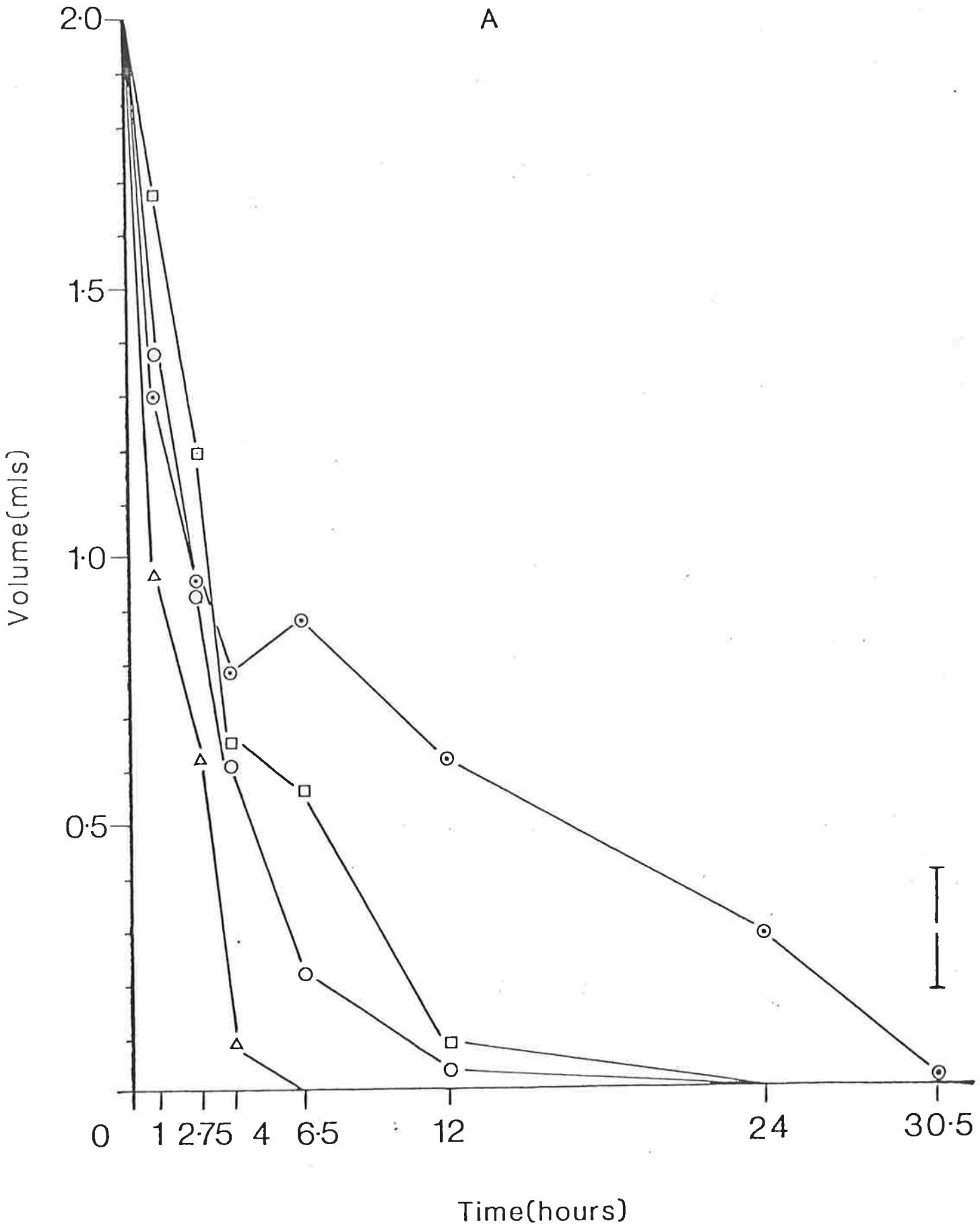
□ - Amino Acids;

○ - PVP;

△ - Saline.

The grand standard errors of all the mean volumes were ± 0.1053 mls for normal and ± 0.1225 mls for coumarin treatment.

These are indicated by: I


A
2·0
1·5
1·0
0·5
Volume(mls)
0
1
2·75
4
6·5
12
24
30·5
Time(hours)

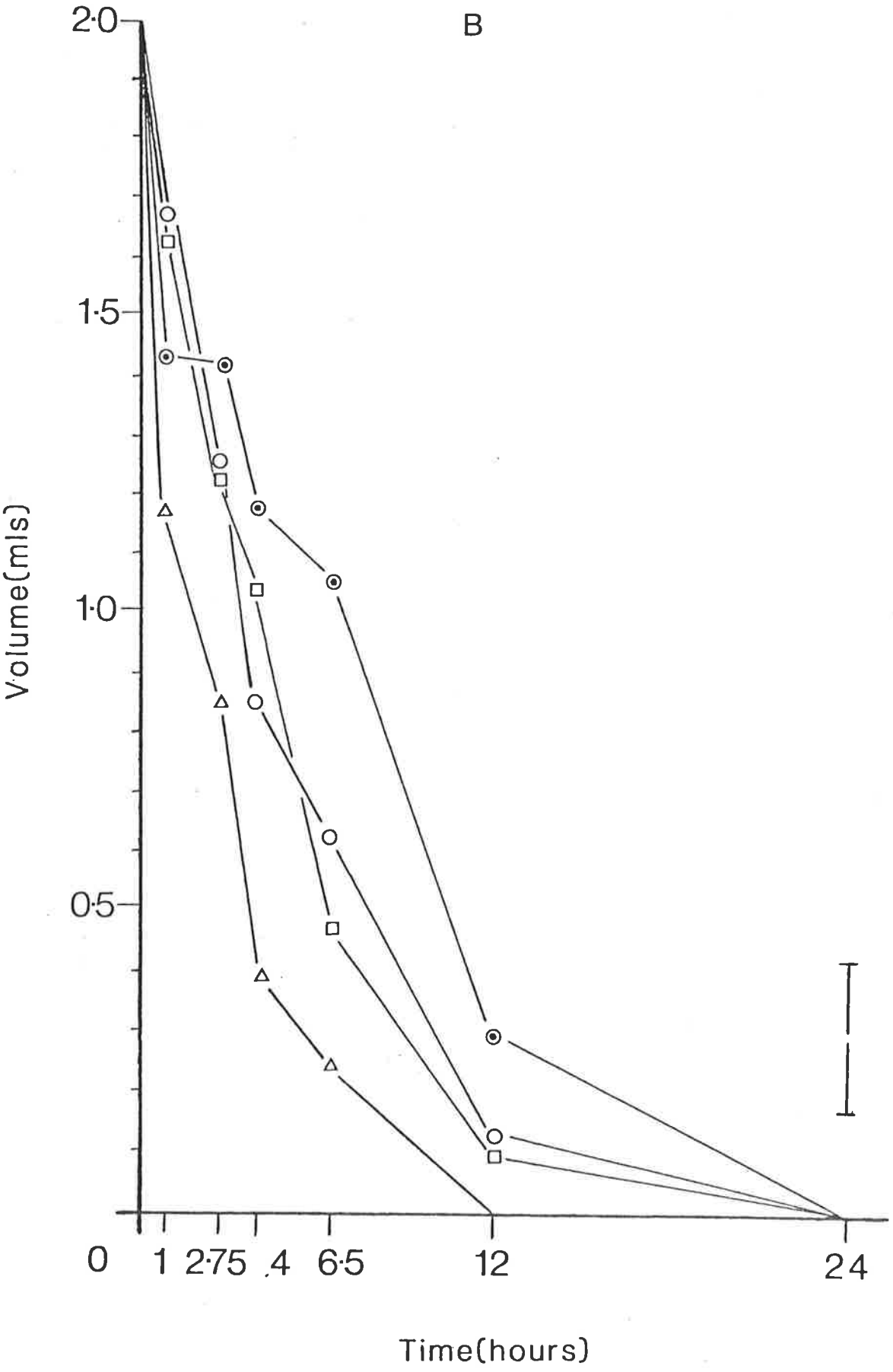

B
2·0
1·5
1·0
0·5
Volume(mls)
0
1
2·75
4
6·5
12
24
Time(hours)

Table 3.1: The Rates of Resolution (k) of Oedema in Rat Legs With Normal Lymphatics and Proteolysis.

TREATMENT	MEAN RATE (hours^{-1})	STANDARD ERROR	NUMBER	TIME TO NORMAL (hours)	SIG OF t-STAT$_a$
Plasma (0 - 4 hrs)	0.0295	.0011	18		
Plasma + Coumarin (0 - 4 hrs)	0.0324	.0032	9		n.s.
Plasma (4 hrs $\longrightarrow$)	0.0050	.0003	18	20.88	
Plasma + Coumarin (4 hrs $\longrightarrow$)	0.0081	.0008	9	16.61	***
Amino Acids	0.0232	.0034	19	9.60	
Amino Acids + Coumarin	0.0225	.0036	10	9.90	n.s.
PVP	0.0320	.0037	17	7.0	
PVP + Coumarin	0.0258	.0046	10	8.65	n.s.
Saline	0.0563	.0040	19	3.96	
Saline + Coumarin	0.0378	.0054	9	5.91	n.s.
COMBINED MEANS WITH AND WITHOUT COUMARIN					
Plasma $\pm$ Coumarin (0 - 4 hrs)	0.0303	.0013	27		
Amino Acids $\pm$ Coumarin	0.023	.0024	29	9.3	
PVP $\pm$ Coumarin	0.0305	.0027	27	7.31	
Saline $\pm$ Coumarin	0.0500	.0032	29	4.46	

a. The t-STAT (t-statistic) is the result of the Student's t-test on the difference between the means. The significance of this t-statistic is denoted by n.s. = not significant; *** = $p < 0.001$

coumarin (Table 3.1 and Figure 3.2). For example, since coumarin had no effect on the rate of resolution of saline oedema, the saline and saline+coumarin groups have been combined and regarded as one group, termed saline±coumarin. The statistical significance of the differences between the combined means is illustrated in Figure 3.2. Coumarin did significantly increase the rate of removal of plasma after four hours ($p < 0.001$).

Saline was removed more rapidly ($p < 0.001$) than all other injectants; approximately 85 percent being removed in the first four hours.

There was no significant difference between the rates of removal of PVP, amino acids and plasma (0 - 4 hours).

After four, hours plasma with or without coumarin was removed at a much slower rate ($p < 0.001$) than all the other injectants, including the amino acids.

3.1.5 Discussion:

Low-Protein Oedema:

Low-protein oedemas can be caused by raised venous pressure, lowered plasma concentration or lowered tissue hydrostatic pressure. The permeability of the blood

Figure 3.2:

The mean resolution of oedema (for animals with normal lymphatics and proteolysis) obtained by fitting:

$$\ln V_t = \ln V_0 - kt$$

to the volume data for each rat. The means for coumarin and non-coumarin treated groups have been combined for saline (△), PVP (○), Amino Acids (□) and Plasma 0 - 4 hours (⊙).

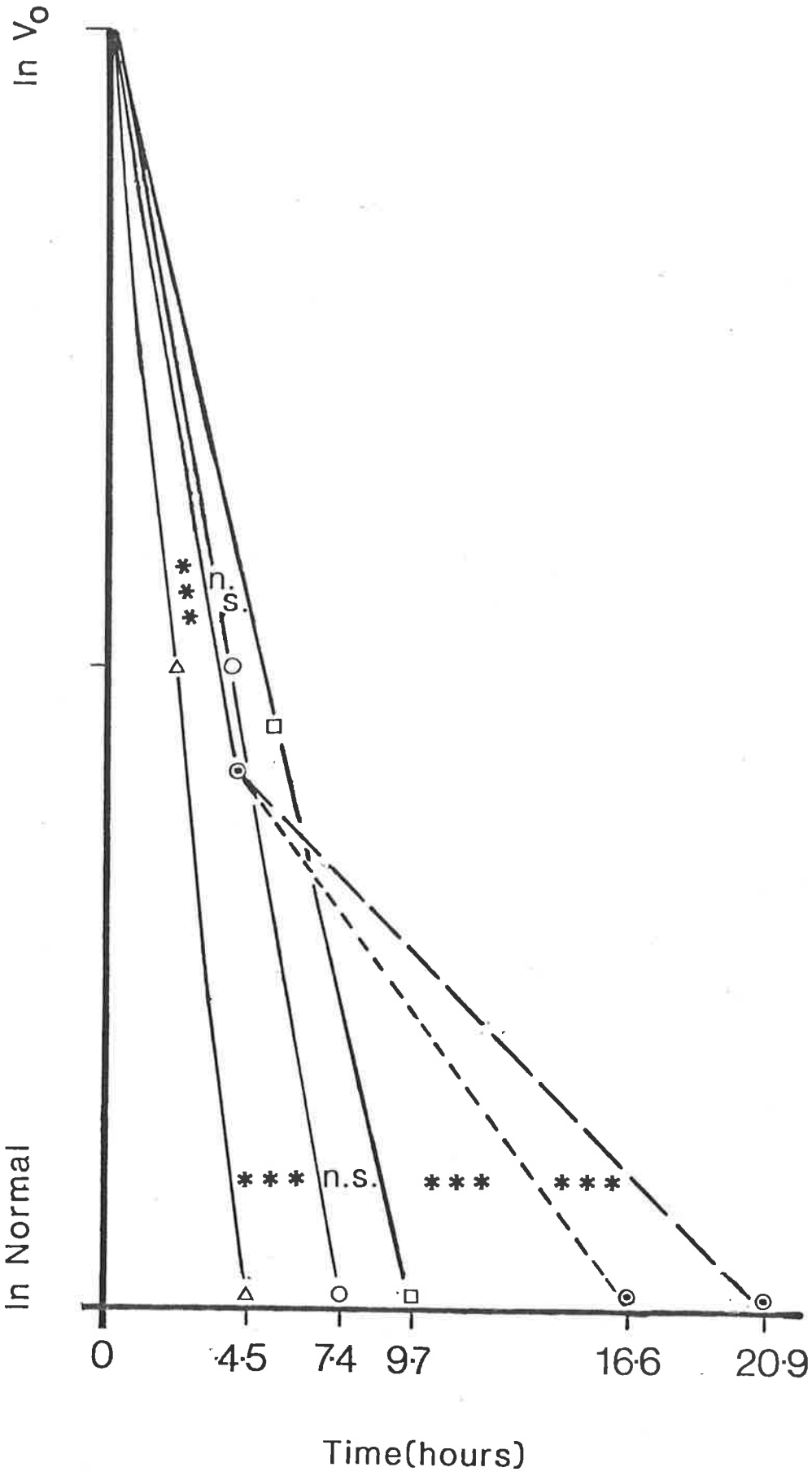
After 4 hours the resolution of plasma is illustrated by —— ⊙ —— , whilst that for plasma+coumarin is illustrated by — — ⊙ — —.

The mean rates of resolution for each group were compared using the Student's t-test. The significances of the t-statistic are indicated by:

n.s.= not significant,

*** = $p < 0.001$.

The graphical representation allows a visual comparison of the rates of resolution of the different oedemas.


ln V_o
ln Normal
***
n.s.
*** n.s.
***
***
0
4·5
7·4
9·7
16·6
20·9
Time(hours)

vascular walls to macromolecules is essentially unaltered, but the increased net pressure forces excess fluid of very low protein content into the tissues. As the fluid accumulates in the tissues the tissue hydrostatic pressure rises causing the lymph flow to increase (Taylor et al., 1973). Oedema occurs when the rate of fluid accumulation exceeds the rate of removal by the blood vessels and lymphatics. Regardless of the net outflow there will still be a greater inflow of small molecules into the blood vessels than into the lymphatics (Casley-Smith and Piller, 1974). In this type of oedema the lymphatics play only a small, though vital, part. If the oedema-reducing ability of the benzopyrones is solely dependent on increased proteolysis they will have no effect in low-protein oedemas.

The saline and amino acid injections were used to simulate low-protein oedemas where the initial cause, the venous pressure or plasma concentration, has returned to normal. Coumarin had no significant effect on their rates of resolution. Coumarin does cause injury to the vascular endothelium and increases the outflow of fluid and protein (Casley-Smith, 1976b). The fact that this does not significantly reduce the rate of resolution when proteolysis is not involved, implies that the injury has no real effect on the drug's oedema-reducing ability. Saline can be removed into the blood more rapidly than

amino acids because of the relatively smaller size of the constituents.

High-Protein High-Flow Oedema:

High-protein high-flow oedemas are caused by traumas or diseases which cause breaks or the appearance of large vacuoles (Casley-Smith and Window, 1976) in the blood capillary endothelium. These cause excessive amounts of protein and fluid to enter the tissues. As in low-protein oedemas, the lymph flow will increase in an attempt to remove the additional fluid and restore the balance between inflow and outflow. Because of the concentration gradient, there can be no net removal of extravascular plasma proteins by the blood. They must be removed by the lymphatics and tissue proteolysis. There is evidence suggesting that normally the lymphatics play the most important part (Casley-Smith, 1982a, b).

The plasma injections were used to simulate high-protein high-flow oedemas where the vascular permeability has returned to normal. Initially the lymphatics would be functioning at their maximum in response to the increased protein and fluid. In 1962, Paz and Spector showed that the migration of monocytes into injured regions is biphasic, reaching maxima at 3 - 5 hours and again at 8 - 24 hours. This probably explains

the biphasic nature of plasma removal seen in this experiment. Until the number of monocytes was greatest, tissue proteolysis may not have increased sufficiently to play a significant part in the removal of the excess protein and fluid. Under these conditions even the increased lymph flow would not be able to remove the excess (Földi and Casley-Smith, 1978). Lymph flow would increase to maximum almost immediately due to the increased tissue and osmotic pressures (Földi and Casley-Smith, 1978) and the initial lymphatics would discharge much lymph into the relatively empty collecting lymphatics. These collecting vessels would become full. Although they would pump more and remove some of this excess, there would still be a greater impedance to flow, especially, at the nodes (Casley-Smith, 1982a). Hence lymph flow would reduce after a time. By four hours the macrophages would have reached maximum numbers and proteolysis would be increasing. This method of removal is more gradual (Casley-Smith, 1982a) and would not compensate for the reduced lymph flow. Hence after four hours the rate of resolution would be slower.

Coumarin had no effect on plasma removal until after four hours. Although coumarin can increase lymphatic flow in normal tissue it has no effect when the lymphatics are functioning optimally (Casley-Smith, 1976b). Proteolysis will remove some of the accumulated

protein and allow some of the fluid to be removed into the blood vessels. This will reduce the lymphatic load and the lymph flow may begin to increase again. It seems unlikely that coumarin could cause any further increase at this time. Coumarin may have caused an increase in the rate of migration of monocytes but its effect would have been masked by the rapid rate of lymphatic removal. It may have caused an increase in the number of monocytes arriving in the inflamed area although there is evidence to suggest that the accumulation of monocytes is due to their selective retention (Harris, 1954). Benzopyrones would be able to increase this retention by reducing connective tissue permeability (Fabianek and Herp, 1965). Coumarin also stimulates macrophage proteolysis (Casley-Smith, 1976b; Dunn et al., 1977; Piller, 1977c, 1978). The finding that these effects of coumarin are not significant until after four hours is consistent with the biphasic nature of plasma removal hypothesized above. It also implies that the drug's oedema-reducing ability is dependent on stimulated proteolysis.

There is, however, evidence to suggest that the benzopyrones may also stimulate proteolysis by fibroblasts and neutrophils (Piller, 1976e, 1977b). Other anti-inflammatory drugs stimulate neutral proteolytic activity in fibroblasts (Houck and Sharma, 1969), beginning at two hours after administration and reaching a

maximum at four hours. If they are similarly stimulated by coumarin, the time of maximum activity would also agree very well with the biphasic plasma removal. Since coumarin causes injury to the vascular endothelium and increases the outflow of fluid and proteins it is possible that in the first four hours this outflow was significant and it was balanced by increased proteolysis. Coumarin would only appear to have no effect during that time. At four hours when proteolysis would be high and the vascular permeability returned to normal (Piller, 1977d), the effect of coumarin would become obvious. These changes could have occurred, but the fact that the increased outflow caused by the drug had no significant effect on the removal of amino acids, PVP or saline indicates that the outflow would not have been significant in plasma treated groups either. This further implies that any increased proteolysis caused by coumarin in the first four hours is also insignificant.

The significantly faster removal of amino acids compared with plasma (with or without coumarin) implies that the splitting of proteins does increase the rate of resolution of oedema. Amino acid removal does not equal the proteolysis phase of coumarin-stimulated plasma removal because of the time delays required to digest and split the proteins into smaller fragments.

The removal of PVP was expected to parallel that of plasma proteins, without coumarin. The rate should have been even slower because it is not metabolizable. Some PVP molecules would be taken up by macrophages, fibroblasts or neutrophils; inactivating them as far as further digestion is concerned. The oedema would be reduced appropriately by this uptake. The rate of removal into the blood vessels should be very slow due to the large molecular size. The lymphatic load should be at least as great as that caused by the plasma injections and the same loss of function should occur. There was no slowing of the rate of removal, indicating that the lymphatics were not overwhelmed. It seems that the PVP molecules must have been removed into the blood more rapidly than expected. As the molecules are long and narrow they may readily pass through the inter-endothelial junctions and tissue channels; and certainly their concentration gradient was directed into the blood vessels. This PVP molecule and any of smaller molecular weight do not behave as non-metabolizable plasma proteins, indicating that they are not suitable as control molecules for proteolysis experiments. The finding that coumarin had no significant effect on the removal of PVP is further evidence which indicates that the drug's oedema-reducing ability is directly related to its ability to increase proteolysis.

3.1.6 Conclusions:

The resolution of high-protein oedema (plasma) was biphasic. The first phase was more rapid and was probably dependent on maximum lymphatic function. The second phase was slower and continued until the tissues returned to normal. It was probably dependent on proteolysis and began when the more central lymphatic collectors became filled, causing local lymph flow to be progressively reduced. The oedema caused by the injection of amino acids was resolved more rapidly than that caused by plasma, showing that the products of proteolysis are removed from the site of oedema more rapidly than the proteins themselves.

The non-metabolizable PVP was not removed more slowly than the plasma but at the same rate as the amino acids; indicating that it was not a suitable control molecule for plasma proteins. The physiological saline was removed more rapidly than all the other injectants.

Coumarin had no effect in the non-protein oedemas, nor in the first four hours after plasma injection. It did increase the rate of resolution in the second (or proteolysis) phase of plasma removal. This further confirms that coumarin enhances proteolysis and reduces high-protein oedema.

3.2 Further Investigations of an Experimental Model of Acute Lymphoedema:

3.2.1 Introduction:

The conclusions drawn from the previous experiment indicated the necessity to investigate more closely the role of lymphatic function in the resolution of high-protein oedemas. The conclusion that the first, more rapid, phase of removal was probably due to maximum lymphatic function was investigated. In order to do this the resolution rates of the oedemas needed to be studied during lymphostasis. Lymphostasis has often been induced using an experimental procedure described by Casley-Smith and Piller (1974). However this procedure for lymphostasis causes lymphoedema and has often been used as a model for acute lymphoedema of the rat hind-limb (Piller and Casley-Smith, 1975; Piller, 1976d; Casley-Smith and Piller, 1977; Piller, 1977a, e). Therefore, it is possible that any effect of lymphostasis in slowing down the rate of removal of oedema would be complicated by an increased swelling due to lymphoedema. A review of the literature, where this model was used, yielded no information on the rate at which lymphoedema developed or its extent. It was merely left 12 hours, acute lymphoedema assumed, and other studies performed. There was also no information regarding the rate of swelling

and/or resolution after that time. Therefore, before continuing with the artificial oedema experiments it was necessary to run a complete study of the time course of the effects of this experimental procedure for lymphostasis.

3.2.2 Creation of Lymphostasis:

Twenty-one male hooded rats of average weight 400±50 gm were randomly assigned to two experimental groups.

Both hind limbs of each rat were closely shaved approximately 24 hours before commencing the experiment. The animals were anaesthetized with intraperitoneal injections of 0.75 mls/100 gm of a 10 percent solution of Sagatal (May and Baker, Aust.) in 10 percent A. R. ethyl alcohol. The first group of eleven animals were given lymphostasis in the right hind limb, under sterile conditions, according to the following method as described by Casley-Smith and Piller (1974).

An incision was made following the line of the groin, on the upper inside region of the hind limb. The glandular and subcutaneous tissues were carefully separated to expose the femoral vein, artery and nerve. A small superficial cutaneous vein was ligated. The femoral

vessels were then completely isolated from the surrounding fascia by careful disection for about 15 mm. Extreme care was taken to ensure that all fascia was removed to eliminate perivascular lymph vessels. Two overlapping ligatures were then placed around the remaining tissues. The first was passed under the femoral vein and artery, through the skin to come out in the back of the thigh. It was then tied very firmly in the groin completely encircling the muscles. The second ligature overlapped the first, passing under the vein and artery in the opposite direction. It was then passed through the muscle and out through the skin on the back of the leg. This ligature was tied very firmly on the upper outer side of the leg, as close to the abdominal wall as possible. When tying the ligatures, care was taken to ensure that the femoral vessels were not constricted in any way. Only the femoral vein, artery and nerve were left uncompressed. The wound was closed with continuous sutures and the external surface was liberally sprinkled with Cicatrin Amino Acid Antibiotic Powder (Burroughs Wellcome, Aust.).

The second group of ten animals was given lymphostasis of the right hind limb but the ligatures were tied less tightly, around a pencil of eight mm diameter. This was done to determine what differences might arise due to any arbitrariness in the tightness of the ligatures. Sham operations were performed on the left hind limbs of all animals.

3.2.3 Plethysmography:

The legs were marked around the upper thigh. The limb volumes, to this mark, were measured by plethysmography immediately after the operation, at hourly intervals for six hours and then at the times specified in Table 3.2. At each time the average of three measurements was recorded. After each measurement the leg and wound area were carefully wiped dry and sprinkled with Cicatrin to minimize the chances of infection. Measurements ceased when the leg volume returned to normal, the wound became infected, the ligatures began to wear through the skin, or until other complications arose.

3.2.4 Percentage Increase in Limb Volume:

The percentage increase from the normal right hind limb volume was calculated for each rat at each time interval. The mean percentage increase and its standard error were then calculated at these times for each group. Measurements outside the range of the (mean$\pm$twice the standard error) were rejected and the means and standard errors recalculated. The results are shown in Table 3.2 and graphed in Figure 3.3. The sham operated legs showed no changes in volume.

Table 3.2: Mean Percentage Increase from Normal Limb Volume of Rats with Lymphostasis:

(The t-STAT (NORMAL) is the result of the Student's t-test comparison with the normal limb volume. The t-STAT (LESS V TIGHT) is the result of the Student's t-test comparison between the types of ligatures. The significance of the differences between the means is indicated by:

n.s. = not significant;

* = $0.05 > p > 0.01$;

*** = $p < 0.001$.

	LESS TIGHT LIGATURES				TIGHT LIGATURES				SIG OF
TIME (HOURS)	MEAN	STAND. ERROR	NO	SIG OF t-STAT (NORMAL)	MEAN	STAND. ERROR	NO	SIG OF t-STAT (NORMAL)	t-STAT (LESS V (TIGHT)
1	1.08	0.51	10	*	1.19	0.66	10	n.s.	n.s.
2	3.41	1.18	10	***	2.14	0.86	9	*	n.s.
3	4.65	1.2	10	***	6.41	1.66	11	***	n.s.
4	5.34	1.17	9	***	5.82	1.80	10	***	n.s.
5	6.59	1.10	8	***	6.68	1.93	10	***	n.s.
6	8.64	2.16	9	***	7.75	2.35	10	***	n.s.
8	8.97	3.12	9	***	15.05	3.77	10	***	n.s.
10	8.61	2.26	9	***	18.11	4.60	10	***	n.s.
12	7.64	1.70	9	***	24.23	5.72	11	***	*
20	7.31	1.70	8	***	22.37	5.01	10	***	*
22	7.13	2.26	8	***	28.69	6.04	11	***	***
24	8.38	2.21	8	***	29.54	5.95	11	***	***
26	12.67	3.16	9	***	29.41	5.91	11	***	*
28	13.83	3.25	9	***	29.19	6.06	11	***	n.s.
30	14.32	3.13	9	***	29.83	5.77	11	***	*
32	14.52	3.36	9	***	29.38	5.73	11	***	*
34	-----	----	-	---	27.04	5.55	11	***	-
36	-----	----	-	---	27.53	5.65	11	***	-
46	19.90	4.59	9	***	31.25	5.45	11	***	n.s.
50	22.69	4.69	9	***	32.35	5.83	11	***	n.s.
52	23.42	4.96	9	***	33.73	5.85	11	***	n.s.
54	23.61	5.02	9	***	34.66	6.22	11	***	n.s.
60	-----	----	-	---	35.26	6.04	11	***	----
67	26.52	4.86	9	***	-----	----	--	---	----
70	29.43	6.13	8	***	36.63	5.09	11	***	n.s.
73	-----	----	-	---	39.29	5.21	11	***	----
76	27.08	5.49	8	***	41.59	5.86	11	***	n.s.
95	37.38	7.04	8	***	47.20	6.78	10	***	n.s.
100	41.74	7.95	9	***	42.33	6.72	8	***	n.s.
120	46.20	9.28	9	***	42.18	7.04	8	***	n.s.
125	45.02	8.96	9	***	-----	----	-	---	----
144	37.83	8.08	9	***	44.35	6.34	6	***	n.s
165	19.83	5.48	8	***	27.78	3.65	5	***	n.s.
190	14.78	7.09	6	n.s.	10.74	6.42	3	n.s.	n.s.
215	5.92	3.87	5	n.s.	8.34	8.34	2	n.s.	n.s.

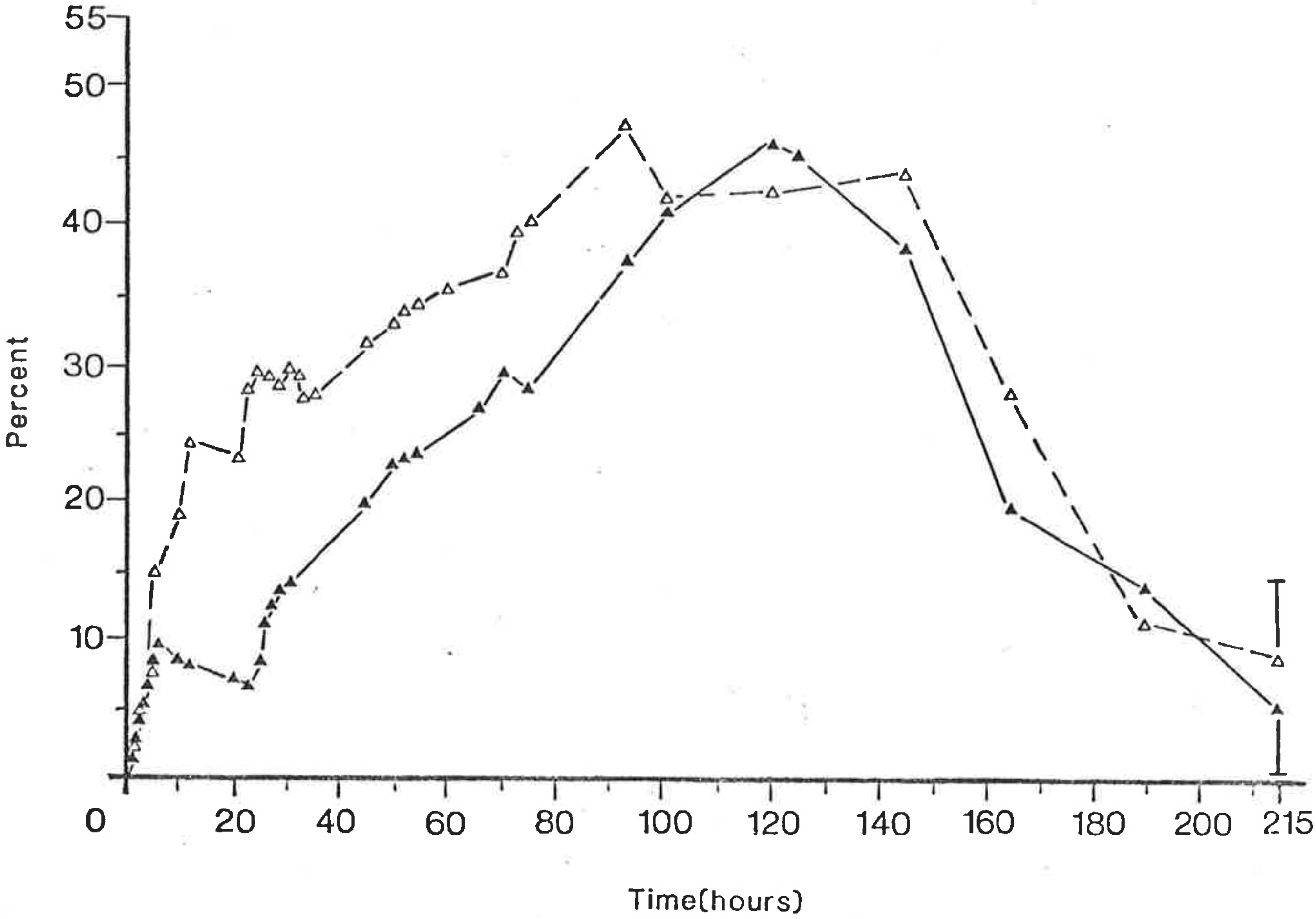


Figure 3.3:

The mean percentage increase from normal limb volume, of animals with lymphostasis (Table 3.2).

The symbols indicate:

△ - tight ligatures;

▲ - less tight ligatures.

The grand standard errors of all the mean percentage increases were ± 5.358 percent for the tight ligatures and ± 4.77 percent for the less tight ligatures. These are indicated by:

△ and ▲ respectively.

The limbs given lymphostasis were observed to swell quite considerably very quickly. As time progressed complications arose in some animals and readings were stopped in those cases. For example, in most cases complications arose after approximately 165 hours (about seven days). At this time the ligatures began to wear through the skin even if the swelling was disappearing. Also around this time several animals chewed their ligatures loose. No animals with the less tight ligatures did this, whilst four of the tight did. More of the less tight ligatures wore through the skin (four). The tightness of the ligatures made no difference to the numbers of animals which returned to normal without complication (four of each). The tight ligatures did lead to two cases of infection at about 100 hours whilst the less tight had no such complications. These infected legs were swollen so large that the ligatures were cut, for humane reasons. This may have been premature as some cases which were swollen to about twice their normal volume, did return to normal later (for both tight and less tight ligatures).

Student's t-test comparisons were made between the means for the two groups and also between each group and the normal (Table 3.2). The degree of tightness of the ligatures had no significant effect on the percentage increase in limb volume until 12 hours. The tight

ligatures caused significantly greater increases from 12 hours until about 34 hours. The tight ligatures caused significant increases from normal limb volumes, from as early as two hours until they returned back to normal by 190 hours. The less tight ligatures caused the percentage increase in limb volume to be greater than zero from one hour and it also returned to normal by 190 hours.

For the tight ligatures the mean percentage increase rose rapidly until approximately 22 hours, followed by a plateau from about 22 - 32 hours. This was followed by a slight reduction from 32 - 34 hours. It then rose less rapidly but at a constant rate, to the maximum before returning to normal. The less tight ligatures caused the same initial rapid increase but reached a plateau earlier at about six hours. The limb volumes appeared to remain stable for approximately four hours followed by a reduction from 12 - 22 hours. They then appeared to increase more rapidly than the tight ligatures to peak shortly after. They returned to normal at a similar rate.

3.2.5 Regression Analysis:

The mean percentage increase from normal limb volume (as illustrated in Figure 3.3) seem to approximate a series of straight lines, for both tight and less tight

ligatures. Therefore, straight line regression analyses were performed on these results for the time intervals suggested above and the results are shown in Table 3.3. It was not possible to fit straight lines to the plateaux. The data variables show a high degree of linear association. This is indicated by the correlation coefficients which are all much greater than 0.75 and almost the perfect ± 1. The analysis will make it possible to predict the relationship between the percentage increase in limb volume and time. The lines were then plotted to find the points of intersection for continuity and this is illustrated in Figure 3.4. Student's t-test comparisons between the slopes were performed and the results are shown in Table 3.3 and Figure 3.4.

The observations described above were confirmed by the regression analyses. For tight ligatures the rate of change from 0 - 22 hours was significantly faster than that from 34 - 95 hours. The actual rate of change from 95 - 215 hours was not significantly different from that of 34 - 95 hours but it was in the opposite direction (i.e. from 95 - 215 hours the swelling decreased at the same rate as it increased from 34 - 95 hours). This pattern was identical to that found for the less tight ligatures.

Table 3.3: Linear Regression Analysis of the Relationship between Percentage Change in Limb Volume and Time.

	TIME INTERVAL (hours)	RATE OF CHANGE (% per hour)	CORRELATION COEFFICIENT	SIG OF t-STAT[a]
TIGHT LIGATURES	0 - 22	1.46	0.96	***
	34 - 95	0.32	0.99	b
	95 - 215	- 0.34	0.93	
LESS TIGHT LIGATURES	0 - 6	1.48	0.99	***
	22 - 120	0.38	0.99	b
	120 - 215	- 0.45	0.98	

a. The significance of the difference between the slopes of the lines using the Student's t-test: *** = $p < 0.001$

b. The actual numerical rates of change are not significantly different but they are in opposite directions.

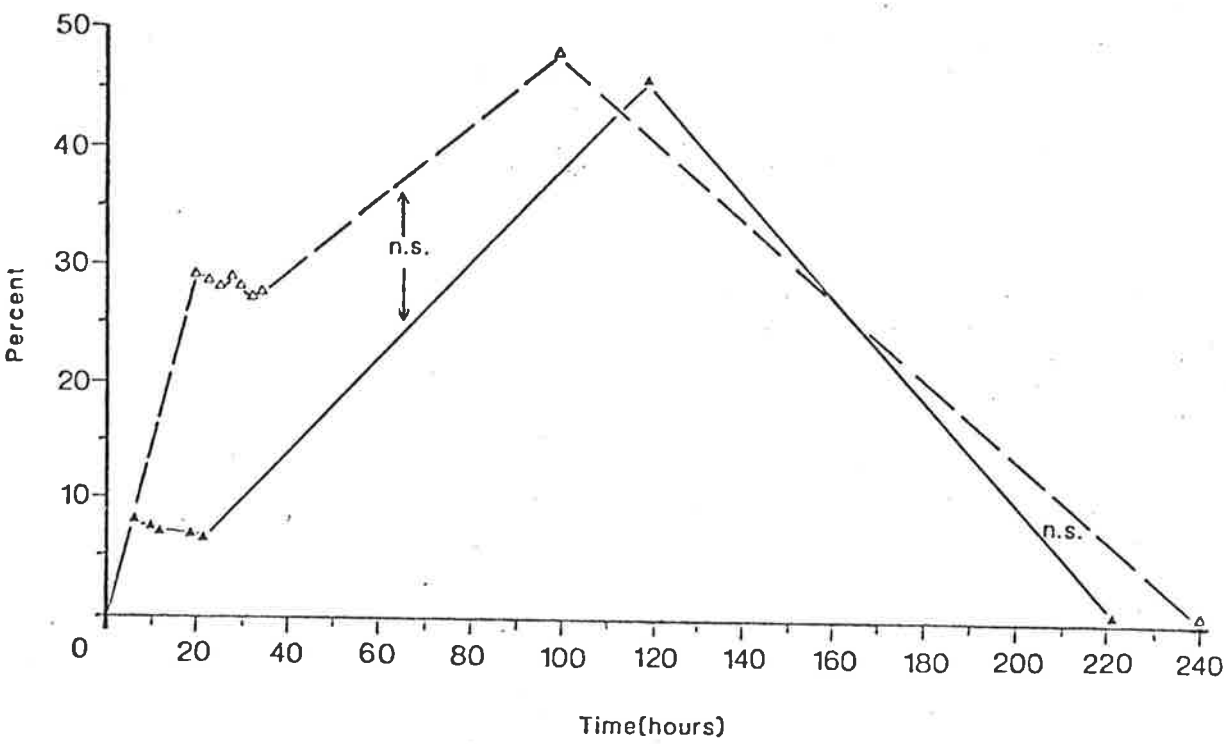


Figure 3.4:

The rates of change in percentage increase from normal limb volume (of animals with lymphostasis) are graphed against time. These rates were obtained by straight line regression analysis of the percentage data as shown in Table 3.2 and Figure 3.3.

The symbols indicate:

△ - tight ligatures;

▲ - less tight ligatures.

The rates of change for the different ligatures were compared using the Student's t-test. The significance of the t-statistic is indicated by:

n.s. - not significant.

In addition, there were no significant differences between the rates of change caused by the tight and less tight ligatures for the comparable time intervals (i.e. 0 - 22 compared with 0 - 6; 34 - 95 compared with 22 - 120 and 95 - 215 compared with 120 - 215). The plateau and slight reduction lasted for 12 hours with tight ligatures and for 16 hours with the less tight ligatures.

This appears to indicate that this lymphostasis leads to four separate, quite distinct phases in the development of acute lymphoedema; independently of the degree of tightness of the ligatures. The tightness of the ligatures only influences the duration of the first two phases.

3.2.6 Lymphoedema:

This experimental procedure for lymphostasis produces a useful model of acute lymphoedema. It can not, however, be used to study chronic lymphoedema for a number of reasons. Firstly, there is a tendency for the oedema to be resolved. This could occur as the result of lymphatic regeneration (Gray, 1940), the development of a collateral lymphatic circulation (Goffrini and Bobbio, 1964), the formation of lymphatico-venous shunts (Malek, 1972) or increased proteolysis. It is possible that chronic lymphoedema could develop later due to

insufficiencies in the natural repair mechanisms but, as mentioned below, the nature of this experimental procedure implies that the condition can not be continued for much longer than a week. The tying of the ligatures on the outside of the skin causes complications, such as rubbing the skin raw which in turn leads to infection, or the animal becomes so agitated that it chews the ligatures (or its own legs) to the point of causing the experiment to be terminated.

The tightness of the ligatures is unimportant to the rates of development of lymphoedema. However, the less tight ligatures allow the intermediate latent phase to occur earlier after a much smaller initial increase in leg volume. They also allow this latent phase to last slightly longer.

3.2.7 The Four Phases of Acute Lymphoedema:

During the first phase the protein and fluid would accumulate in the tissues at a constant rate. The tight ligatures cause this phase to last for 22 hours. At this time macrophage numbers are probably at a maximum and proteolysis could stabilize the situation temporarily (for 12 hours). This is the second phase. For a short time, proteolysis may allow the oedema to be resolved, at the same rate as new material accumulates in the tissues.

During the third phase proteolysis would still be occurring but its effectiveness would gradually be reduced. The macrophages would no longer be able to compensate for the increasing oedema. Eventually a point is reached when the system is able to resolve the oedema and return the tissues to normal (or apparently normal). Lymphatic removal could be increased, for the reasons described above, or macrophage numbers could be further increased hence increasing proteolysis. However, it seems most likely that some combination of these effects allows the return to normal. With the less tight ligatures it is probable that lymphatic function was not completely stopped, initially. Combined with proteolysis, the rate of formation of lymphoedema can be temporarily halted at a much earlier stage. However, this residual lymphatic removal is rapidly overwhelmed and the third and fourth stages of acute lymphoedema are identical with those caused by the tight ligatures.

It is interesting to note, that both types of ligature caused the peak to be reached after the same period of time (95 - 98 hours) and that the maximum increases were approximately equal (46 - 48 percent). Possibly this is the period of time required for the formation of the new lymphatic circulation. It is possible that stretching of the tissues gradually increases the total tissue pressure. The tissue

hydrostatic pressure would also increase and oedema formation would be gradually reduced. At the point where proteolysis again becomes effective the rate of formation of lymphoedema would be reduced. It is most likely that a combination of these effects allows the return to normal.

3.3 The Effect of Lymphostasis on Oedema Resolution:

3.3.1 Introduction:

As concluded from Section 3.1, the resolution of high-protein oedema was biphasic; with the first more rapid phase probably dependent on maximum lymphatic function and unaffected by the benzopyrone, coumarin. To confirm this role of the lymphatics, the resolution rates of the artificially induced protein and amino acid oedemas were studied during lymphostasis. The experimental procedure for lymphostasis described in Section 3.2.2 caused statistically significant increases in leg volumes from as early as one hour after surgery. Therefore, when using this model in these resolution studies it was necessary to compensate for the increase in leg volume due to the development of lymphoedema.

3.3.2 Injection and Measurement Procedures:

Male hooded rats of average weight 360±40 gm were

used for this experiment. All animals were given lymphostasis of the right hind limb as described in Section 3.2.2. The ligatures were tied tightly. These animals were divided into four groups and received artificial oedemas by the injection of homologous rat plasma, six percent amino acids, six percent PVP in physiological saline or physiological saline alone. Each of these groups was divided into two subgroups, one of which was treated with coumarin. The injection solutions and procedures were identical with those described in Section 3.1.2. Limb volumes were measured by plethysmography immediately after the surgery for lymphostasis (i.e. immediately before the injections), then half hourly for four hours with plasma, and for four to six hours with the amino acids, PVP and saline. Again, at each time the average of three measurements was recorded.

Every volume (V) measurement for each rat was corrected to compensate for the increased swelling due to the development of lymphoedema, using the percentage increase in limb volume for that particular time (t) obtained in Section 3.2.4. The correction was calculated as follows; corrected V_t = measured V_t - (percentage increase in V at t due to lymphoedema) x (initial V + 2.0 mls injected i.e. V_0).

Exponential decay curves $V_t = V_0 e^{-kt}$ were

fitted to the corrected data for each rat. Mean rates of resolution were calculated for each group and were compared using the Student's t-test.

3.3.3 The Rates Of Resolution of Oedema:

The mean corrected volume changes for each treatment are shown in Figure 3.5.

The exponential decay curve gave a good fit (correlation coefficients > 0.75) to the experimental data for all groups and the mean rates of resolution appear in Table 3.4.

Coumarin had no significant effect on the rates of removal of saline, PVP, amino acids or on the first four hours of plasma removal (Table 3.4). Therefore, the mean rate of resolution for each treatment without coumarin has been combined with the mean rate for that treatment with coumarin (Table 3.4 and Figure 3.6) as described in Section 3.1.4 (e.g. saline$\pm$coumarin). The statistical significance of the difference between each pair of treatment means is also illustrated in Figure 3.6.

Again, saline was removed more rapidly than all other injectants and there was no significant difference between the rates of removal of plasma (0 - 4 hours) and

Figure 3.5:

The mean volume of injectant remaining in the legs as measured by plethysmography for animals with lymphostasis and proteolysis.

The curves in part A illustrate the untreated responses to oedema whilst those in part B illustrate the coumarin treated responses.

The symbols indicate the type of injectant:

⊙ - Plasma;

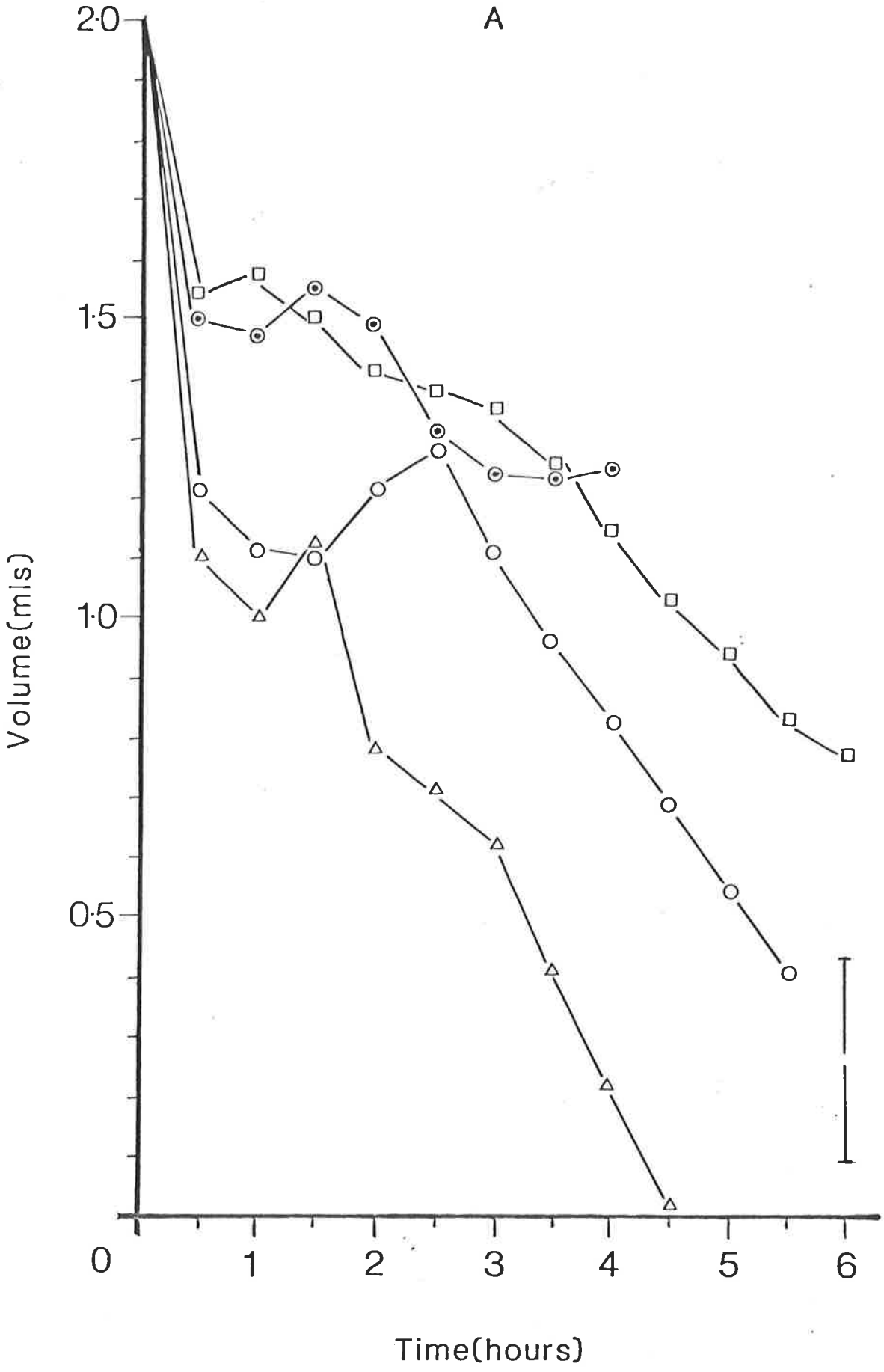
□ - Amino Acids;

○ - PVP;

△ - Saline.

The grand standard errors of all the mean volumes were ± 0.1680 mls for the untreated groups and ± 0.1481 mls for coumarin treatment.

These are indicated by: I


A
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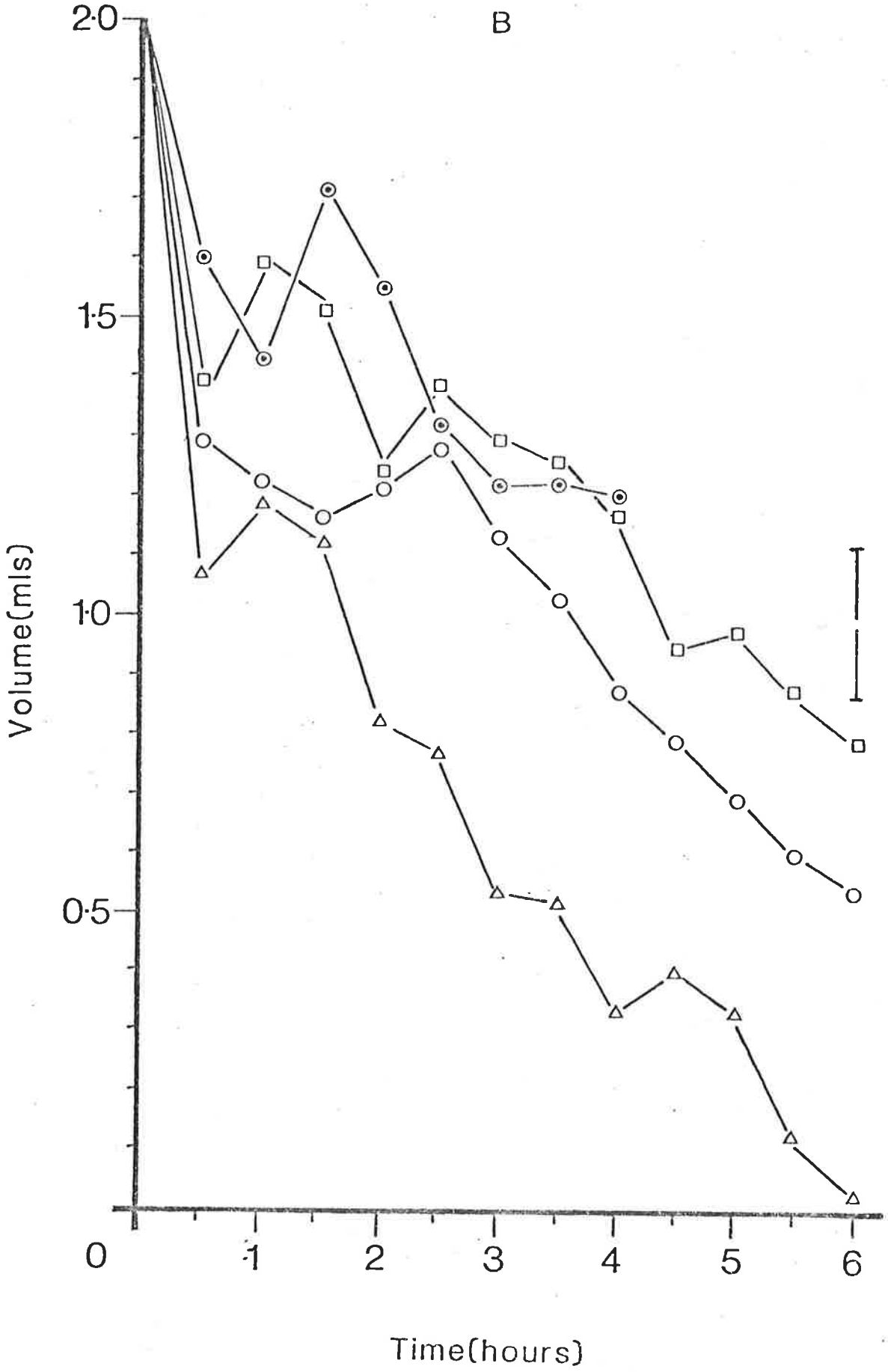
4
5
6
Volume(mls)
Time(hours)


B
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3
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5
6
Volume(mls)
Time(hours)

Table 3.4: The Rates of Resolution (k) of Oedema in Rat Legs With Lymphostasis and Normal Proteolysis.

TREATMENT	MEAN RATE ($hours^{-1}$)	STANDARD ERROR	NUMBER	TIME TO NORMAL (hours)	SIG OF t-STAT[a]
Plasma (0 - 4 hrs)	0.0189	.0021	9		
Plasma + Coumarin (0 - 4 hrs)	0.0185	.0025	8		n.s.
Amino Acids	0.0224	.0029	10	9.95	
Amino Acids + Coumarin	0.0210	.0026	9	10.65	n.s.
PVP	0.0316	.0042	10	7.06	
PVP + Coumarin	0.0297	.0045	10	7.52	n.s.
Saline	0.0486	.0070	9	4.59	
Saline + Coumarin	0.0396	.0069	10	5.63	n.s.
COMBINED MEANS WITH AND WITHOUT COUMARIN					
Plasma ± Coumarin (0 - 4 hrs)	0.0189	.0015	17		
Amino Acids ± Coumarin	0.0217	.0019	19	10.29	
PVP ± Coumarin	0.0309	.0029	19	7.23	
Saline ± Coumarin	0.0439	.0049	19	5.1	

a. The significance of the difference between the means using the Student's t-test: n.s. = not significant.

Figure 3.6:

The mean resolution of oedema (for animals with lymphostasis and proteolysis) obtained by fitting:

$$\ln V_t = \ln V_0 - kt$$

to the volume data for each rat. The means for coumarin and non-coumarin treated groups have been combined for saline (△), PVP (O), Amino Acids (□) and Plasma 0 - 4 hours (⊙).

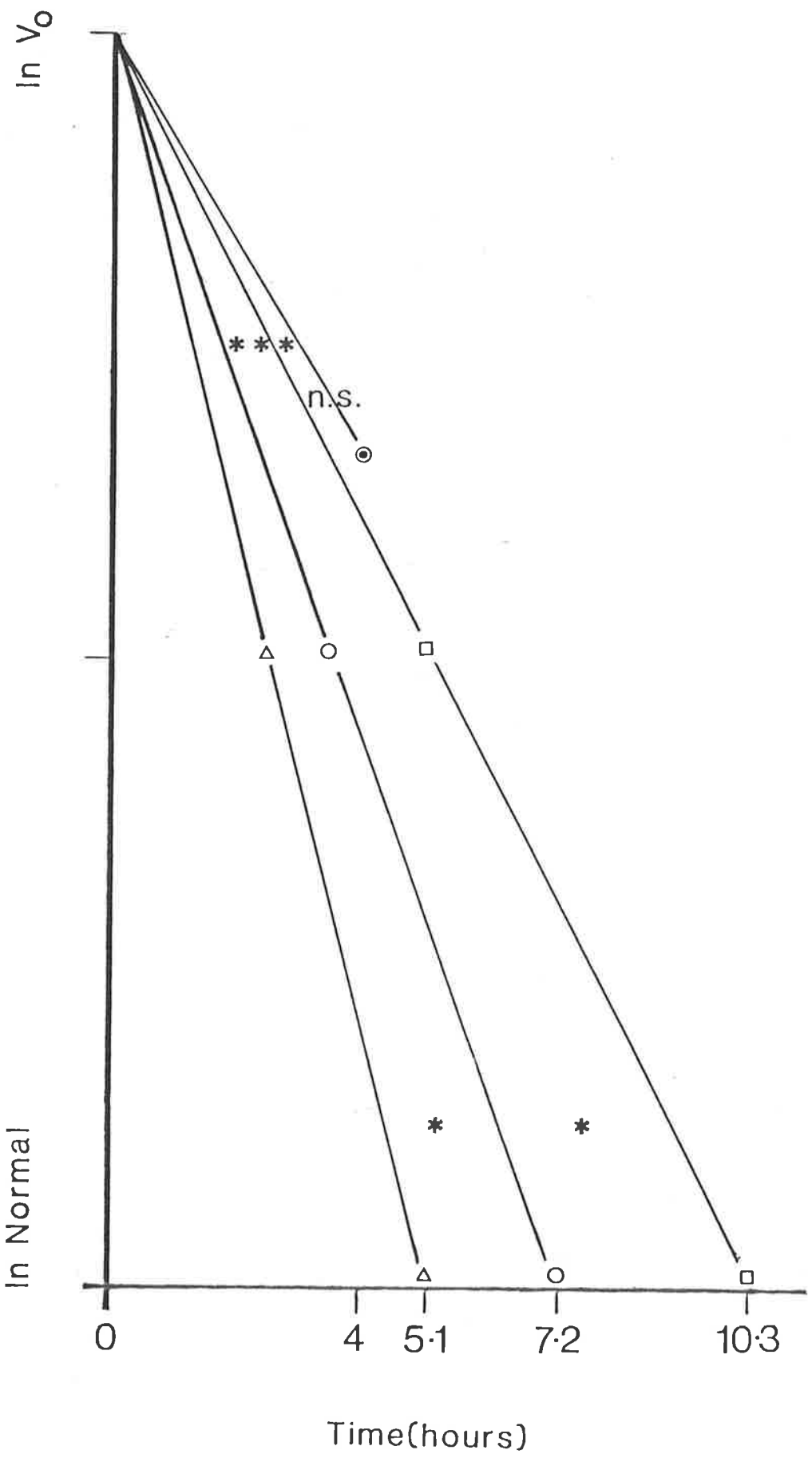
The mean rates of resolution for each group were compared using the Student's t-test. The significances of the t-statistic are indicated by:

n.s. = not significant,

* = $0.05 > p > 0.01$,

*** = $p < 0.001$.

The graphical representation allows a visual comparison of the rates of resolution of the different oedemas.


In V_o
***
n.s.
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In Normal
0
4
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7·2
10·3
Time(hours)

amino acids. PVP±coumarin was removed significantly faster than amino acids and plasma, but slower than saline. (The uncombined rates were not significantly different from the amino acids. Without coumarin, PVP was removed more rapidly than the plasma whilst with coumarin there was no significant difference.)

3.3.4. Discussion:

The Effect of Coumarin:

Again, coumarin had no effect on the rates of resolution of these low-protein oedemas. This was expected as the oedema-reducing effect of the benzopyrones is believed to be due to their ability to increase proteolysis and not to some other effect such as enhancing fluid removal.

Coumarin again had no effect on those first four hours of plasma removal indicating that significantly increased proteolysis had not commenced. The ineffectiveness of coumarin indicates that its oedema-reducing ability is not due to increased fluid or large molecule removal via the blood vessels.

The Effect of Lymphostasis:

Student's t-test comparisons were performed between each pair of treatments with lymphostasis and without. The results for the uncombined means were identical with those for the combined means and therefore only the latter are tabled here (Table 3.5).

Lymphostasis had no effect on the rates of resolution of the low-protein oedemas, simulated by the saline and amino acid injections, reinforcing the previous conclusion that the route for removal of these small molecules is via the blood vessels rather than the lymphatics.

Lymphostasis caused a significant reduction in the rate of resolution of high-protein oedema simulated by the injection of plasma. The rate was reduced by 40 percent. Hence the lymphatics do play a very vital part in those initial stages of oedema resolution. The fact that the oedema is still resolved at a faster rate than that seen from four hours onwards, and ascribed to proteolysis, suggests that some fluid was removed by the blood vessels. Some protein may have been removed by macrophages or other cells (such as fibroblasts or neutrophils) already in the tissues. This would allow some of the fluid to be removed by the blood vessels. The

Table 3.5: The Effect of Lymphostasis on Oedema Resolution.

	MEAN RATES OF RESOLUTION ($hours^{-1}$)		SIG OF t-STAT [a]
	NORMAL	WITH LYMPHSTASIS	
Plasma ± Coumarin (0 - 4 hrs)	0.0303	0.0189	***
Amino Acids ± Coumarin	0.023	0.0217	n.s.
PVP ± Coumarin	0.0305	0.0309	n.s.
Saline ± Coumarin	0.0500	0.0439	n.s.

a. The significance of the difference between the means using the Student's t-test: n.s. = not significant; *** = $p < 0.001$.

rate of removal of PVP was not altered by lymphostasis. This implies that the PVP molecules were indeed removed into the blood as suggested in Section 3.1.5 and that its behaviour does not parallel that of the plasma proteins.

3.4 The Effect of the Macrophages on Oedema Resolution:

3.4.1 Introduction:

In Section 3.1.6 it was concluded that the second, slower, rate of removal of high-protein oedema was due to proteolysis (perhaps by macrophages) and that the earlier faster removal was due primarily to the lymphatics. This second conclusion was justified to a certain extent by the results of Section 3.3.

However, for further clarification of both conclusions the experiment was repeated again but with the macrophages destroyed. This allowed their effectiveness in oedema resolution to be ascertained. The macrophages were destroyed by using the tridymite form of silica which is known to have selective toxicity for them, resulting in a 'substantial depletion of the total population' (Kessel, Monaco and Marchisio, 1963; Allison, Harington and Birbeck, 1966; Nadler and Goldfischer, 1970; Pearsall and Weiser, 1970).

3.4.2 Destruction of Macrophages:

Male hooded rats of average weight 360±40 gm were randomly divided into four groups.

The macrophages were destroyed by the intraperitoneal injection of a solution of the tridymite form of silica (average particle size 5 μm) in physiological saline (Piller, 1976e). The silica was suspended in the saline to give 10 mg/ml and then 1 ml of this solution was injected per 100 gm body weight, daily, for eight days prior to the simulation of oedema.

3.4.3 Injection and Measurement Procedures:

Each group was divided into two subgroups, one of which was treated with coumarin for three days prior to the oedema injections (Section 3.1.2). The experimental groups then received artificial oedemas by the injection of plasma, amino acids, PVP and saline as described in Section 3.1.2.

Limb volumes were measured by plethysmography immediately before the injections, then at 1, 2, 3, 4, 6, 12.5, 24 and 30 hours. Again, the average of three measurements was recorded and the exponential decay curve $V_t = V_0 e^{-kt}$ was fitted to the volume data for each

rat. This yielded the rate (k) of resolution of the oedema, i.e. the rate at which the leg volume returned to the initial value. The mean rates were calculated for each group and were compared using the Student's t-test.

3.4.4 The Rates of Resolution of Oedema:

The mean volume changes for each treatment are shown in Figure 3.7.

The exponential decay curve gave a good fit (correlation coefficients > 0.75) to the data for all groups. As in Section 3.1.4 separate curves were fitted to the data for the plasma injected animals; from zero to four hours and from four hours onwards. The mean rates of resolution appear in Table 3.6.

Coumarin had no significant effect on the rates of removal of saline, PVP, amino acids or on the first four hours of plasma removal (Table 3.6). Therefore, the mean rate of resolution for each treatment without coumarin has been combined with the mean rate for that treatment with coumarin (Table 3.6 and Figure 3.8), as described in Section 3.1.4 (e.g. saline$\pm$coumarin). The statistical significance of the difference between each pair of combined treatment means is illustrated in Figure 3.8. Coumarin did significantly increase the rate of removal of

Figure 3.7:

The mean volume of injectant remaining in the legs as measured by plethysmography for animals with normal lymphatics and without proteolysis.

The curves in part A illustrate the untreated responses to oedema whilst those in part B illustrate the coumarin treated responses.

The symbols indicate the type of injectant:

⊙ - Plasma;

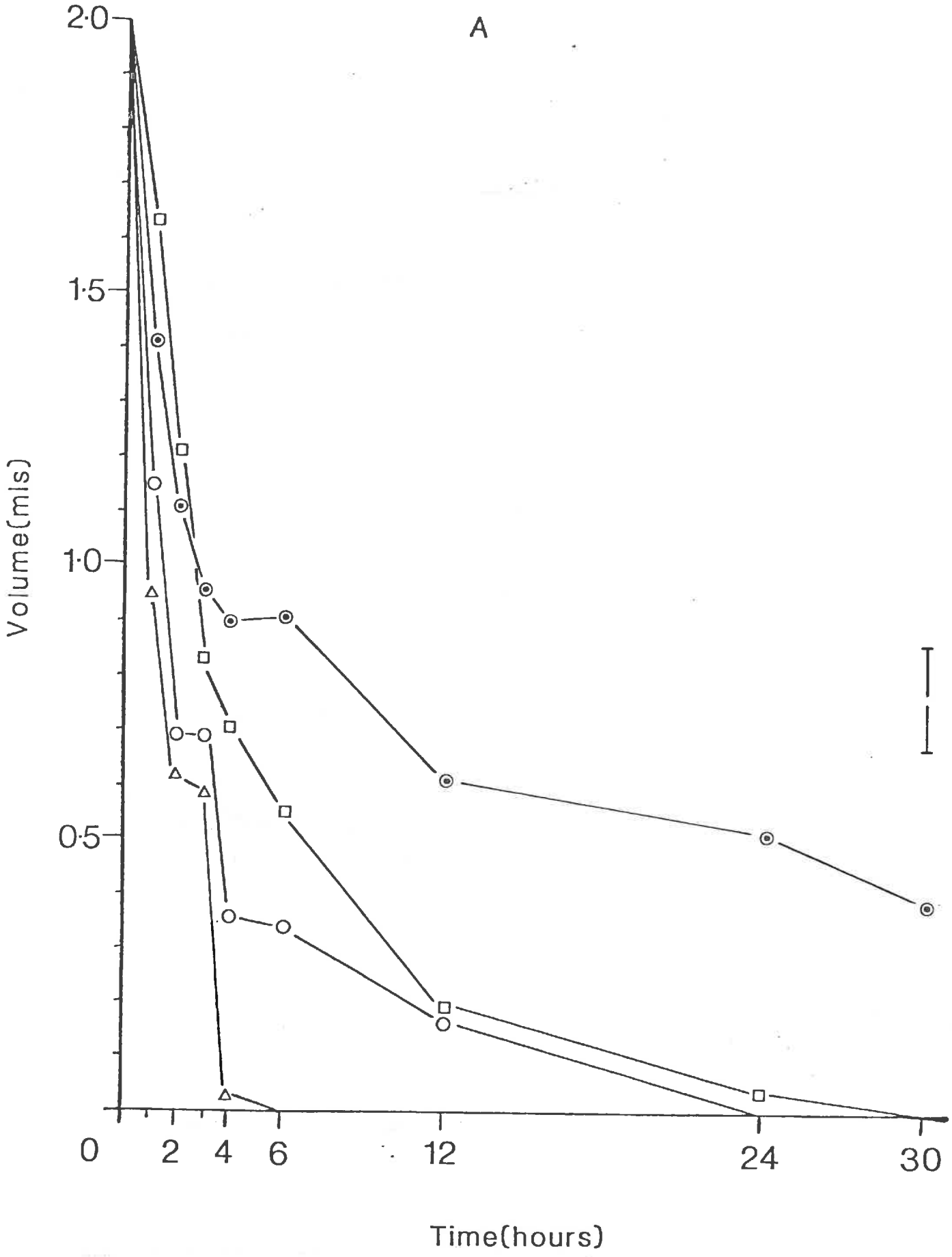
□ - Amino Acids;

○ - PVP;

△ - Saline.

The grand standard errors of all the mean volumes were ± 0.0936 mls for the untreated groups and ± 0.1279 mls for coumarin treatment.

These are indicated by: I


A
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Volume(mls)
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6
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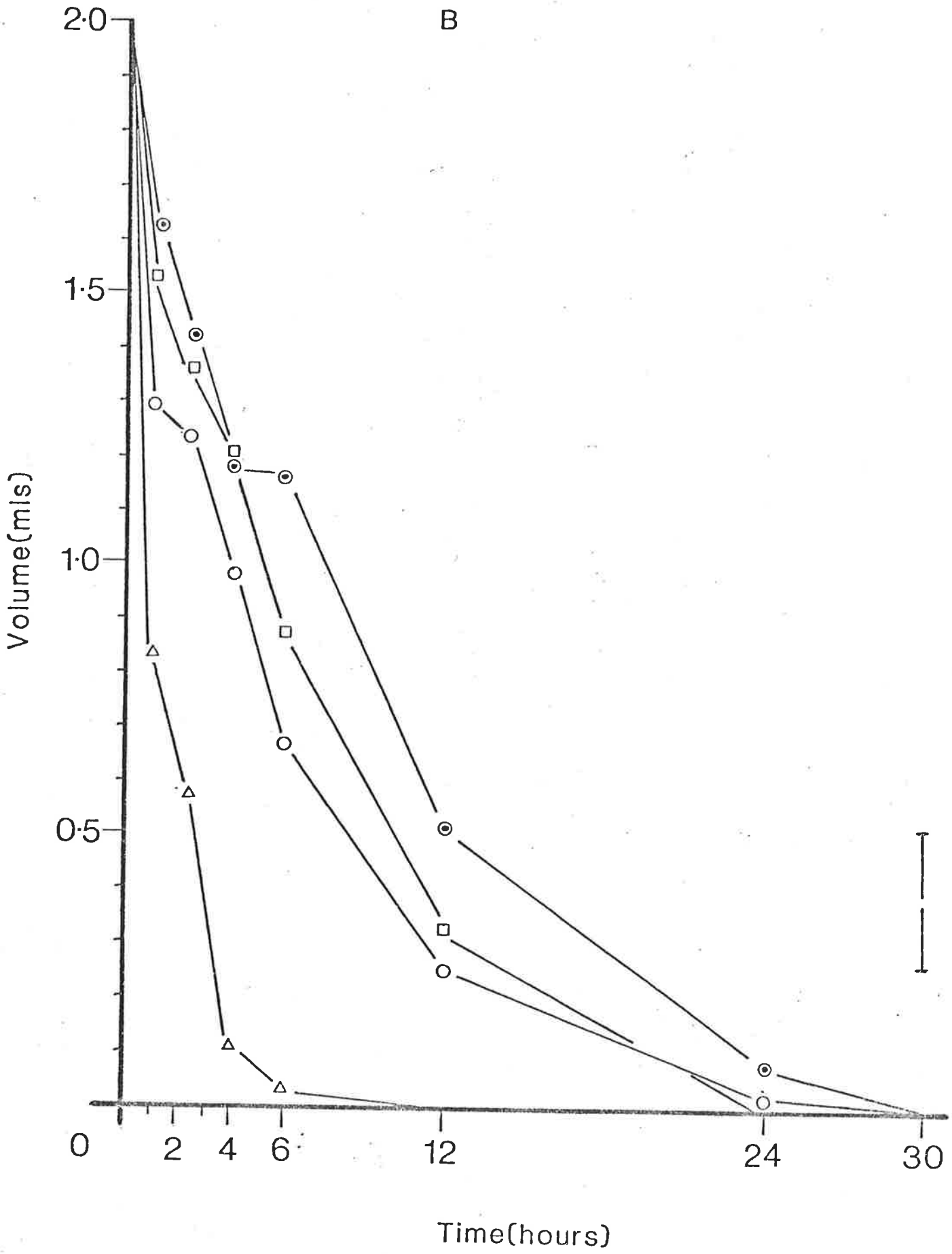
24
30
Time(hours)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1·0
0·5
Volume(mls)
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2
4
6
12
24
30
Time(hours)

Table 3.6: The Rates of Resolution (k) of Oedema in Rat Legs With Normal Lymphatics and Without Macrophages.

TREATMENT	MEAN RATE (hours^{-1})	STANDARD ERROR	NUMBER	TIME TO NORMAL (hours)	SIG OF t-STAT$_a$
Plasma (0 - 4 hrs)	0.0272	.0030	10		
Plasma + Coumarin (0 - 4 hrs)	0.0302	.0042	10		n.s.
Plasma (4 hrs ⟶)	0.0034	.0005	10	34.05	
Plasma + Coumarin (4 hrs ⟶)	0.0093	.0014	9	15.04	***
Amino Acids	0.0259	.0048	10	8.61	
Amino Acids + Coumarin	0.0192	.0025	10	11.61	n.s.
PVP	0.0300	.0044	10	7.45	
PVP + Coumarin	0.0258	.0063	9	8.66	n.s.
Saline	0.0563	.0066	9	3.96	
Saline + Coumarin	0.0441	.0075	9	5.07	n.s.
COMBINED MEANS WITH AND WITHOUT COUMARIN					
Plasma ± Coumarin (0 - 4 hrs)	0.0287	.0025	20		
Amino Acids ± Coumarin	0.0226	.0027	20	9.89	
PVP ± Coumarin	0.0285	.0037	19	7.83	
Saline ± Coumarin	0.0502	.0051	18	4.45	

a. The significance of the difference between the means using the Student's t-test: n.s. = not significant; *** = $p < 0.001$.

Figure 3.8:

The mean resolution of oedema (for animals with normal lymphatics and without proteolysis) obtained by fitting:

$$\ln V_t = \ln V_0 - kt$$

to the volume data for each rat. The means for coumarin and non-coumarin treated groups have been combined for saline (△), PVP (○), Amino Acids (□) and Plasma 0 - 4 hours (⊙).

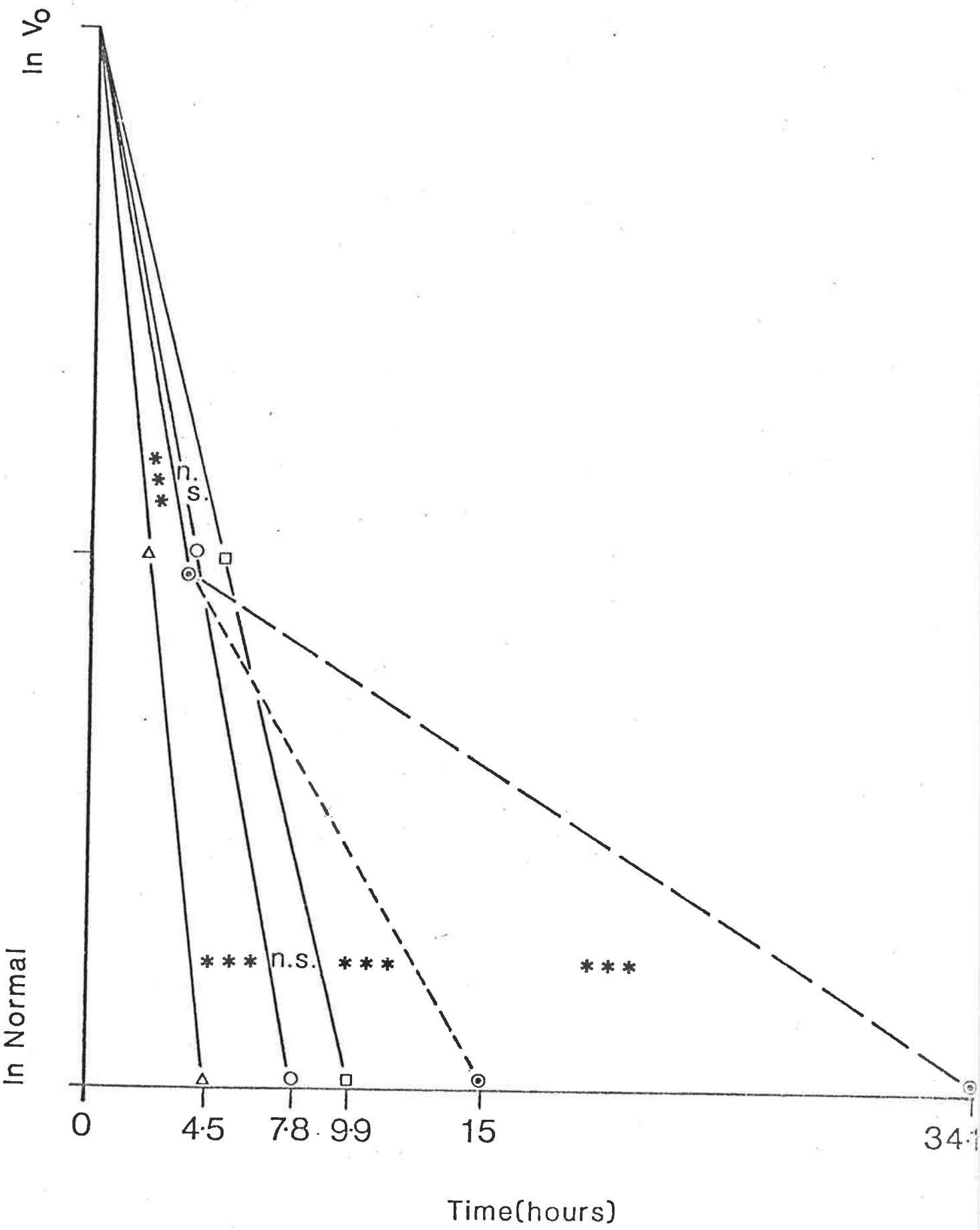
After 4 hours the resolution of plasma is illustrated by ——⊙——, whilst that for plasma+coumarin is illustrated by ——⊙——.

The mean rates of resolution for each group were compared using the Student's t-test. The significances of the t-statistic are indicated by:

n.s. = not significant,

*** = $p < 0.001$.

The graphical representation allows a visual comparison of the rates of resolution of the different oedemas.


ln Vo
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n.s.
*** n.s. ***
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7·8
9·9
15
34·1
Time(hours)

plasma after four hours ($p < 0.001$) as it did under normal conditions (Section 3.1.4).

As seen in Sections 3.1 and 3.3 saline was again removed more rapidly than all the other injectants. Similarly to the normal condition there were no significant differences between the rates of removal of PVP, amino acids and plasma (0 - 4 hours).

After four hours plasma with or without coumarin was removed at a much slower rate ($p < 0.001$) than all the other injectants, including the amino acids.

3.4.5 Discussion:

The Effect of Coumarin:

Again, coumarin had no effect on the rates of resolution of the low-protein oedemas as was expected. It also had no effect on the the first four hours of plasma removal as predicted. Coumarin did however, still increase the rate of removal of plasma after four hours even though the macrophages were destroyed. It has been repeatedly shown that the drug's oedema-reducing effect is due to its ability to increase proteolysis, rather than simply enhancing the the removal of fluid or small or large molecules. That this effect was still present when

the macrophages were destroyed indicates that the drug is capable of stimulating proteolysis by other cells, perhaps neutrophils or fibroblasts as suggested in Section 3.1.5.

The Effect of Macrophages:

To determine the effect of macrophages on oedema resolution the rates under normal conditions were compared with those obtained after silica treatment (i.e. after destruction of macrophages). Student's t-test comparisons were performed on each pair of treatment means With macrophages and without. The results for the uncombined means were identical with those for their respective combined means. The latter are listed in Table 3.7.

From this Table it can be seen that macrophages had no effect on the rates of resolution of low-protein oedemas, on the first four hours of plasma removal or, surprisingly, on the coumarin treated removal of plasma after four hours. However the destruction of the macrophages did cause a significant reduction in the rate of resolution of the high-protein oedema after four hours, without coumarin treatment.

It appears that the macrophages normally play a very vital part in the resolution of high-protein oedema after four hours, but that their destruction does not reduce the effectiveness of coumarin. Perhaps coumarin enhances proteolysis by other cells.

Table 3.7: The Effect of Macrophages on Oedema Resolution.

	MEAN RATES OF RESOLUTION ($hours^{-1}$)		SIG OF
	NORMAL	WITHOUT MACROPHAGES	$t\text{-STAT}_a$
Plasma ± Coumarin (0 - 4 hrs)	0.0303	0.0287	n.s.
Plasma (4 hrs ⟶)	0.0050	0.0034	***
Plasma + Coumarin (4 hrs ⟶)	0.0081	0.0093	n.s.
Amino Acids ± Coumarin	0.023	0.0226	n.s.
PVP ± Coumarin	0.0305	0.0285	n.s.
Saline ± Coumarin	0.0500	0.0502	n.s.

a. The significance of the difference between the means using the Student's t-test: n.s. = not significant; *** = $p < 0.001$.

SECTION 4

EXCESS PLASMA PROTEINS AS A CAUSE OF CHRONIC INFLAMMATION AND LYMPHOEDEMA

4.1 Introduction:

Willoughby and Di Rosa (1970) hypothesized that the accumulation of altered proteins in the tissues could cause both acute and chronic inflammation. They suggested, especially in the case of chronic inflammation, that these altered proteins could fix complement and lead to the release of many mediators, partly via the coagulation system. Therefore, the plasma proteins were implicated as well. This concept had not been tested experimentally.

Lymphoedema is caused by the functional incapacity of the lymphatic system, and the tissue proteolytic systems (Földi, 1975, 1977; Casley-Smith, 1976b, 1982b; Földi and Casley-Smith, 1978), to remove the plasma proteins which normally pass to the tissues. The colloidal osmotic pressure of these excess proteins causes oedema to occur. Many of the changes seen in inflammation have been observed in latent and chronic lymphoedema; namely, alterations in the lymphatics themselves (Kalima, 1971; Casley-Smith, Földi-Börcsök and Földi, 1974;

Casley-Smith, 1977a, 1982b; Altorfer, Hedinger and Clodius, 1977; Casley-Smith et al., 1977a, 1978; Olszewski, 1977; Casley-Smith, Clodius and Piller, 1980), in the adventia of the large lymphatics, in the surrounding tissue and in the blood vessels. Increased fibrosis and collections of the cells typical of inflammation (Pfleger, 1964; Veress et al., 1966; Rusznyák, Földi and Szabó,1967; Kalima, 1971; Huth, 1972, 1982a and b; Cremer, Müller and Louven, 1973; Asano, 1974; Casley-Smith, Földi-Börcsök and Földi, 1974; Casley-Smith et al., 1977a, 1978; Casley-Smith, Clodius and Piller, 1980; Cremer, Müller and Bechtelsheimer, 1974; Altorfer, Hedinger and Clodius, 1977; Casley-Smith, 1977a, 1982b; Olszewski, 1977) and acute inflammatory episodes, usually without bacterial involvement (Clodius, 1981) have also been observed. Therefore, it has been suggested that lymphoedema is a form of inflammation (Casley-Smith, 1976b, 1979b, 1982b; Földi and Casley-Smith, 1978). It is possible that the accumulated 'normal' plasma proteins in the tissues, as a result of lymphostasis, could become altered, or cause alterations in the tissue proteins (perhaps via induced cellular activities) and could then be responsible for all the pathological changes found in lymphoedema, as hypothesized for inflammation.

Willoughby and Di Rosa's hypothesis and its possible implications in lymphoedema were tested by injecting repeated doses of plasma into the subcutaneous

tissues of immunologically-tolerant rats for up to 64 days. In effect, lymphoedema was produced "without lymphostasis".

The effects of the benzopyrone, coumarin, were investigated. As described in Section 3, this drug greatly reduces high protein oedemas in animals (Casley-Smith, 1976b, 1977a, 1982b; Dunn et al., 1977; Földi and Casley-Smith, 1978; Koh, Parente and Willoughby, 1978a and b; Piller, 1976a, b, c, d, and e, 1977b) and in humans (Casley-Smith, 1976b; Piller and Clodius, 1976; Clodius and Piller, 1978). Its effectiveness as a treatment for chronic inflammation, in general, is of great interest.

Biochemical estimations of various components of the tissues were performed in order to examine the macroscopic changes that occurred. In addition, quantitative electron microscopy was performed by J. R. Casley-Smith (Casley-Smith and Gaffney, 1981).

4.2 Materials and Methods:

Forty-nine female Wistar rats (250 $\pm$ 50 gm) from an inbred colony were allocated to each of six experimental groups. These groups were divided into pairs. One group from each pair received intraperitoneal

injections of coumarin (2.5 mg/ml in physiological saline per 100 gm body weight) daily, commencing three days before beginning the experimental injections described below.

To test for immuno-tolerance, 18 rats, similar to the experimental ones, were given three skin-grafts from each other, plus one from themselves, using the technique described by Billingham and Silvers (1961). After 21 days, all the self-grafts had taken, while 43 out of the 54 non-self ones had. This latter was an 80 percent take, with a Standard Error (S.E.) of 5.4 percent. No extra rejection occured over the next 50 days. Additional proof that the changes observed were caused just by the excess protein, and not by immunological mechanisms, was the minimal lymphocyte response, with the absence of lymphoblasts (Casley-Smith and Gaffney, 1981).

It was decided to inject the solutions into the subcutaneous tissue at the nape of the neck to avoid the animals being able to bite the injection site. Immediately prior to each injection the neck circumference was measured using a tape measure. One pair was injected with 1 ml of physiological saline (0.85 percent). The second pair received 1 ml of 6 gm/100 ml PVP (m. wt. 40,000 Daltons) in physiological saline. The remaining pair received 1 ml of homologous plasma.

This plasma was obtained under sterile conditions, by cardiac puncture using heparinized syringes. Siliconized equipment was used throughout to avoid activating the Hageman Factor. The blood was centrifuged at 3,000 rpm for ten minutes and the plasma was syringed off. To inactivate the complement system, the plasma was treated in an enclosed tube with 1M ammonium hydroxide in the ratio 40:1, at 37 ^{o}C for 60 minutes. It was then dialyzed overnight against at least 1000x volume of physiological saline at 4 ^{o}C.

Injections were repeated every four days until the animals were killed. Seven rats from each group were killed by breaking their necks after 60 minutes, i.e. "0", and also after 1, 4, 8, 16, 32 and 64 days. In addition, ten other rats were killed and processed as normal controls as well as ten which had received three days of coumarin treatment (coumarin controls).

For the biochemical estimations, the area of skin covering the swollen area and all the underlying fascia were removed, weighed and processed separately. Where no swelling was evident a constant area equal to that taken at 60 minutes (15 mm square) was removed. All water, fat and elastin were removed from accurately weighed portions, of approximately 50 mg, from each tissue sample (Jackson and Cleary, 1967; Cleary, personal communication, 1978).

The quantities of each were recorded. The biochemical estimation of collagen was then carried out on these purified portions according to Woessner (1961, 1976).

Small portions of skin and fascia from the injection sites were fixed by immersion in 4gm/100ml glutaraldehyde in Millonig's (1961) buffer for one hour at $4^{o}C$. The skin was examined deep to the germinal layer. After a brief wash, the blocks were post-fixed in 2gm/100ml OsO_4 in the buffer, dehydrated in acetone and embedded in araldite. Sections were stained with lead (pH 10) and Uranyl acetate. They were examined by J. R. Casley-Smith (Casley-Smith and Gaffney, 1981).

4.3 Method of Analysis:

The difference in neck circumference from the initial value was converted to the increased thickness of the injection site using the approximation:

$$T_t = \frac{C_t - C_o}{\pi}$$

where T_t is the increased thickness at time t; C_t is the neck circumference at time t and C_o is the initial neck circumference.

Because of the large volume of data obtained and the even larger amount of information which could be

derived from it, a Fortran program was written to perform all the required calculations and to print the tables and the graphs of the results for the skin, fascia and for the skin+fascia.

Firstly using the 50 mg specimens the following calculations were made for skin, fascia and skin+fascia for each rat. As is usually done, the collagen content was originally expressed as a percentage of the wet weight, dry weight, dry fat free weight and the dry fat free-residue weight of the specimens. Also, the ratios:

mg of water/mg of wet tissue,

mg water/mg dry tissue,

mg water/mg dry fat free tissue,

mg dry tissue/mg wet tissue,

mg dry fat free tissue/mg wet tissue,

mg dry tissue/mg dryfat free tissue

and mg dry fat free tissue/mg dry tissue

were calculated. The means and standard errors were calculated for each group at each time interval. Any values outside the range of the mean$\pm$twice the standard error were discarded and the means and standard errors recalculated. Graphs of the means for each group were plotted against the time at which the sample was taken. Student's t-test comparisons were made between all pairs of groups and also with the normal and coumarin controls at each time interval. Where necessary comparisons were

also made between time intervals for the same group .

The Ineffectiveness of Analysis by Biopsy Alone:

It became obvious that neither the percentages nor the ratios were an effective way of expressing the constituents of an inflamed or swollen area. The ratios appeared very erratic and almost impossible to interpret. The percentage collagen content decreased with time (an example of this is illustrated in Figure 4.1) to well below normal values at 64 days when, especially with plasma injection, it was quite obvious to the naked eye that the net amount of fibrous tissue had increased. In inflammation, injury or oedema, any given volume of normal tissue (say 50 mg) is swollen with fluid to many times this weight. The other constituents of this tissue are dispersed in a larger volume. Therefore, if only 50 mg of this swollen tissue is analyzed it is probable that the amounts of these other constituents will be less than normal. The percentage content in a 50 mg specimen would probably appear to be less than normal. Any increase in the amounts of other constituents would be masked by this much larger increase in the water content.

Therefore, when analyzing constituents from a swollen area the results must be related back to the total affected area; i.e. to the total weight or volume of the

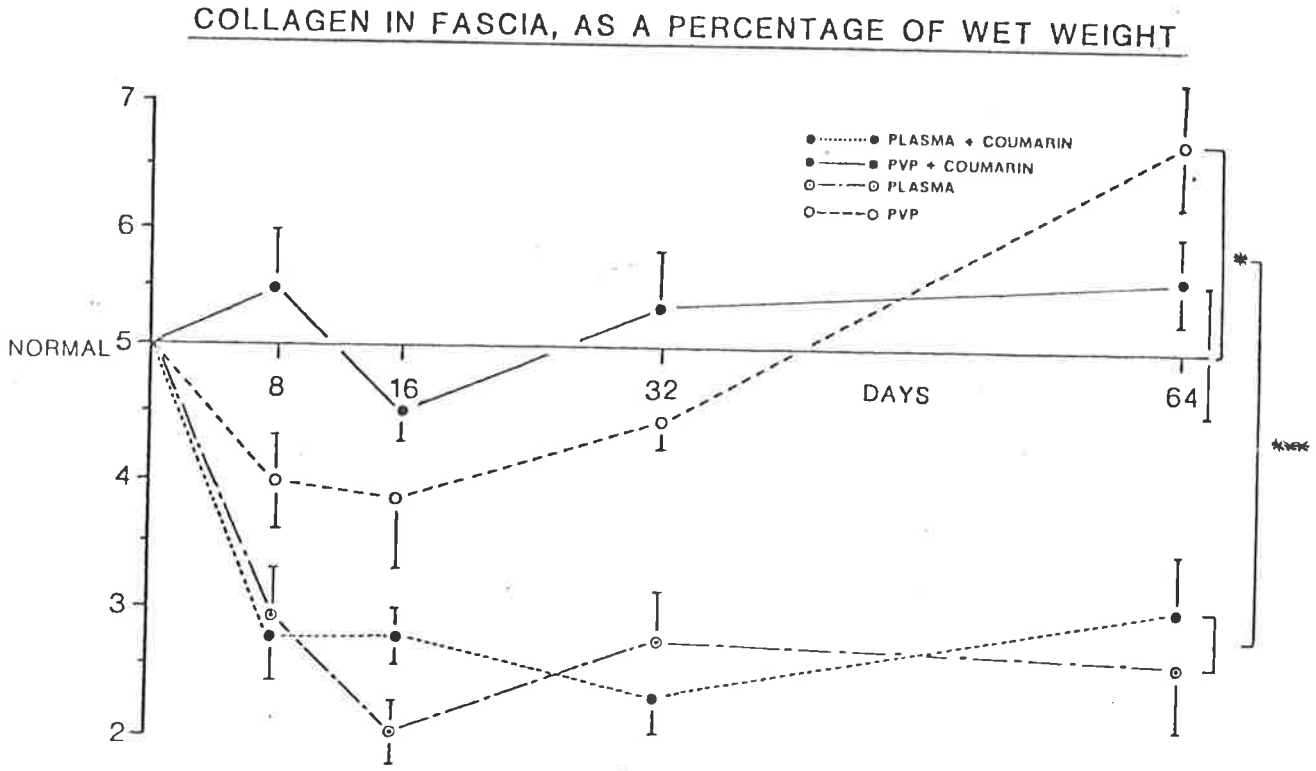

Figure 4.1:

The normal, normal plus coumarin, saline and saline plus coumarin did not differ from each other and are represented by the normal line. All the standard errors are illustrated.

Any significant differences between the means at 64 days are shown;

$*$ = $0.05 > p > 0.01$;

*** = $p < 0.001$.

Plasma showed a decrease below normal throughout.

swollen area, before any meaningful conclusions can be drawn. The biopsy method of analysis of oedematous areas is inconclusive. An example can be seen in Piller (1976d). The extent of the error depends on the extent of the swelling.

In this experiment the amounts of collagen, water and fat in the specimens of skin, fascia and skin+fascia were corrected by the total sample wet weight, taken superficial to the 15 mm square of deep fascia, to yield the total sample content for each rat. In addition the total dry weight, the total dry fat free weight and the total collagen+water of the inflamed skin, fascia and skin+fascia were calculated for each rat.

As described above, (for skin, fascia and skin+fascia) means and standard errors were calculated for each group at each time interval, Graphs of the means were plotted and Student's t-test comparisons were made.

4.4 Results:

In all the measurements made in this experiment; the results for normal animals and normal, but coumarin treated, animals did not differ significantly. Therefore, they have been combined into one "normal" group. Similarly, the results for saline-injected animals (with

and without coumarin) did not differ significantly from normal at any time. They also have been included in the "normal" group. In most cases the individual results for 60 minutes, one and four days varied widely and yielded large standard errors. Therefore, they have been omitted from the graphs and the discussions to improve clarity. The Student's t-test results for 64 days are illustrated in the graphs and discussed in the text, as by this time the predominant trends were obvious. Any differences of particular significance at earlier times will be mentioned. The results for the skin and fascia are discussed separately as there were marked differences in their responses to the injections.

Visual Observations and Injection Site Thickness:

It was quite obvious, by eye, that the necks were greatly increased in the animals given plasma, compared with those injected with PVP and those injected with saline. At one day the plasma-injected necks were still swollen with fluid whilst those which received PVP and saline appeared normal. The plasma-injected necks seemed to have returned to normal by four days but swellings became obvious at eight days and increased in size with time. Coumarin gave no visible effect.

The increased thicknesses of the injection sites (Figure 4.2) agree very well with these observations.

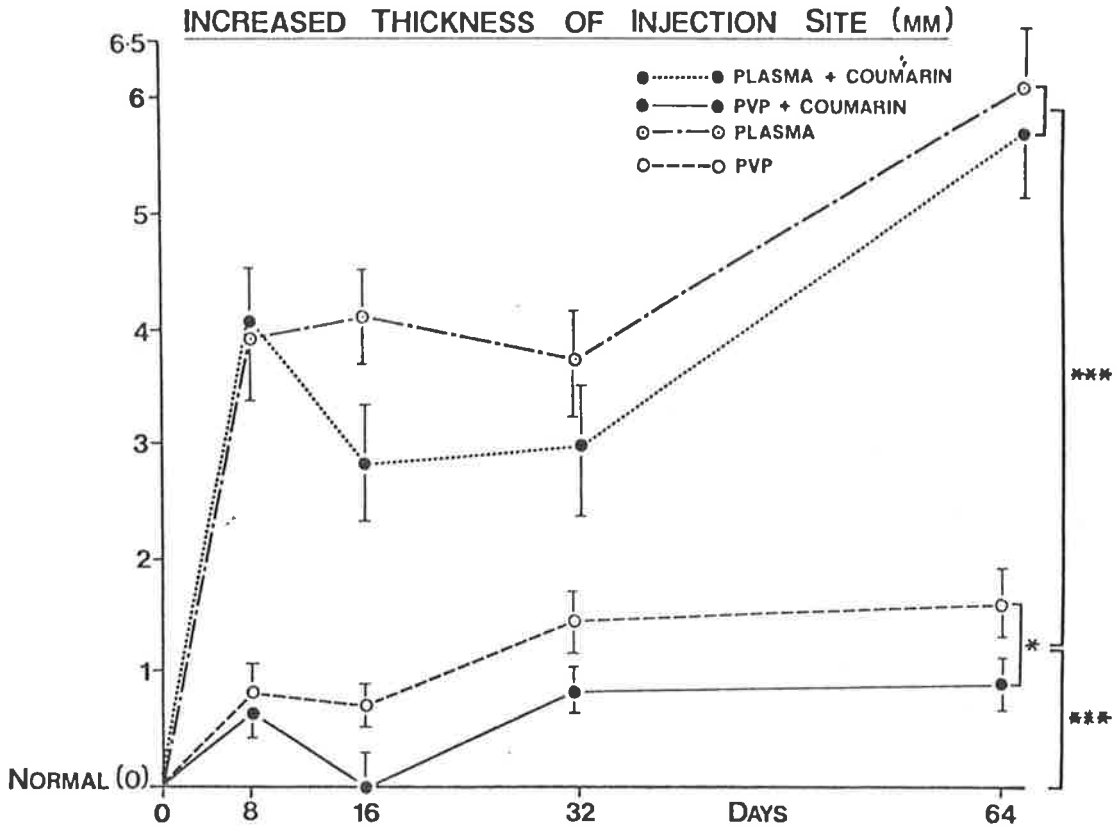

Figure 4.2:

The normal, normal plus coumarin, saline and saline plus coumarin, did not differ from each other and are represented by the normal line. All the standard errors are illustrated.

Any significant differences between the means at 64 days are shown;

$* = 0.05 > p > 0.01;$

$*** = p < 0.001.$

Plasma caused a 4 times greater increase than that caused by PVP. At 64 days coumarin reduced this with PVP only.

In addition, the measurements indicated that the thicknesses of the PVP-injected sites (with and without coumarin) were increased significantly above normal at 32 days. These were further increased at 64 days. This effect was significantly lessened in the coumarin treated group at both times.

These observations and measurements were further clarified when the tissue samples were excised. The skin from plasma-injected groups did not seem to increase in thickness with time. Most of the increased thickness of the injection site appeared to be due to swollen fascia. At one and four days, the fascia contained sacs of red fluid and at eight days these were surrounded by red stained fibrous tissue. The size of the pools of blood decreased whilst the volume of fibrous tissue increased with time. At 64 days dark brown granules were found within the pools. From one day the sacs or lumps could be completely excised without causing any leakage of their contents and the remaining tissue had a yellowish tinge. There was no visible difference in the coumarin treated animals.

The skin and fascia of the PVP- and saline-injected groups did not appear different from normal, with the following exception. At 64 days the skin from PVP-injected groups seemed thicker and tougher than

normal. The volume of fascia seemed greater and it was white compared with clear earlier. These observations were consistent with the measured increases in the thicknesses of the injection sites.

Wet Weight:

The wet weights of the excised tissue samples confirmed and further clarified the observations and measurements described above. The skin samples (Figure 4.3) from plasma-injected groups weighed significantly more than normal from eight days and increased slightly with time. Coumarin significantly reduced the skin weight at 64 days only. The weight of fascia (Figure 4.4) was very significantly greater than that from PVP and normal groups from as early as one day. These differences were much larger than those in the skin. The skin weight was approximately twice normal throughout, whilst the fascia rose from about 12 times normal at one day to about 32 times normal at 64 days. Coumarin had no significant effect on the fascia weight of these plasma-injected sites.

The skin from PVP-injected groups did not weigh significantly more than normal until 32 days and this increased by 64 days to about 1.5 times normal. This was consistent with the observed increased toughness and the measured increased thickness of these skin samples at 64

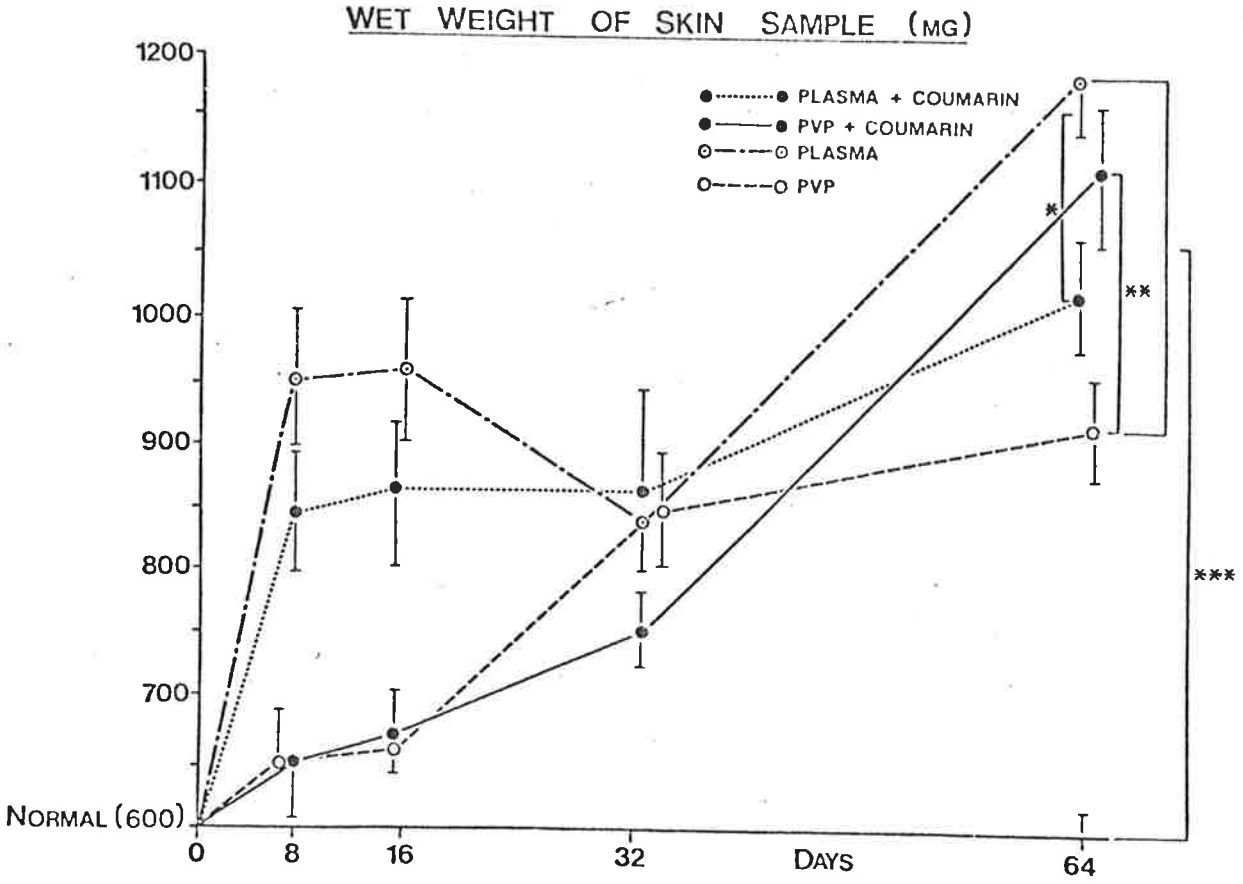

Figure 4.3:

The normal, normal plus coumarin, saline and saline plus coumarin, did not differ from each other and are represented by the normal line. All the standard errors are illustrated.

Any significant differences between the means at 64 days are shown; * = $0.05 > p > 0.01$;

** = $0.01 > p > 0.001$;

*** = $p < 0.001$.

PVP and plasma caused moderate increases. That caused by plasma was more immediate. Coumarin caused a reduction with plasma but caused a further increase with PVP.

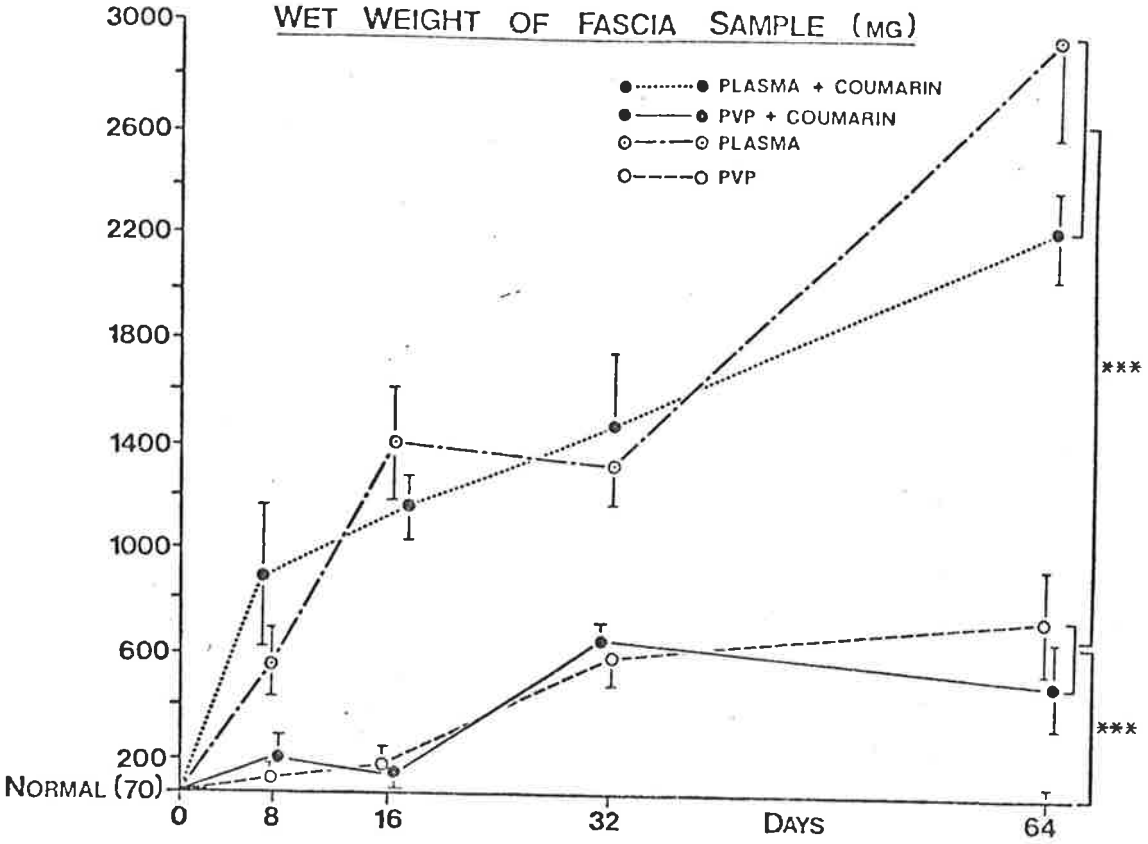

Figure 4.4:

The normal, normal plus coumarin, saline and saline plus coumarin, did not differ from each other and are represented by the normal line. All the standard errors are illustrated.

Any significant differences between the means at 64 days are shown;

*** = $p < 0.001$.

With PVP this was greater than normal from 32 days, whilst plasma caused a more immediate and much greater increase. Coumarin had no effect.

days. Coumarin further increased the skin weight of these PVP-injected sites to about twice normal. The fascia from PVP-injected groups did not weigh significantly more than normal until 32 days and increased to about 11 times normal at 64 days. This was consistent with the increased volume of fascia observed at 64 days. Coumarin had no significant effect on the fascia weight of PVP-injected sites.

The relative contributions of the skin and the fascia to the increased thickness of the injection site at 64 days is illustrated in Figure 4.5. It can be seen that the fascia contributed approximately 2.5 times the skin to the total weight of the injection site in plasma-injected groups. The skin and fascia contributed equally to the total weight of the untreated PVP-injected sites whilst the skin weighed about twice the fascia in the coumarin-treated PVP sites.

Water Content:

The graphs of the mean weights of water in these tissue samples followed exactly the same shape as those of the total tissue sample weights. The significant differences were identical. Therefore, these graphs are not included here as it appears that the total weight of the tissue samples approximately reflects their water

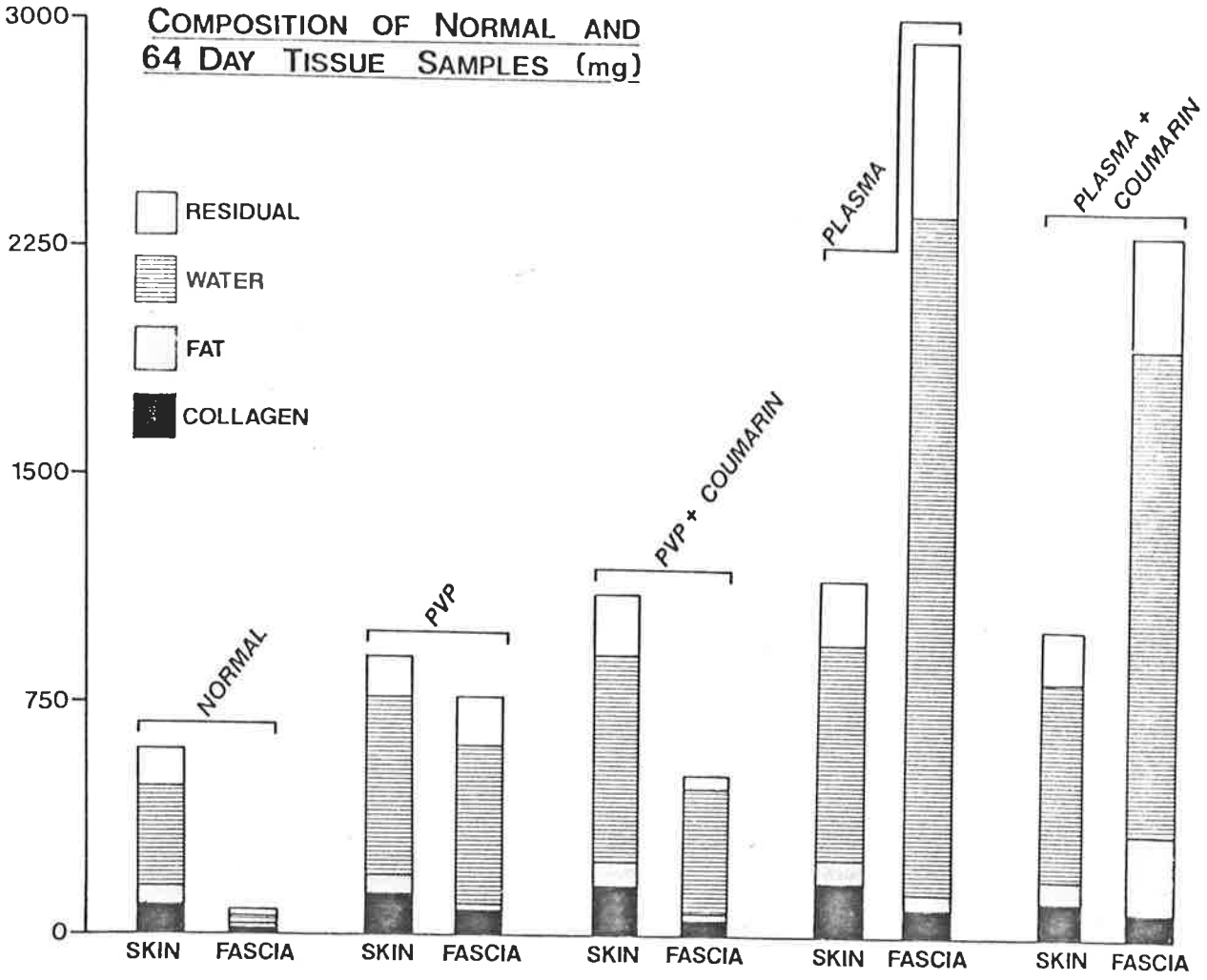

Figure 4.5:

The contributions of collagen, fat, and water to the total wet weight of the excised tissue samples are illustrated.

The massive increases in the water content of the fascia compared with smaller increases in the skin can be seen, especially with plasma injection.

content. This is confirmed by examining Figure 4.5. It can be seen that the main component of all the tissue samples was water and that the changes in that water content were mainly responsible for the observed and measured differences between the groups described above. The water content of the skin of plasma-injected animals (without coumarin) was about twice normal at 64 days whilst that of the fascia rose steadily to about 45 times normal. Coumarin significantly reduced the water content of the skin at 64 days only but had no effect in the fascia. The magnitude and direction of changes in the water content of the skin and fascia of PVP-injected sites was exactly as described above for the total tissue weights.

Fat Content:

The weight of fat in the skin (Figure 4.6) and the fascia (Figure 4.7) from plasma-injected sites (without coumarin) was significantly greater than normal until 64 days. Coumarin reduced the amount in the skin to normal at 16 and 32 days; having no effect at 64 days. Coumarin also significantly reduced the amount in the fascia at 16 and 32 days but the level was still significantly greater than normal. At 64 days coumarin very significantly increased the weight of fat in the fascia of the plasma-injected samples approximately five times. The

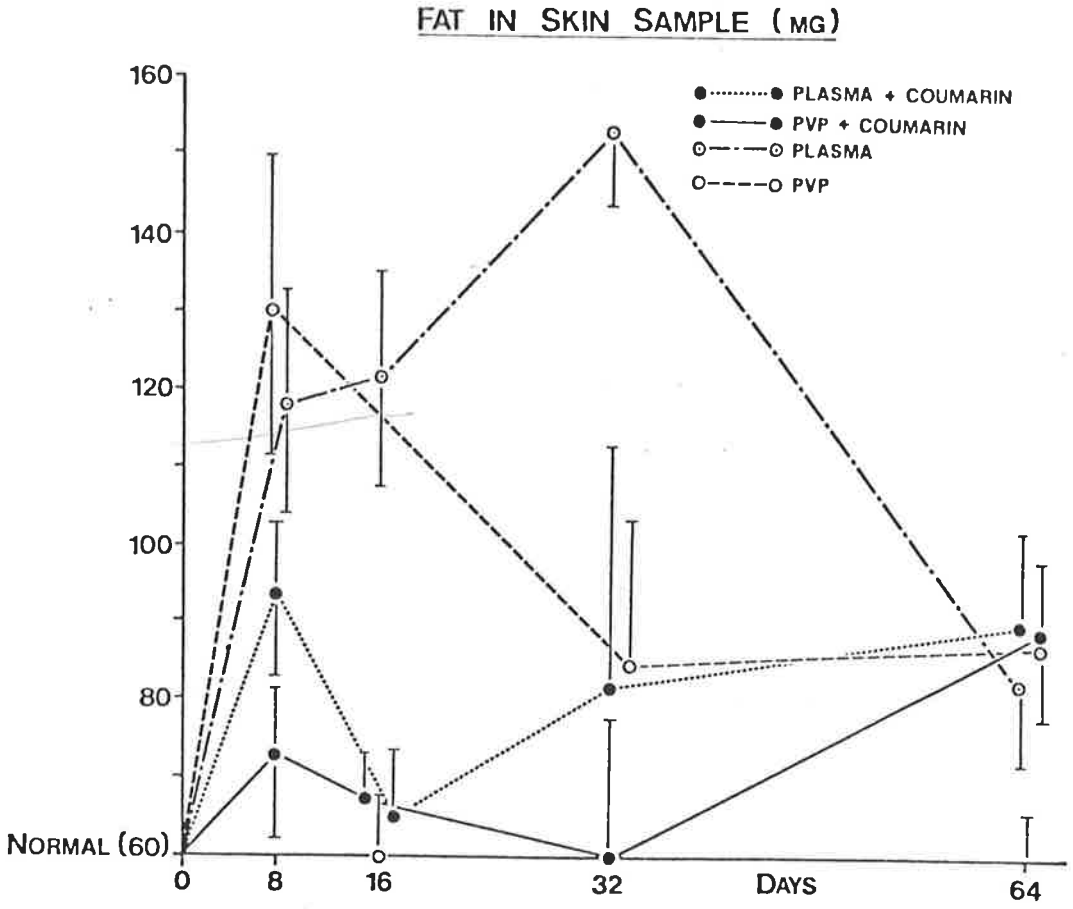

Figure 4.6:

The normal, normal plus coumarin, saline and saline plus coumarin, did not differ from each other and are represented by the normal line. All the standard errors are illustrated.

There are no significant differences between the means at 64 days.

PVP did not cause any change from normal whilst plasma caused a large increase which returned to normal by 64 days. Coumarin had no effect at 64 days.

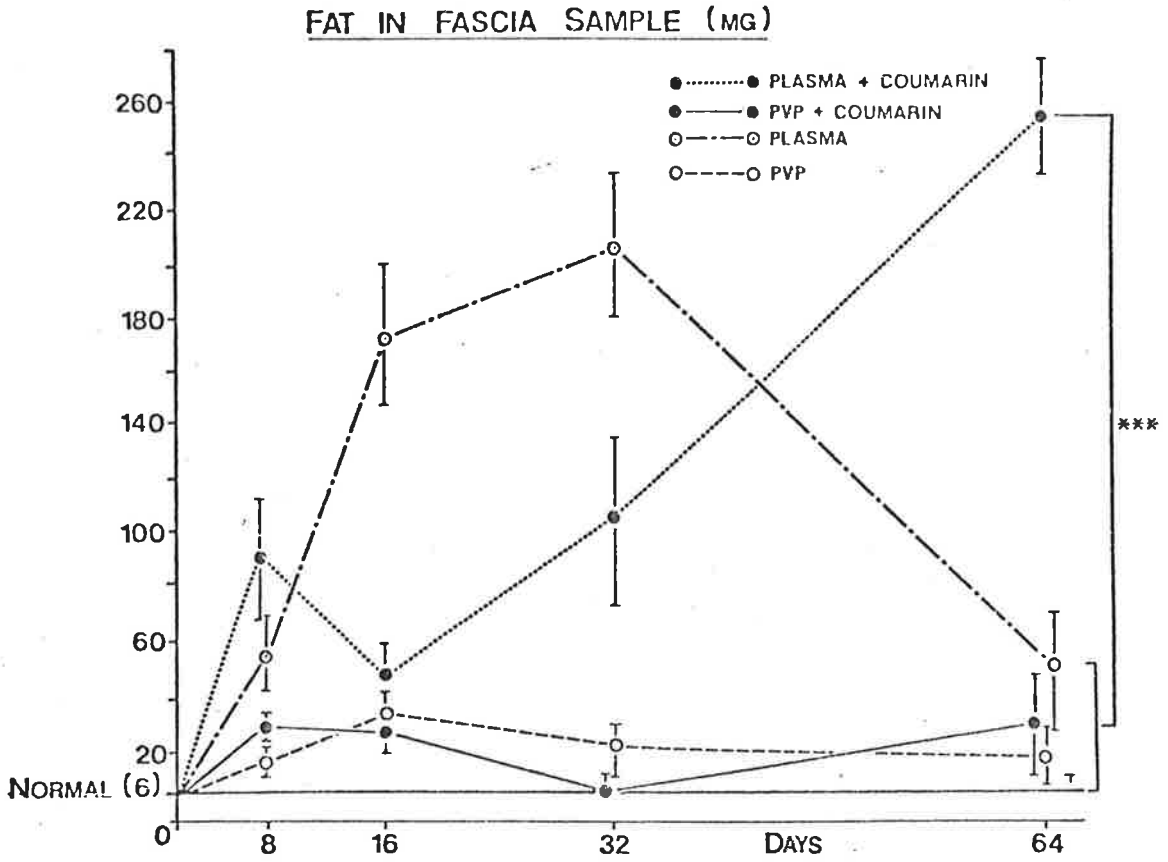

Figure 4.7:

The normal, normal plus coumarin, saline and saline plus coumarin, did not differ from each other and are represented by the normal line. All the standard errors are illustrated. Any significant differences between the means at 64 days are shown; *** = $p < 0.001$.

PVP caused a slight increase which returned to normal by 32 days and coumarin had no effect. As with the skin, plasma caused a large increase which returned to normal by 64 days. Coumarin caused a very large increase above normal at 64 days.

increased fat content of the skin and fascia from plasma-injected sites (without coumarin) contributed to their increased weights and hence to the increased thickness of the injection site prior to 64 days. In the coumarin treated group the fat content did not contribute to the increased weight of the skin samples. It did contribute to the increased weight of the fascia and hence also to the increased thickness of the injection site, especially at 64 days.

The weight of fat in the skin samples from PVP-injected sites (without coumarin) was normal from 16 days whilst that of the fascia was normal from 32 days. At earlier times the mean weights were greater than normal but still significantly less than with plasma. Coumarin had no effect on the amount of fat in either tissue sample at any time. Therefore, fat content did not contribute to the increased tissue weights or to the increased thickness of the injection sites mentioned above for these PVP-injected groups. At 64 days, the fat content of the skin from all the experimental groups was normal whilst in the fascia only the group which received plasma and coumarin differed from normal.

Collagen Content:

The weight of collagen in the skin (Figure 4.8)

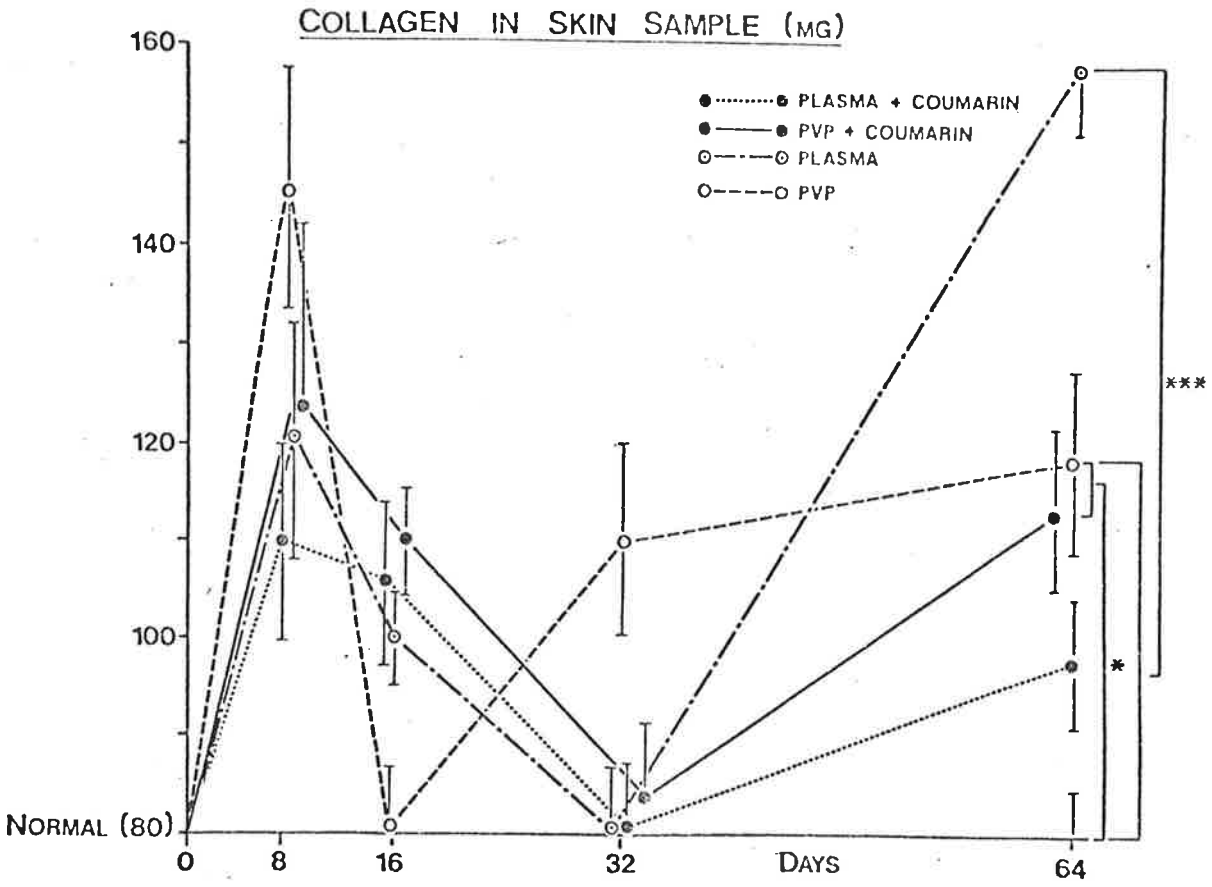

Figure 4.8:

The normal, normal plus coumarin, saline and saline plus coumarin, did not differ from each other and are represented by the normal line. All the standard errors are illustrated.

Any significant differences between the means at 64 days are shown; * = $0.05 > p > 0.01$;

*** = $p < 0.001$.

With PVP this was greater than normal at 64 days. Coumarin had no effect. At 64 days plasma caused a much greater increase which was reduced to normal by coumarin.

from plasma-injected sites (without coumarin) was significantly greater than normal, except at 32 days. At 64 days the amount was twice the normal value. Coumarin had no significant effect until 64 days when it reduced the amount to normal. The weight of collagen in the fascia (Figure 4.9) from plasma-injected sites (with and without coumarin) was significantly greater than normal at all times, especially at 64 days when in both groups the level was about 17 times normal. Coumarin had no significant effect at any time. These results were consistent with the weights of the excised tissue samples discussed above and also with the increased thicknesses of the injection sites measured for these groups. The observed increase in the fibrous nature of the fascia in both the plasma-injected groups was confirmed by the measured increase in their collagen content.

The weight of collagen in the skin from PVP-injected groups fluctuated considerably, as did the results for the t-test comparisons between them and with normal and plasma-injected groups. At 64 days the skin from both PVP-injected groups contained significantly more collagen tham normal and significantly less than that from the plasma (without coumarin) group. Coumarin had no significant effect at this time. The weight of collagen in the fascia from the PVP-injected groups was significantly greater than normal from 32 days. At 64

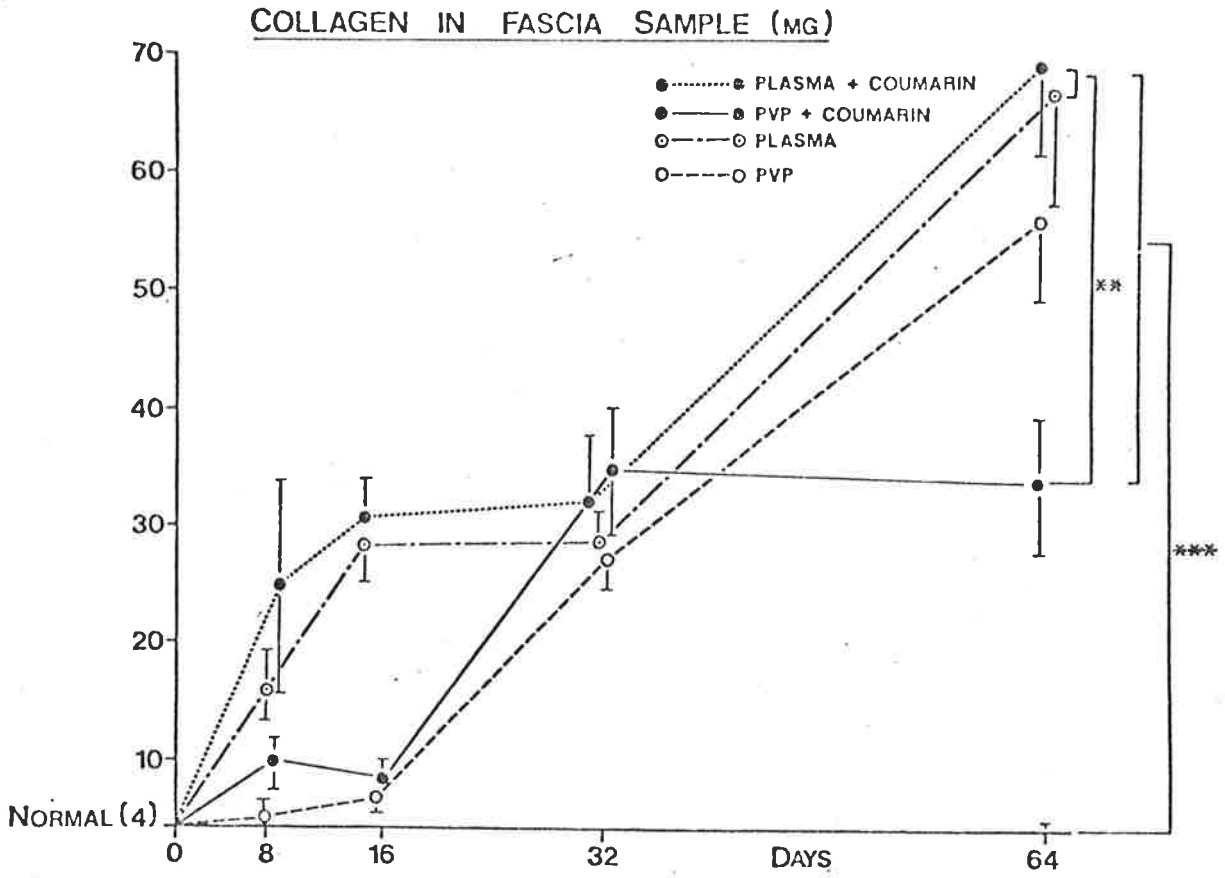

Figure 4.9:

The normal, normal plus coumarin, saline and saline plus coumarin, did not differ from each other and are represented by the normal line. All the standard errors are illustrated.

Any significant differences between the means at 64 days are shown; ** = $0.01 > p > 0.001$;

*** = $p < 0.001$.

Plasma caused an immediate increase which rose to about 17 times normal at 64 days. Although PVP did not cause any increase until after 16 days this rose sharply to be approximately the same as that caused by plasma. Coumarin had no effect with either injection.

days this reached about 16 times normal in the PVP-only group and about eight times normal in the PVP-plus-coumarin group. Coumarin had no statistically significant effect at this or any other time. It appears that the collagen content contributed to the increased weights of the tissue samples and to the increased thickness of the injection sites found in these PVP groups. It also appears that differences between the plasma- and PVP-injected groups can not be accounted for by differences in the collagen content (Figure 4.5).

4.5 Chronic Inflammation:

The repeated injection of plasma caused changes similar to those seen in chronic inflammation, in both the skin and the fascia. The thickness of the injection site was quite remarkably increased rising to about six times normal by 64 days. Large swellings appeared as early as eight days. The weights of the total tissue samples, and their water and collagen contents were greatly increased. The magnitudes of these changes were much greater in the fascia than in the skin. In the skin all these constituents increased to twice normal by 64 days. The extent of the increases varied in the fascia, with the increase in the water content being approximately twice that of the collagen content. In addition haemorrhage, presumably caused by blood vessel damage, and increased

fibrosis were observed. The fat content of both the skin and fascia were normal at 64 days, although it was greater at earlier times. None of these changes occurred in the saline controls. In these the tissues remained as normal throughout the repeated injections.

From 32 days, the PVP injections caused smaller increases in the thickness of the injection site, the weight of the tissue samples, their water content, and the collagen content of the skin. The differences between the plasma- and PVP-injected groups were greater for the fascia. The collagen content of the fascia also increased from 32 days to be the same as that found in plasma-injected samples at 64 days. The fat content of both the fascia and the skin remained normal throughout. It appears that PVP is also capable of causing some of the changes typical of chronic inflammation, although there was no evidence of haemorrhage (i.e. of blood vessel damage) in either of the PVP-injected groups. PVP takes approximately 32 days longer than plasma to initiate these changes, which explains the smaller magnitude of these found here. These results seem to imply that PVP is not a very good control molecule in this type of experiment.

4.6 Excess Plasma Proteins as the Cause:

The alterations could not have been caused by an immunological reaction to the repeated injections of

foreign proteins or PVP. In the case of the plasma, care was taken not to activate the Hageman Factor and to inactivate the complement system. The strain of rats was tested and found to be immuno-tolerant. No lymphoblasts or other evidence of an immunological response were found in either the plasma- or the PVP-injected tissue samples (Casley-Smith and Gaffney, 1981). Therefore, it is concluded that the accumulation of excess plasma proteins in the tissues can cause chronic inflammation, as hypothesized by Willoughby and Di Rosa (1970). This may occur by the release of mediators, possibly due to direct cell damage. Although PVP is approximately the same molecular weight as plasma proteins it appears that it can be removed from the tissues more rapidly (Gaffney, 1981 and Section 3.1). Therefore, it will take much longer for it to accumulate in the tissues to the extent necessary to cause cell damage, the release of mediators or the initiation of an inflammatory reaction.

4.7 Chronic Lymphoedema:

The changes described above and the morphological changes described by Casley-Smith and Gaffney (1981) are identical with those found in acute and chronic lymphoedema (Rusznyák, Földi and Szabó, 1967; Kalima, 1971; Huth, 1972, 1982a and b; Asano, 1974; Cremer, Müller and Bechtelsheimer, 1974; Altorfer, Hedinger and Clodius,

1977; Casley-Smith, 1977, 1982b; Casley-Smith et al., 1977a; Clodius, Köhnlein and Piller, 1977; Földi, 1977; Olszewski, 1977; Piller and Clodius, 1978; Casley-Smith, Clodius and Piller, 1980); even to the haemorrhage and fat accumulation (Földi, 1977). Therefore, it appears that the accumulation of plasma proteins, as the result of lymphostasis, is the stimulus which is responsible for all the other alterations which occur in lymphoedema. This has implications which may explain the latent phase of as many as 20 years between acute and chronic lymphoedema (Gregl et al., 1967; Olszewski, 1977; Clodius and Piller, 1978). Even though an acute attack may appear to have been resolved, small amounts of excess proteins may not have been removed from the tissues. The lymphatics may not have returned to normal; perhaps functioning just below normal during this latent phase, causing a very slow accumulation of protein and fluid in the tissues, especially those adjacent to the collecting lymphatics. A relatively minor injury, causing only a small extra amount of protein to enter the tissues could be sufficient to cause the changes seen in this experiment, i.e. to initiate chronic inflammation. The already poorly functioning lymphatics would be overwhelmed (Földi and Casley-Smith, 1978), leading to chronic lymphoedema. Similarly, after the passage of enough time, the protein and fluid accumulation around the vessels would be sufficient to initiate fibrosis and lead to the chronic phase.

4.8 The Effect of Coumarin:

With plasma injection, coumarin had no visible effect on the size or composition of the swellings on the necks or on the extent of fibrosis or blood vessel damage. It also had no effect on the measured increased thickness of the injection site. It had no effect on the fascia weight or on its water and collagen content. It reduced the fat content at earlier times but very significantly increased this at 64 days. Coumarin did reduce the skin weight and both its water and collagen content at 64 days. It also reduced the fat content to normal at earlier times, having no effect on the already normal level at 64 days. Therefore, in the presence of excess proteins, coumarin significantly reduces the extent of the inflammatory reaction in the skin but not (by the tests reported here) in the fascia.

This may have been due to the fact that the inflammatory changes in the fascia were more extensive than those found in the skin. A significant effect may be more difficult to obtain under these circumstances. Perhaps if the experiment had been continued for a longer period of time the drug may have been more effective in the fascia. It has been shown clinically and experimentally that long term treatment, of 4 - 6 months, was necessary to reduce the fibrosis of chronic

lymphoedema (Piller and Clodius, 1976; Clodius and Piller, 1978; Földi and Casley-Smith, 1978). It is possible that less collagen was actually formed as coumarin decreased the number of fibroblasts and increased the number of fibrocytes in the skin of these plasma-injected animals (Casley-Smith and Gaffney, 1981). Alternatively, the same amount of collagen may have been formed in both cases, but it may not have been converted to mature collagen in the coumarin-treated animals. It was found electron microscopically (Casley-Smith and Gaffney, 1981) that fixable collagen was greatly reduced by coumarin. Presumably non-fixable collagen molecules are equally detectable biochemically as mature collagen, but have not formed fibrous tissue. Thus, no doubt, they are more readily lysed by the proteolytic cells.

Coumarin very significantly increased the numbers of macrophages in both the skin and fascia (Casley-Smith and Gaffney, 1981) which is consistent with other findings (Dunn et al., 1977; Piller, 1978; Piller and Clodius, 1979; Matisons, 1982). Their coumarin-stimulated proteolytic activity (Casley-Smith, 1976b, 1977, 1982b; Földi and Casley-Smith, 1978; Koh, Parente and Willoughby, 1978a, b; Piller, 1976c, e, 1977b, 1978) would have removed some of the excess proteins and fibrous tissue in both the skin and the fascia. The fragments readily leave via the blood (Gaffney, 1981 and Section 3.1) in place of

the proteins having to be, slowly, removed via the lymphatics. Other anti-inflammatory drugs are known to stimulate neutral proteolytic activity in fibroblasts and possibly skin epithelial cells (Houck and Sharma, 1969) or neutrophils. No neutrophils were found in the skin or fascia of any of the experimental groups studied here and coumarin decreased fibroblast numbers (Casley-Smith and Gaffney, 1981).

Coumarin has been shown to increase the levels of neutral proteases in the skin and serum (Piller, 1976e, 1977b). There is some evidence that neutral proteases are secreted by stimulated macrophages (Unkeless, Gordon and Reich, 1974; Davies, 1976), but when the macrophages were destroyed by silica, coumarin still elevated neutral protease levels (Piller, 1976e). This stimulated activity was sufficient to slow the rate of swelling and decrease the maximal swelling volume of the macrophage-free thermally injured rats. The conclusion that coumarin-stimulated lysis of abnormal protein ceased when the macrophages were destroyed does not seem to reflect all the evidence.

This work seems to indicate that coumarin was capable of stimulating cells (other than macrophages, neutrophils or fibroblasts) to cause a significant decrease in the amount of collagen deposited in the skin.

The decreased blood capillary vascularity and less vascular damage found in the skin (Casley-Smith and Gaffney, 1981) after coumarin treatment, would have contributed to the reduced oedema and fibrosis by allowing less leakage of proteins and fluid into the tissues. This, and the reduced lymphatic vascularity, also indicates that there was less chronic inflammation.

The greatly increased amount of fat found in the fascia at 64 days, with coumarin treatment, may have been due to gluconeogenesis followed by lipid formation (Guyton, 1971). In the presence of excess protein, some amino acids would have been converted by deamination to glucose and, in turn, converted to fat. Coumarin, presumably, enhanced this effect by causing more proteolysis.

4.9 Conclusions:

The repeated injection of plasma proteins caused changes typical of chronic inflammation in the skin and to a much greater extent in the fascia. Coumarin significantly reduced the extent of this inflammatory reaction in the skin but not in the fascia. It increased the numbers of fibrocytes in the skin and may have stimulated them to lyse the excess protein. After 32 days, PVP injection caused some of the changes seen in

chronic inflammation, but to a much lesser extent than those found with plasma. The only effect of coumarin was to reduce the water content of the skin (and hence the skin weight).

It is concluded that the accumulation of excess proteins in the tissues can cause chronic inflammation as hypothesized by Willoughby and Di Rosa (1970). In particular, chronic lymphoedema is a form of chronic inflammation and the slow accumulation of fluid and protein in the tissues may explain the latent phase often seen before the onset of chronic lymphoedema. It is also concluded that in the presence of excess protein the benzopyrone, coumarin, is capable of stimulating cells, other than macrophages, fibroblasts and neutrophils, in the skin.

SECTION 5

GENERAL DISCUSSION

5.1 The Protein Balance of Normal Tissues:

The protein balance of normal tissue is maintained by a complex relationship between vascular endothelial permeability, tissue proteolysis and lymphatic removal. Net protein flux occurs (Section 2), from the blood to the tissues, by vesicular transport and also across close and open junctions (and fenestrae, if present). This continuous leakage of protein provides the tissues with the new material necessary for their normal function. Protein must also be continually removed from the tissues to prevent accumulation to the extent where oedema arises. Some, or most (Section 2), of this protein is removed back into the blood, against its concentration gradient, by the same avenues mentioned above for the net outflow. The remainder is removed by the lymphatics and tissue proteolysis.

Coumarin treatment has no significant effect on the protein balance or normal functioning of the blood-tissue-lymph system (Sections 3 and 4). It is important to note that long term administration of the

drug has no detrimental effects on normal tissues. In particular, there was no evidence of significantly increased vascular permeability in the short (Sections 3.1, 3.3 and 3.4) or long (Section 4) term.

5.2 The Tissues in Low-Protein Oedema:

Low-protein oedema in the tissues is resolved quite rapidly (Section 3.1). The accumulated fluid and small molecules pass back into the blood across the vascular endothelium, mainly across endothelial junctions. The rate of resolution is directly related to the size of the molecules in the fluid. The fluid itself passes back into the blood very quickly. Neither the lymphatics (Section 3.3), nor tissue proteolysis by macrophages (Section 3.4) play a significant part in the the resolution of low-protein oedema.

Long term maintenance of low-protein oedema in the tissues does not lead to chronic inflammation in experimental animals (Section 4) or humans. There is no evidence of increased blood vessel damage, increased vascular permeability or fibrosis; no alterations to the lymphatics and no abnormal accumulations of cells (Section 4). The blood vessels cope very well with moderate leakage of fluid and small molecules into the tissues;

preventing any disruption to the protein balance.

The resolution of low-protein oedema is unaffected by coumarin therapy. The short (Section 3) or long (Section 4) term administration of the drug is neither advantageous nor detrimental to the protein balance or function of a blood-tissue-lymph system with low-protein oedema.

5.3 The Protein Balance of Injured Tissues:

Protein often accumulates in injured tissues, leading to high-protein high-flow oedema. Tissue proteolysis and lymphatic removal increase and combine with the vascular permeability (Section 2) in an integrated attempt to restore the protein balance of the tissues to normal. These all function throughout the return to normal. Impairment of one, alone (Section 3), will not prevent the resolution of the high-protein oedema, only retard it. Even lymphoedematous tissues can be returned to "normal" (Section 3.2). Each method of removal is dominant at a different stage in the process and each is vital to the normal protein balance of the tissues. During the initial stage in high-protein high-flow oedema, when vascular permeability is increased, most of the protein removal from the tissues occurs across

the vascular endothelium (Section 2) back into the blood. This takes place against its concentration gradient by vesicular transport and also across the opened junctions (Section 2). The smaller proportion of protein (Section 2) is removed by tissue proteolysis and the lymphatics. After approximately 30 minutes, when vascular permeability returns to normal, lymphatic removal dominates the resolution of the high-protein oedema (Section 3.3). This situation persists for approximately four hours, when macrophage accumulation in the affected tissues reaches maximum numbers. Tissue proteolysis then plays the dominant role (Section 3.4), breaking the proteins into their constituent amino acids. These can be removed more rapidly (Section 3.1), back into the blood, across the vascular endothelium.

In high-protein oedema, coumarin therapy does not affect lymphatic or blood vessel removal of protein, amino acids or fluid (Sections 3.1, 3.3 and 3.4). It enhances tissue proteolysis, but only when macrophage accumulation in the tissues reaches a maximum (Sections 3.1 and 3.4). When the macrophages are destroyed (Section 3.4), coumarin therapy still significantly increases this third phase in the resolution of high-protein oedema. Possibly the drug is capable of stimulating proteolysis by cells other than macrophages.

The maintenance of large non-metabolizable foreign particles in the tissues increases vascular permeability, after a significantly longer period of time than protein accumulation (Section 4). Even then, blood vessel damage does not occur to the same extent. High-protein oedema is initiated and fibrosis increases. Therefore, coumarin should also be able to reduce the extent of this inflammatory reaction after an appropriate period of treatment.

5.4 The Tissues in Chronic Inflammation:

If abnormally high amounts of protein are maintained in the tissues, vascular permeability is increased (Section 4). Even though more protein may be removed back into the blood vessels under these conditions, net flux is still directed towards the tissues (Section 2). Hence the protein balance of the tissues is further disrupted and oedema formation continues. The excess protein allows increased fibrosis and attracts abnormal accumulations of cells, eventually causing more vascular damage (Section 4). Blood vessel damage occurs to the extent where erythrocytes escape into the tissues, die and are broken down there. After a time, alterations to the lymphatics occur, lymphatic removal is impaired and protein accumulation in the tissues is increased even further. Increased tissue proteolysis can not compensate

for the ineffectiveness of blood vessel amd lymphatic removal. This chronic inflammatory state will persist and worsen unless protein removal can be increased to a greater rate than protein accumulation.

By stimulating tissue proteolysis, coumarin therapy is capable of restoring this protein balance in favour of removal. Hence it is capable of reducing the extent of chronic inflammation (Section 4).

5.5 The Protein Balance of Lymphoedematous Tissues:

The main function (Section 3.3) of the lymphatics is protein removal. As described above, the lymphatics are not the only, nor (quantitatively) the most important avenue of protein removal from the tissues. However, they are of vital importance to the maintenance of the protein balance of the tissues. Lymphostasis causes high-protein oedema by allowing protein to accumulate in the tissues. Therefore, the changes seen in high-protein high-flow oedema would also occur in lymphoedema.

The chronic inflammatory reaction commences after betwen four and eight days of high-protein oedema (Section 4). Lymphoedema volume is at a maximum after four days of lymphostasis and returns to normal by nine days (Section 3.2). It seems probable that any additional stress on the

protein balance of the tissues within that period would initiate chronic lymphoedema.

Coumarin stimulated proteolysis is known to be extremely successful in reducing the effects of chronic lymphoedema. As expected, it has now been shown to produce a similar result in chronic inflammation (Section 4). However, a longer period of coumarin therapy may be required for optimum efficiency in chronic inflammation, as was found to be the case in the lymphoedema trials.

SECTION 6

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SECTION 7

APPENDICES

7.1 Experimental Procedures for Section 2:

Capsule Implantation:

The open ends of plastic, perforated cylindrical hair curlers were drawn together with strong cotton thread. Therefore, the capsule shape was ellipsoidal with dimensions 2.5 cms high, 1.5 cms wide and 5 cms long. The perforations were approximately 2 mm square and were separated by larger areas of plastic. Prior to implantation the capsules were sterilized in 80 percent A. R. grade alcohol. Twenty-six female hooded rats of average weight 200 $\pm$ 25 gm were anaesthetized with aether and the capsules were implanted with appropriate sterile technique.The capsules were implanted subcutaneously, dorsally, from the midline of the right side and parallel to the spine. After the skin was sown back in place the wound was liberally sprinkled with Cicatrin Antibiotic Powder (Burroughs Wellcome, Australia). To prevent infection 0.1 gm of streptomycin sulphate in 0.5 mls of injection water was injected intradermally, adjacent to the capsule, daily for a week after implantation.

Intracapsular pressure:

Intracapsular pressure measurements were commenced after one week and were made at intervals of ten days to minimize the possibility of imbalance and infection due to the measurement technique itself. These pressures were determined by a direct manometric method using a needle inserted through one of the capsular perforations. The animals were not anaesthetized during the measurements but were placed in wire isolation cages which prevented movement. After removing the needle from the capsule the site of penetration was sprinkled with Cicatrin. Measurements were stopped when the pressure remained constant or when the capsule became too badly infected, or chewed open. Infection was recognized by the appearance of pink-brown coloured fluid in the manometric tubing during measurements.

During the period of measurement eight animals died or chewed their wounds open and their capsules were immediately excised and examined visually. Tissue had grown through the perforations to line the inside of the capsules. Blood vessels had also grown to the inside. In a capsule examined after seven weeks the intracapsular cavity was approximately 1.5 cms high, 7 mm wide and 4 cms long.

The mean intracapsular pressures were calculated and are illustrated in Figure 7.1. The final value for the ten uninfected animals was -3.82 cms H_2O with a standard error of 0.163. Seven of these animals reached their constant value by 47 days and the remainder by 57 days. These ten animals were chosen for the protein flux experiment.

The intracapsular pressure results are in accord with those of Guyton. All pressures were positive after seven days when some inflammation due to the surgery was probably still present. Although the mean pressure was negative after 17 days the very wide range of values, both negative and positive, agrees with the "erratic" pressures measured by Guyton (1963) after this period of time. Infection caused the intracapsular pressures to become positive in every case, in agreement with Guyton's work. These pressures were relatively constant (between +2.0 and +3.0 cms H_2O) which may be some indication of the degree of infection. From 27 days all the non-infected capsules had negative pressures. The non-infected intracapsular pressures were all constant after 57 days but the mean was quite different to that for dogs (Guyton, 1963) and that for white rats (Strömme, Maggert and Scholander, 1969). This was expected as in this experiment a different species and different measuring techniques were used. Also the animals were not anaesthetized.

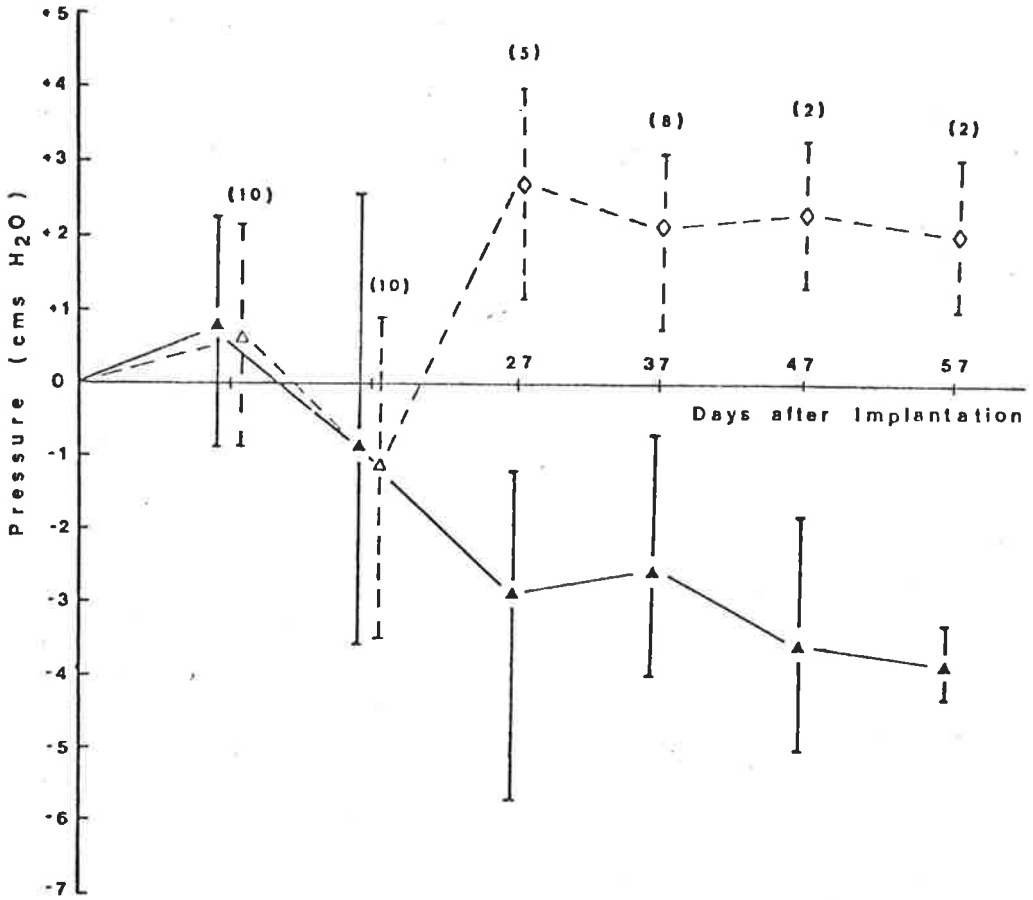

Figure 7.1:

The mean intracapsular pressures and the range of values are illustrated.

The symbols indicate;

▲ = non-infected capsules;

◊ = infected capsules;

△ = non-infected capsules which became infected at a later stage.

The non-infected means were calculated from 11 animals whilst the numbers in the other groups are indicated in brackets e.g. (10).

Injection and Sampling of Radioactive Tracers:

The radioactive tracers used in this experiment were RISA (^{125}I) and chromic chloride (^{51}Cr) both obtained from the Radiochemical Centre, Amersham, U.K.. The ^{51}Cr was bound to albumin by mixing with homologous rat plasma in the volume ratio 1:1 so that 0.5 mls of the ^{51}Cr-labelled plasma had an activity of 25 µci. The ^{51}Cr has been shown to have a high affinity for albumin; 95 - 98 percent binding within the first five minutes of mixing (Beierwalters, 1965). Therefore, the solutions were mixed at room temperature for ten minutes. To remove any free ^{51}Cr and adjust tonicity the mixture was dialysed against 50 percent whole blood in isotonic saline for two days at 6 - 8 ^{o}C. The dialysate was changed after one day. On both days a sample of dialysate was counted on a Packard Tri Carb Scintillation Counter for free ^{51}Cr but in each case there was no significant elevation above background count levels (< 5 percent). Evan's Blue was then added to the mixture to a concentration of 0.5 percent.

Five of the rats were anaesthetized with aether and 0.1 ml of RISA with an activity of 5 µci was injected into the blood by heart puncture. Before the ^{51}Cr was injected into the capsule, 0.6 ml of capsular fluid was removed to ensure that the capsule volume remained

constant. The 0.5 ml dose of ^{51}Cr was injected into one end of the capsular cavity, together with 0.1 ml of physiological saline. Simultaneously, fluid was withdrawn from the opposite end of the capsule. To ensure complete mixing this fluid was re-injected, simultaneously withdrawn from the other end and re-injected again until the dye was homogenous at both ends.

Samples of 0.1 ml were taken from the blood and the capsule at 5, 20, 45, 80, 320 and 1440 minutes. Each capsule sample was replaced with 0.1 ml of the previously removed capsular fluid and its activity was corrected for this dilution woth unlabelled material. The chromium and iodine activities of each sample were counted simultaneously for one minute.

Moderate histamine injury was induced in the capsular capillaries of the other five rats by adding three ugm of histamine acid phosphate (Casley-Smith and Window, 1976) to the 0.1 ml of physiological saline in the capsule injectant. The rest of the experimental procedure was the same as that described above for the controls.

After ten days, the treatments for these two groups were reversed. Additional samples of blood and capsular fluid were taken prior to the injection of tracers and these counts were regarded as the background

for all subsequent samples. Technical difficulties caused a few animals to be discarded but essentially they served as their own controls. No significant differences were found between the groups given histamine first and those given it second.