FIBRIN NETWORK STRUCTURE - CRITIQUE ON DETERMINANTS AND CLINICAL APPLICATION

by

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STATEMENT

EXCEPT WHERE ACKNOWLEDGED, THE INVESTIGATIONS DESCRIBED IN THIS THESIS ARE MY OWN ORIGINAL WORK.

Chenicheri Hariharan Nair.

FOR MUM, DAD, SIDDHA AND MEERA

Les thèories qui reprèsentent l'ensemble de nos idèes scientifique sont sans doute indispensables pour reprèsenter la science. Elles doivant aussi servir de point d'appui à des idèes investigatrices nouvelles. Mais ces thèories et ces idèes n'etant point la verità immuable, il faut être toujours prêtàles abondonner, à les modifer ou à les changer dès qu'elles ne reprèsentent plus la rèalitè. En un mot, il faut modifier la thèorie pour l'adapter à la nature, et non la nature pour l'adapter à la thèorie.

Claude Bernard.
Introduction àl'êtude la
mèdicine expèrimental.
Paris: J.B. Baillièreet Fils,
1865, p.70.

The theories which embody our scientific ideas as a whole are, without doubt, indispensable as representations of science. They should also serve as support for new investigative ideas. But since these theories and ideas by no means constitute an immutable truth, one must always be ready to abandon them, to modify them, or to change them, as soon as they cease to represent reality. In a word, we must adapt theory to nature, but not nature to theory.

Claude Bernard.

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ABBREVIATIONS

Å - Angström

ACT - Australian Capital Territory

ATIII - Antithrombin III

C - Fibrinogen concentration

Cn - Network protein content

HMW Kininogen - High molecular weight kininogen

λ - Wavelength

LMW kininogen - Low molecular weight kininogen

Mw - Weight average molecular weight

nm - Nanometer

PFP - Pure fibrinogen solution

PPP - Platelet poor plasma

SEM - Standard error of mean

τ - Permeability, or Darcy Constant

u/ml - Units per ml

u_P - Mass-length ratio derived from permeability

u_T - Mass-length ratio derived from turbidity

ABSTRACT

Recently available methods of characterizing fibrin network structure were used in a critical study of its determinants under physiological conditions. It was found that these methods are highly reproducible and reliable in detecting alterations in network structure in response to physiological conditions of clotting. In addition it was found that kinetics of network development determine the final network structure through influencing events preceding the appearance of visible fibrin.

They were found to be theoretically acceptable when applied to characterization of networks in plasma. However, remarkable differences were found between networks developed in plasma and in fibrinogen solution. Albumin, γ-globulin, fibronectin, ATIII and cellular elements of blood were shown to alter network characteristics. But no one component was found to account for all the differences in network characteristics in the two systems.

Applicability of these methods in sequential studies in clinical situation has been investigated. In any one individual the methods were found highly reproducible

from one day to the next. A study was undertaken on the effect of operation and dextran infusion. It was found that surgery as well as dextran can alter network characteristics in a consistent fashion.

CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

Haemostatic mechanisms against haemorrhage involves three processes. One is the contraction of blood vessel walls reducing blood flowing through vessels. The second involves platelets adhering to each other and to the damaged vessel wall. The third process of haemostasis occurs in blood. In minor injuries, vasoconstriction and the formation of a platelet plug may suffice to stop blood loss. However, in the event of major injury these two mechanisms cannot by themselves cope because of the transient nature of vascular spasm and the instability of the platelet plug. Structural strengthening of the plug is achieved by the formation of fibrin through an enzyme cascade system. Formation of a clot begins in 15 to 20 seconds if the trauma to the vascular wall has been severe, and in one or two minutes if the trauma has not been severe (Sixma, 1981).

It is generally accepted that coagulation occurs in a cybernetic manner (Bick, 1982) and that the outcome of normal haemostasis *vis-a-vis* increased fibrin deposition (thrombosis) or increased fibrino(geno)-lysis

(haemorrhage) depends upon a delicate balance between the procoagulant system and associated inhibitors, as well as the fibrino(geno)-lytic system and its associated inhibitors (Mammen, 1971; Seegers, 1971; Harpel and Rosenberg, 1976; Murano, 1978;).

1.2 THE MECHANISM OF COAGULATION

The coagulation system is a biological amplifier system. Briefly, the plasma protein fibrinogen is converted into fibrin through the proteolytic action of thrombin which splits four small polypeptides from each molecule. The fibrin monomer then polymerizes into fibrin strands - a process greatly accelerated by calcium ions. Fibrin monomers are usually joined by covalent bonds through the action of Factor XIII or Fibrin Stabilizing Factor. The formation of thrombin is possible via two different pathways; the intrinsic pathway and the extrinsic pathway. The intrinsic pathway involves the participation of a number of agents and contact activation through interaction of Factor XIII (Hageman Factor), Factor XI (Plasma Thromboplastin Antecedent) and components of the Kallikrein-kinin System, i.e. prekallikrein and high molecular weight kininogen (HMW Kininogen) (Ogston,1981).

Human Factor XII (Chan and Movat, 1976) is a single polypeptide chain of molecular weight 80,000 (Vanucchi *et al*, 1982). Most studies on this enzyme have been made on the bovine variety which has a molecular weight of 74,000 - 78,000. The actual concentration in plasma is approximately 30ug/ml, as determined by radial immunodiffusion. The site of synthesis of Factor XII is unknown although the liver has been suggested as a possibility because of decreased plasma concentration of Factor XII in patients with hepatic cirrhosis.

Activation of Factor XII takes place on contact of normal plasma with a large variety of negatively charged surfaces, such as glass and kaolin. Physiologically, collagen and platelet membranes and even, at least in the rabbit, cultured epithelial cells have been shown to be capable of activating Factor XII (Ogston, 1981). There seems to be some controversy over this mode of activation. One proposal is that binding of Factor XII to a negatively charged surface results in conformational change in the molecule, which exposes its active sites and accompanying hydrophobic sites. The alternative

proposal suggests a cleavage into a two-chain form (McMillan *et al*, 1974). Apart from solid phase activation of Factor XII on contact with negatively charged surfaces, fluid phase activation may also be induced by incubation of Factor XII with proteases like kallikrein and plasmin. The fragments thus produced have the capability of converting prekallikrein to kallikrein (Kaplan and Austen, 1971).

Activation of the intrinsic coagulation mechanism takes place on contact of normal plasma with a surface, but a mixture of highly purified XII and XI incubated with kaolin does not result in clot forming activity (Ogston, 1981). This led to the finding that both prekallikrein and HMW kininogen are also required in the coagulation process, but their precise roles in the intrinsic pathway are still not fully established. Kallikrein is an enzyme which releases the pharmacologically active polypeptide kinin from its precursors, called kininogens, or prokinin. There are two types of kallikreins in plasma - plasma kallikrein and glandular kallikrein. There are also two forms of kininogen, i.e. High Molecular Weight kininogen (HMW kininogen) and Low

Molecular Weight kininogen (LMW kininogen). Glandular kininogen preferentially cleaves LMW kininogen with the release of lysyl-bradykinin (kallidin) , while plasma kallikrein cleaves HMW kininogen to release bradykinin.

Experiments show that maximal rates of activation of procoagulant activity of Factor XI require Factor XII, HMW kininogen and a surface. On the basis of several studies Griffin and Cochrane (1976) , and Griffin (1978) have formulated the hypothesis of reciprocal activation of Factor XII and prekallikrein. Recent evidence suggest that kallikrein itself may influence the role of HMW kininogen in contact activation. Both the extrinsic and intrinsic pathways are required for haemostasis, and one pathway does not compensate for the loss, if any, of the other. The extrinsic pathway has two major components, i.e. tissue thromboplastin (Tissue Factor) and Factor VII . Tissue Factor is a separate mixture of protein and lipids, the protein fraction being apoprotein III. Particularly rich sources of tissue factor are brain, lung, placenta and the thyroid glands. The protein component can be synthesized by monocytes and macrophages, and has procoagulant activity. Apoprotein

III by itself, though, has no proteolytic activity unless associated with a phospholipid, like phosphatidyl choline.

Factor VII is a single chain polypeptide of molecular weight 48,000. It is a vitamin K-dependent clotting factor with eight to ten α-carboxyl glutamic acid residues. Factor VII can be activated by Xa, IXa, and XIIa and it can be seen from this one example of the interaction of the haemostatic integration.

Both the intrinsic and extrinsic pathways start off enzyme cascades which ultimately activate Factor X. Factor Xa, with the help of calcium ions, phospholipids and Factor Va, converts prothrombin,a single-chained glycoprotein with molecular weight 70,000, to thrombin. Thrombin then cleaves two small polypeptides from the NH_2 position of the α and β chains of fibrinogen, which is a dimer with a molecular weight of 340,000. The cleaved fibrinopeptides Aα polymerize end to end. The β peptide then permits side to side polymerization and ultimately the platelet plug is stabilised. The cross-linking of fibrin is catalysed by Factor XIIIa which is found in the activated form in circulation and in

platelets and requires the presence of calcium ions.

1.3 FORMATION OF A FIBRIN NETWORK

1.3.1 Fibrinogen Structure.

Fibrinogen was crudely separated by Denis in the mid-ninteenth century and later by Hammersten in 1879. It has been commonly described as the most important and abundant clotting factor in plasma (Ratnoff, 1977; Esnouf, 1984) and its hepatic origin was suggested by Doyon and Kareff as early as 1904.

Fibrinogen is a glycoprotein of molecular weight 340,000 ± 20,000 (Caspary and Kelwick, 1957), as calculated from sedimentation studies which also support the view that it is a rod-shaped molecule. Support for this view also came from Hall and Slayter (1959) who obtained electronmicrographs using a shadow-casting technique, which showed a molecule consisting of two globular spheres (60A) connected by a "thread like " region to a central sphere (50A).

Fibrinogen is a dimer composed of three separate polypeptide chains α, β and ɣ with molecular weights of 63,000; 56,000 and 47,000 respectively (McKee *et al*, 1970) . The α chain contains 610 amino acids (Doolittle *et al*, 1979) ; the β chain 461 (Watt *et al*, 1970) and the ɣ chain 411 (Henschen and Lottspiech, 1977) .

The fibrinogen molecule also contains four carbohydrate chains with a combined molecular weight of 10,000: one on each of the β and ɣ chains (Review: Blomback *et al*, 1978). It also contains 29 disulphide bonds (Henschen, 1964) which hold together six peptide chains of fibrinogen. Fibrinogen monomers are covalently linked in the N-terminal region by a disulphide bond between the two Aα chains at Cys28. Similarly the two ɣ chains are joined by a pair of bonds between Cys8 and Cy9. Blomback *et al* (1976) described this region as the N-terminal disulphide knot, which is contained within the plasmin degradation product, Fragment 6. The next group of structurally significant disulphide bonds are present in the thread joining fragment D and E regions. Remaining bonds form interchain disulphide loops in the β

and γ chains in the fragment D region and in the chain outside fragment D region (Bouma *et al*, 1978). A growing acceptance of the trinodular form as the true conformation of fibrinogen has stemmed from work on fibrinogen structure by several independent investigators (Fowler and Erickson, 1979; Estis and Haschmeyer, 1980; Price *et al*, 1981; Wiesel *et al*, 1981; Mossesson *et al*, 1981, Plow and Edington, 1981).

1.3.2 Thrombin.

Thrombin is the physiological substance which converts fibrinogen to fibrin. Sequence homology and secondary structure of thrombin is similar to that of chymotrypsin. This has a considerably broader specificity than the other activated clotting factors, predominantly cleaving Arg and neutral or hydroxyl amino acid bonds in a wide range of proteins, including itself. It increases explosively when the coagulation cascade is activated (Mann & Downing, 1976).

Thrombin has multiple roles in the coagulation cascade. It converts fibrinogen to fibrin, activates factors XIII,

V and VIII. The activation of the latter two provides a positive feedback, while simultaneously forming part of a negative feedback loop by protein C activation, which is a potent inactivator of Factor Va and VIIIa. It also causes platelets to aggregate and bind the components of the prothrombin converting complex (Esnouf, 1984).

1.3.3 Fibrin Assembly and Crosslinking.

Fibrin formation is characterized by four distinct steps; namely the activation of fibrinogen by thrombin, end to end polymerization of fibrin monomers to protofibril lateral association of protofibrils to fibrin fibres and finally to covalent cross-linking of fibrin by Factor XIIIa.

Fibrinogen conversion to fibrin by thrombin involves the rapid proteolytic cleavage of the Aα-chains of fibrinogen at the Arg-gly bond between residues 16 - 17. This is followed by the slower cleavage of the Arg-gly bond at the β peptide of the Bβ chain at residue 14 (Esnouf, 1984). The sequential removal of these peptides suggests

that following the loss of the A peptide,a conformational change must take place exposing the β peptide to attack by thrombin (Blomback *et al*, 1978). It is surprising that after years of study some uncertainty still remains about the mechanism of polymerization. As early as 1942, Bailey *et al* suggested that "fibrin is no other than an insoluble modification of fibrinogen without any fundamental change in molecular plane but is in a higher state of aggregation as evidenced by its greater internal cohesion." The first exhaustive investigation of the highly elongated intermediate polymers in fibrin formation was reported by Schulman and Ferry (1951). In 1952 Ferry proposed that polymerization was driven by hydrogen bond formation and dipole-dipole interactions. In that investigation, and in another in 1954, Ferry formed the basis for the theory of polymer formation through "staggered overlapping" (Ferry, 1954) .

Other authors (Bang, 1967; Kay and Cuddigan, 1967) proposed similar models for the aggregation process involving various degrees of overlap. Hall and Slayter (1959) suggested that the first oligomers are end-to-end aggregates of fibrin molecules which shrink lengthwise

during polymerization. Koppel (1967) and Marguerie *et al* (1973) described aggregates as pearl necklaces of petagondodecahedrons, or of spheres.

A more sophisticated view of polymerization propounds that certain peptides make specific polymerization sites. This view also embodies the charged peptide concept. Heene and Mathias (1973) showed that fibrinogen had sites available to bind to fibrin. Fragment D was shown to bind to fibrin-sepharose (Kudryk *et al*, 1973), and thrombin treatment of the N-terminal disulphide knot region coupled to sepharose would also bind fibrinogen and Fragment D (Kudryk *et al*, 1974). These observations suggest that unmasked binding sites are present in the Fragment D region and that masked sites exist in the N-terminal region.

In 1976, Brass *et al* reported that fibrin induction involves an "inductional phase" during which no fibrin formation occurs, and a phase of rapid polymerization. Wiltzius and Kanzig (1981)have shown that on comparison of calculated data for different models using static and dynamic light scattering, polymerization proceeds as an

end-to-end aggregation of elongated and possible flexible molecules.

Fibrin formed in purified fibrinogen solution dissolves in urea but fibrin formed in plasma does not (Robbins, 1944). The reason for this difference was found to be due to the presence of human plasma Factor XIII (Lorand and Jacobsen, 1958). Factor XIII is a tetramer of molecular weight 32,000 (Esnouf, 1984) and circulates in plasma in an inactive form until activated by thrombin or other proteolytic enzymes. Activation occurs by the cleavage of the a subunit of the tetramer which is of an a2b2 molecular structure (Schwartz *et al*, 1973). The cleaved protein has no biological activity until the addition of Ca^{++} (Cooke, 1974). Ca^{++} also induces the dissociation of the modified a subunits from the b subunits and unmasks a cysteine residue at the active centre of the catalytic subunit. Factor XIIa (the active enzyme) catalyzes the formation of the two isopeptide bonds between specific glutamine and lysine residues and adjacent fibrinogen chain (Doolittle *et al*, 1972). γ -γ isopeptide bonds form soon after clot formation, followed by a slow cross-linking of the α chains to give

α polymers (McKee *et al*, 1970). Cottrell *et al* (1979) have also shown the presence of glutamyl acceptors in the α chains which are Gln328 and Gln366.

1.4 FIBRIN NETWORK STRUCTURE.

Using dark field illumination in a study with the light microscope, Howell (1941) observed that the product of fibrinogen conversion to fibrin was "a meshwork of beautiful needles" (Bang; 1967). Interestingly, he claimed that thorough investigations into formation of this meshwork was impossible since smaller particles aggregating to form the needles were well beyond the range of vision for the light microscope.

Wolpers and Ruska (1939) studied electronmicrographs of fibrin networks produced in human plasma and they described the networks as micellar bundles in a parallel arrangement forming thicker strands tied together in a network of communicating bundles. The ultrastructure of fibrin pellicles obtained from the spinal fluid of cases of tubercle meningitis were described to have the same gross structure as plasma fibrin networks (Ruska and

Wolpers, 1940; Schmitt and Holsen, 1944) but with a characteristic cross-striation within larger fibres. First thought of as a special characteristic in fibrin of spinal origin, this periodic structure was shown to be present in all fibrin networks (Hawn and Porter, 1944; Hall, 1949). Hall in fact showed that the macro-axial period averaged 230A, separating lighter stained bands from heavier stained bands.

Ferry and Morrison (1947) used solutions of low concentrations of impure fibrinogen, and using opacity ratio and rigidity measurements concluded that basically there were two distinct clot types: coarse and fine. The coarse clot is opaque, non-friable, plastic and readily compactible whilst the fine clot is transparent, gelatinous, very friable and tended not to collapse under the influence of gravity (i.e. compactible). Studies also showed that in artificial media containing fibrinogen the pH, ionic strength, temperature and ionic composition all influenced the final network characteristics (Ferry *et al*, 1947; Belitzer *et al*, 1968; Schulman and Ferry, 1950; Ferguson *et al*, 1981; Okada and Blomback, 1983). Low pH and low ionic strength resulted in coarse clot formation from an increased electrostatic

attraction between intermediate polymer macromolecules. High pH and high ionic strength produced a fine clot. At a pH of 5.5 or above, gelation did not take place (Ferry, 1954).

1.4.1 Techniques Used To Elucidate Network Structure.

Standard clinical tests on coagulation rely principally on the gel point, i.e. the time taken for fibrinogen to convert to visible network, and for the network to trap sufficient protein to turn from a liquid phase into a solid phase (Bang, 1967). Studies based on the measurement of gel point have been extremely valuable in the investigation of haemostatic disorders (Austen and Rhymes, 1984) , in the measurements of coagulation factors (Austen and Rhymes, 1975) , and to lesser extent in defining the role of platelets and cellular elements of blood in coagulation. However, the conversion of fibrinogen to fibrin continues beyond the gel point (Ferry and Morrison, 1947). The gel point must be regarded as a somewhat arbitrary point in the conversion of fibrinogen. In the recent past it has become known that fibrin network develops within the liquid phase of

fibrinogen solution as the enzyme action of thrombin progresses, and that the polymerization of fibrin monomer proceeds end to end as well as side to side through covalent protein-protein interactions (Hermans and McDonagh, 1982). The exact relation between gel point and the amount of fibrin converted has been examined somewhat cursorily in the past. Even so, it is clear that the gel point is variable and depends upon the concentration of the various proteins in the solution. Thus, for instance, the gel point in plasma corresponds to conversion of approximately 6% of fibrinogen to fibrin, whereas in fibrinogen solution 40% of fibrin converts before the gel point is reached (Abilgaard, 1965). Furthermore, it is not known whether gel points vary with the characteristics of fibrin network itself: its tensile strength, permeation, fibre thickness, and other physical characteristics.

Since the early studies of Ferry and Morrison and the early electronmicroscopic work, our understanding of fibrin networks has advanced greatly with a variety of techniques based on different principles. Morrison and Scudder (1952) used electronmicroscopy, White (1960)

used permeability and Muzaffar *et al* (1972) used turbidity measurements. More recently, several other techniques such as dynamic rigidity, loss modulus, thromboelastography, variable frequency and viscoelasticity (Fukuda and Kaibara, 1973; Gerth *et al*, 1974), creep and creep recovery have been used for such investigations (Nelb *et al*, 1976; Nelb *et al*, 1981). In general, such studies on fibrin networks have concentrated on elucidating biochemical and biophysical mechanisms in network development. Such studies have not been directed towards clarifying the physiological significance of fibrin network structure. The concentrations of fibrinogen and thrombin,ionic strength, ionic composition, temperature and pH used in all these studies has been in an unphysiological range. Furthermore, such techniques as were available only allowed a semi-quantitative or qualitative assessment of size and density of fibrin fibres in the network. Importantly, many of the techniques used were not readily available and their applicability to clinical studies was limited and sometimes questionable.

In the last decade methods based on turbidity and

permeability were developed (Carr *et al*, 1977; Carr and Hermans, 1978) and these have allowed the measurement of mass-length ratio (i.e.a measure of fibre thickness) of fibrin fibres. The determination of fibre thickness from turbidity (known as μ_T) and from permeability (known as μ_P) allowed a more quantitative assessment of fibrin network characteristics. Shah *et al* (1982) used these techniques and showed that fibrin fibres were distributed within any fibre network in a bimodal fashion. They showed that there were two categories of fibres within any given fibrin network: major, thicker fibres and minor fibres in the interstitial spaces of the major fibres. Their study also employed the method of compaction (or collapsibility of a network under a specified gravitational force) developed initially by Dhall *et al* (1976). Compaction is a semi-quantitative technique based on the tensile behaviour of fibrin fibres, and this technique adds the dimension of behaviour characteristics to that of fibre thickness in the assessment of network structure. This study emphasized that the categorization of clots first proposed by Ferry and Morrison with two extreme types was an oversimplification.

1.5 THE PHYSIOLOGICAL RELEVANCE OF FIBRIN NETWORKS

It is becoming increasingly clear that fibrin networks may assume significance in a number of physiological processes other than haemostasis. Nolf (1908) described fibrin deposition and removal as a continuous process taking place upon the endothelial lining of normal blood vessels. This statement may not be completely true. Nevertheless, these two processes probably occur in many sites in the body in response to microtrauma caused by daily wear and tear. In the following review an attempt will be made to describe the varying physiological roles of fibrin.

1.5.1 Fibrin and the Haemostatic Plug.

In minor injuries, vasoconstriction and the formation of a platelet plug may suffice to prevent blood loss. In the event of a major trauma, however, these mechanisms may not be adequate because of the transient nature of the vascular spasm and the instability of the platelet plug. The structural strength of the plug arises from its

fibrin content. The early haemostatic plug is characterized by firmly interdigitated degrannulated platelets in the plug centre (Sixma, 1981). At this stage, fibrin is only observed as tiny strands at the periphery of the plug between platelet remnants or translucent vesicles. Small fibrin fibres are apparent later, after about 30 minutes, between empty platelets, and these are quite thick at the periphery of the plug.

1.5.2 Fibrin and Fibrinogen.

In vitro, plasminogen (especially lys-plasminogen) attaches preferentially to fibrinogen. Plasminogen activators also have an affinity for fibrin: their activity is greatly enhanced by contact with fibrin. Therefore, the present theories on fibrinogen claim that fibrin structures are the main sites of conversion of plasminogen to plasmin. Because the formed plasmin has its lysine binding sites occupied in a complex formation with fibrin and its catalytic site engaged in fibrin degradation, it reacts only very slowly to antiplasmin (Wiman and Collen, 1978). In vivo, thus fibrin orchestrates its own destruction.

Haemorrhage results if fibrin becomes removed prematurely from a tear in a blood vessel. However, this removal - long thought to be due to hyperfibrinolysis - is probably largely attributable to imperfect fibrin clot formations, due to the presence of large amounts of split products interfering with fibrin polymerization (Brommer *et al*, 1981). Thus, the importance of fibrin in haemostasis is further highlighted.

1.5.3 Fibrin and Platelets.

It has been reported that fibrinogen plays an essential role in activating platelets (Weiss and Rogers, 1971; Owen *et al*, 1975). It is now known that polymerizing or polymerized fibrin can activate platelets (Niewarowski *et al*, 1972; Michaeli and Orloff, 1976; Orloff and Michaeli, 1976). It has been shown by immunological and ultrastructural studies that fibrinogen is secreted relatively rapidly from washed platelets following thrombin stimulation and the secreted fibrinogen is subsequently deposited as fibrin on membrane surfaces of

aggregated platelets. This fibrin has a bridging role in platelet-platelet attachments (Chao *et al*, 1980; Shirasawa *et al*, 1972).

1.5.4 Malignancy and Fibrin.

Clotting abnormalities have long been observed in patients with malignant neoplasia. Fibrin deposits found in and around tumours have led to the suggestion that blood coagulation plays an important role in tumour growth and metastatic processes (Donati *et al* 1981). Fibrin deposits have been reported to surround primary and transplanted tumours in man and animals (Dvorak *et al*, 1979). However, since O'Meara (1980) suggested a nutritional role, the biological significance has remained a mystery. Two hypotheses, however, exist. First, that fibrin, coating tumour cells, acts as a glue facilitating the adhesion of the cell so that the cells of the primary tumour mass are protected from being disseminated into circulation (Weiss, 1977; Donati *et al*, 1981). The second theory is that fibrin represents a biological barrier against the hosts' cell defence mechanisms by retarding diffusion of antigens to the host

lymphoid tissue (Dingemans,1974; Donati *et al*, 1981). It has also been suggested that fibrin deposits may promote tumour growth by assisting in angiogenesis (Marx, 1981). Dvorak *et al* (1979) have shown that in hepatic carcinoma in guinea pigs neovascularisation - the stimulation of inflammatory cell responses and finally fibrinoplasia - could be induced by subcutaneous implantation of fibrin. This suggests activation of clotting and/or fibrinolytic systems by tumour cells may provide sufficient stimulation for ingrowth of new blood vessels without the requirement of a separate angiogenic factor. Conversely, it has been proposed (Donati *et al*, 1981) that fibrin deposits around circulating tumour cells can favour their arrest and lodgement (Wood, 1958).

1.5.5 Fibrin and Injury.

It is generally believed that fibrin facilitates responses to injury in a variety of ways. Walter and Israel (1974) suggested that fibrin assists by forming a union between severed tissues, thus limiting the exudative process and providing a haemostatic barrier. The mesh of fibrin may also act as a barrier to bacterial

invasion. This localisation may facilitate phagocytosis of organisms by invading leucocytes (Walter and Israel, 1974). Products released during the formation of fibrin and fibrinogen can attract additional inflammatory cells (Marx, 1982). Dvorak *et al* (1979) have found that fibrin deposits promote angiogenesis (in the same way as in cancer growth) and although this is part of normal healing, it could also serve to facilitate the entry of inflammatory cells into the damaged area.

Fibrin also supports the growth of certain tissues. Fibroblasts and neutrophils are among the first cells to migrate or grow into fibrin rich regions. These cells repeatedly alter morphology on contact with fibrin in cell cultures, and adhere and grow along fibrin fibres.

A fibrin adhesive system has been reported in bone healing.

1.5.6 General Comment.

The early view that the role of fibrin is specific to haemostasis is no longer true. While the arrest of haemorrhage requires a mesh through which red cells

should not be able to escape, fibrin network also provides a scaffold for reparative processes to proceed. It is clear from the above evidence that fibrin serves a variety of physiological needs. Investigations in the past have not examined the possible modification of fibrin network in meeting the complex and varied roles of fibrin in physiology. A study needs to be undertaken to establish whether physiological variables, which may alter in certain disease states, modify network structure, and if so what relevance these modifications have in physiology.

1.6 PROBLEMS FOR STUDY

Fibrin network structure has not been extensively studied since the pioneering work of Ferry and Morrison four decades ago. When interest in network structure was recently rekindled, techniques employed were similar to those used forty years ago. Previous studies on network structure, as mentioned earlier, were more suitable for the examination of the biophysics of the network. Little regard was paid to physiological interpretations of findings. The first comprehensive attempt at correlating

network structure to physiology was made by Ferguson (1982) who examined fibrin network characteristics using opacity ratio and mass-length ratio derived from permeability. Opacity ratio was calculated from clot turbidity measured at two wavelengths of incident light in much the same way as first used by Ferry and Morrison in 1947. This technique has also been used by other investigators, (Dhall and Bryce,1970; Muzaffar *et al*, 1972; Dhall *et al*, 1976). However,it is a qualitative method of measuring fibre diameter. Shah *et al* (1984) have more recently demonstrated several disadvantages to the use of opacity ratio as a technique. In a comparative study of two turbidimetric measures - opacity ratio and μ_T - they found lack of correlation between the two, particularly when the concentration of fibrinogen was increased within the pathophysiological range. The reason for this lack of correlation was found in the theoretical basis of the turbidimetric techniques which states that turbidity of a clot varies linearly with $1/\lambda^3$ where λ is the wavelength of incident light. It was observed that linear proportionality between the two methods break down as fibrinogen concentration was increased - especially at the smaller wavelengths, i.e.

at higher values of $1/\lambda^3$. Opacity ratio has generally been measured at 350 nM and 608nm. Shah *et al* (1984) also showed that the measured turbidity at 350nM was an underestimate of actual turbidity for the very turbid clots which form with high fibrinogen concentration. Further, they found the discrepancy between the measured and theoretical turbidities increased proportional to the increase in fibrinogen concentration.

It should be noted that in his studies Ferguson used an SP1800 spectrophotometer for turbidimetric studies. This equipment allowed measurement of optical density up to 2.0 absorbance units, and this is equivalent to a turbidity of 4.6 (i.e. 2 x 2.303). Thus, as the concentration of fibrinogen increases,the optical density at 350nM is at first an underestimate of the theoretical value and then attains a maximum constant value. Concurrently the turbidity of the same sample measured at 608nM is rapidly increasing. A ratio of the two results in an artifactual decrease in opacity ratio, interpreted as the formation of thicker fibres. These theoretical limitations of the opacity ratio technique have been found to give rise to erroneous interpretation. A further

source of inaccuracy in studies by Ferguson was a lack of standardization of technique. In a number of experimental studies he had failed to ensure that an equilibrium state had been reached and maximal conversion of fibrinogen to fibrin had occurred. This source of error was most obvious when thrombin concentrations were varied and networks with different fibrin content were compared. Furthermore, although most of his studies on networks were performed on those developed in pure fibrinogen solution, he used 0.15u/ml thrombin as the final concentration, basing this on results of a study by Schumann and Majerus (1976) who showed that the concentration of thrombin in vivo at the instant of clotting was 0.15 u/ml. Using 0.15u/ml thrombin for clotting, Ferguson incubated fibrinogen and thrombin for 30 minutes before making turbidimetric and permeability measurements. However, as early as 1947, Ferry showed that strengthening of the clot continued for many hours. Thus, although some fibrinogen converted to fibrin after 30 minutes, Ferguson did not ensure that networks were fully formed. Furthermore, thrombin generation is an explosive process and in the microenvironment of a clot thrombin concentration could be many times 0.15 u/ml, a

concentration Ferguson used for most of his studies.These major inadequacies of an otherwise comprehensive study question the results of such investigations.

It is accepted that the aims of Fergusons' investigations were sound and thus a need still remains for a full and comprehensive study of determinants of fibrin network structure under physiological conditions. However, before such a study may be undertaken it is first necessary to establish that methods currently available can indeed measure fibre characteristics consistently. Assuming that methods are reliable and reproducible,re-examination of network determinants is necessary to establish whether factors such as pH, ionic strength, temperature, and fibrinogen and thrombin concentration affect network structure when altered, not in the extreme pharmacological range already investigated, but in a narrow pathophysiological range. If currently available methods (and this would seem reasonable to suggest from Ferguson's work) are capable of detecting alterations in network structure in response to small changes in experimental conditions, then investigations should be directed to explore the possibility of using currently

available methods to characterize networks in plasma. The background theoretical basis rests on the assumption that these techniques may only be used in dilute fibrinogen solutions. A preliminary examination is required to determine whether the theoretical basis of the methods could be acceptably extended to plasma. If this is so then the methods need to be tested for their recovery and reproducibility when used in plasma. If this evaluation proves that these techniques can be used in plasma, any differences between networks developed in fibrinogen solution and in plasma should be fully examined to determine the role of various protein components. The more common proteins such as albumin, ɣ-globulin, fibronectin and antithrombin III should be examined critically to determine their contribution to network structure. Also, because a thrombus is composed of cellular elements, the influence of red cells and platelets should be evaluated.

If these methods are capable of being used in plasma reproducibly, then they should be examined for their applicability in sequential studies in clinical situations to determine whether when so used differences

may be demonstrated between individuals and whether a reproducible measurement characterizes one individual. Methods of venepuncture, handling of blood, anticoagulants used, time between venepuncture and measurements, will require standardization. These methods would be applicable to clinical studies if they can be shown to be capable of detecting changes in network structure in sequential studies.For investigations into this area of study, these methods will be used to detect the widely-known alteration in network characteristics induced by dextran (Dhall *et al*) both in vitro and after infusion. Surgery is known to induce dramatic changes in haemostatic and fibrinolytic characteristics. These methods will be used before and after operation to determine their applicability to sequential clinical studies.

CHAPTER 2

MATERIALS AND METHODS

In this chapter, general methods used in the thesis are described. Methods and materials used in special investigations are described in the appropriate chapters.

2.1 MATERIALS

All reagents used were of Analar quality.

2.1.1 Fibrinogen.

4gm of human fibrinogen (Grade 1, Kabi, Stockholm) was dissolved in 50ml of 0.3M sodium chloride and dialysed using cellulose casing dialysing tubing (Visking Co., Chicago, Illinois, USA) against 0.3M NaCl at 4°C for 20 hours with three changes. After dialysis the solution was centrifuged at 30,000g for 20 minutes and stored in 3ml aliquots in plastic tubes at -77°C. Fibrinogen concentration was determined from absorbance at 282nm.

2.1.2 Radio-Labelled Fibrinogen

0.9mg of I^{125}-labelled fibrinogen (Amersham, U.K.) 40.7mBq (110 µci) was dissolved in 1ml distilled water. Trace amounts of the labelled fibrinogen were added to fibrinogen solution or plasma at the start of

each experiment. This allowed the measurement of fibrinogen conversion, as described later.

2.1.3 Thrombin.

Bovine thrombin (Parke Davis, USA) was dissolved in 0.15M NaCl solution and stored in 0.3ml aliquots in polyvinyl tubes at -77°C.

2.1.4 Tris-saline Buffer.

50mM tris-HCl buffered saline (pH 7.35, ionic strength 0.153) was used to perfuse clots developed in permeation tubes.

2.1.5 Other Reagents.

Except where stated all other chemicals were of analytical reagent grade and were obtained from BDH (Melbourne) or Sigma (St.Louis, USA). Always distilled water or 0.15M saline was used to prepare solutions.

2.2 APPARATUS

2.2.1 Containers.

Disposable plastic test tubes were used wherever possible.

2.2.2 Glassware.

All glassware was cleaned by soaking in Pyroneg solution (Diversay, Sydney). This was followed by scouring with a bristle brush and extensive rinsing under tap water and three rinses with distilled water. Equipment was oven-dried at 80°C.

2.2.3 Cuvettes.

Pye Unicam glass-quartz cuvettes (10 mm FO3) were used.

2.2.4 Spectrophotometer.

Pye Unicam SP1800 ultraviolet spectrophotometer, with a Unicam SP1805 Programme Controller (Cambridge, England) was used for all turbidimetric studies.

2.2.5 γ-Counter

An Autologic γ-Counter (Abbott Laboratories, N.Chicago, Illinois 60064, USA) was used for radioactive counting.

2.3 METHODS

2.3.1 Fibrin Network Developed in Fibrinogen Solution.

Unless otherwise stated, fibrinogen solution containing I^{125}-labelled fibrinogen was clotted at 22°C ± 2°C. pH of the clotting mixture was 7.35, ionic strength 0.153 (0.05 due to TRIS, and the rest due to NaCl). Ionic strength was calculated on the basis of molarity. Activity coefficients, degree of calcium binding and contributions of added macromolecular substances were not taken into account. Fibrinogen concentration was, unless otherwise stated, 2.5mg/ml which corresponded to the mean physiological concentration of fibrinogen in blood, obtained from patients and blood donors at the Woden Valley Hospital, Canberra. When fibrinogen solution was used, it was degassed for 10 minutes using a vacuum pump. This ensured that no bubbles formed in the cuvettes when the solution was clotted. Inadequate degassing led to

microbubbles which interfered with light scattering studies.

2.3.2 Determination of Fibrin Conversion.

Trace amounts of I^{125} was added to fibrinogen solution or plasma. The fibrinogen solution or plasma was then clotted in glass tubes used for the determination of the mass-length ratio from permeation. Approximately 50µl of the perfusate was counted using a γ-counter. 50µl of the unclotted solution was also counted. The following formula was then used to calculate the percentage of fibrin conversion:

$$\text{CONVERSION } (\%) = \frac{A - B}{C} \times 100$$

where A is the CPM from unclotted fibrinogen solution or plasma, B is the CPM from the perfusate serum, and C is the CPM from unclotted fibrinogen solution or plasma.

2.3.3 Fibrin Network Developed in Plasma.

Blood from healthy volunteers was obtained by atraumatic venepuncture from antecubital veins using 30ml plastic syringes, and 21 gauge needles, and mixed in a

ratio 9:1 with 3.8% Tri-sodium citrate. 35µl of 10,000 KIU/ml Trasylol (Bayer, Germany) was added to inhibit fibrinolytic activity. The blood was centrifuged at 2400g at room temperature for ten minutes to obtain platelet-poor plasma (PPP) , which was then centrifuged at 4000g for a further five minutes to obtain essentially platelet-free plasma, with a platelet count of 3000 platelets per ml, or less. Platelet count in plasma was made using a Coulter S plus 4 Particle Counter (Coulter Electronics, Florida, USA). It was observed that a platelet count above 3000 platelets/ml caused clot retraction in cuvettes and therefore made optical measurement technically unreliable.

2.3.4 Fibrinogen Concentration in Plasma.

Fibrinogen concentration was determined by one of the following methods. The method used is indicated in the appropriate section in this thesis.

2.3.4.1 Heat Estimation (Modified from Watson, 1961)

This test was used as an initial estimation of fibrinogen concentration in plasma and is based on the principle of

selective precipitation of fibrin at 56°C. Plasma was divided into two aliquots of 0.3ml. To one was added 2.7ml saline (blank), and to the other 2.7ml of buffer (test). The buffer contained 8.42g $NaH_2PO_4.H_2O$, 8.9 g $NaHPO_4.12H_2O$, 4.25 g/l NaCl. The test solution was heated in a 56°C water bottle for ten minutes. The optical density (O.D.) of each tube was then read in a Pye Unicam SP6-350 visible spectrophotometer (Cambridge, England). The results were obtained using the following equations:

$$\text{Fibrinogen concentration (mg/ml)} = \frac{OD_T - OD_B}{OD_R - OD_{RB}} \times Rcon$$

Where OD is optical density, R is reference, T is test, RB is reference blank and Rcon is concentration of reference.

2.3.4.2 Hickman's Gravimetric Method (1971).

1ml of PPP was added to two 5.0ml plastic tubes containing 2.0ml of phosphate saline buffer (pH 6.0) and 40 NIH units of thrombin. The samples were allowed to clot for 2 hours at room temperature. The clot was then removed by a straightened paper clip and washed

first in saline for 30 minutes, then in distilled water for 30 minutes and finally, after removal from the paper clip, for 30 minutes in acetone. The acetone was drained from the clot and the clot was placed in a glass dish and baked for 48 hours at 90° ± 5°C. The clot was weighed immediately after removal from the oven, and this was taken as the concentration of fibrinogen per ml of plasma.

2.3.4.3 Modified Ratnoff and Menzie (1951).

This assay is based on the measurement of tyrosine residues in plasma derived from the breakdown of fibrinogen. To each of two 15ml conical centrifuge tubes was added 0.5g of glass beads, 5ml of 0.9% NaCl and 0.1ml platelet-poor plasma. The solution was mixed by inversion and 0.025ml of 1000 NIH u/ml thrombin added. The contents were mixed again and allowed to stand for 20 minutes. The tubes were then centrifuged for 20 minutes at 1200 - 1500g. The supernatant was removed leaving the fibrin enmeshed on the glass beads. The fibrin was washed three times in normal saline and centrifuged at 1200 - 1500g for 15 minutes between washes. The saline was removed, 0.5ml of 1000 g/l NaOH

added to the fibrin enmeshed in the glass beads which were placed in boiling water, both for 15 minutes. After the beads had cooled, 3.5ml of water was added to each tube and mixed well. The contents were then transferred to 150 x 16mm glass tubes, and 1.5ml of 200 g/l $NaHCO_3$ added. To this mixture was added 0.5ml of Folin-Ciocalteu reagent, and the tubes allowed to stand for 20 minutes for the development of blue colour.

Two test blanks containing 0.5ml of 1000 g/l NaOH glass beads are treated similarly. Standard curves drawn after optical density of standard mixture were read at 650nm containing:

0.1ml of Tyrosine standard, 0.5ml of 100 g/l NaOH, 3.4ml of distilled H_2O, 1.5g/ml of 200 g/l Na_2CO_3, and 0.5ml of Folin Ciocalteu solution.

The standard blanks contained no tyrosine. The optical density of the preparation was then read at 650nm and the concentration of fibrinogen read off the standard curve.

2.3.5 Network Development.

Development of network was followed by recording increase in optical density at 800nm with a Unicam AR25 linear recorder (Pye Unicam Ltd., Cambridge, England) coupled to the spectrophotometer, until turbidity reached a plateau and showed no further increase for 20 minutes. These recordings of network growth were also used to derive information on the kinetics of networks development.

2.3.6 Preparation of Networks in Cuvettes for Mass-length Ratio from Turbidity (μ_T)

0.9ml of platelet-poor plasma or pure fibrinogen was pipetted into glass tubes. 0.1ml of the appropriate thrombin was added, and the solution stirred with a glass rod. After turbidity reached a plateau, optical density measurements were made at wavelengths of 608, 650, 700, 750 and 800nm , using untreated plasma or pure fibrinogen solution in the reference cell.

2.3.7 Turbidity Technique.

The optical density of fibrin networks developed in a

cuvette with path length of 1cm was measured at a range of wavelengths using a spectrophotometer. Unclotted fibrinogen solution or plasma was used in the reference cell.

Carr and Hermans (1978) showed that turbidity of clots made from fibrinogen is given by the equation:

$$T = 44\ \pi K\ c\ \lambda\ \mu / 15n \qquad (1)$$

where c is the concentration of the fibrin fibres in mg/ml, λ the wavelength, n is the refractive index of the solution, and μ is the mass-length ratio of the fibres. The constant K is given by:

$$K = 2\pi^2 n^2 \ (dn/dc)^2 \ / \ N\lambda^4 \qquad (2)$$

where dn/dc is the specific refractive index increment of the solute in the solvent, and N is the Avogadro number.

The equation is valid provided the diameter of the fibrin fibres is small compared to the wave length of the incident light.

At 633nm:

$$\frac{44}{15} K\lambda^4/n = (44/15)\, 2\pi^3 n\, (dn/dc)^2 \;/\; N = 1.48\times10^{-23} \text{ daltons/cm} \qquad (3)$$

Thus, ignoring the slight wave length dependence of n and dn/dc, the average mass-length ratio of the fibrin fibres is calculated from measurements of turbidity at 800nm, μ_T (800nm) is given by:

$$\mu_T\ (800nm) = \frac{T\lambda^3}{1.48c} \times 10^{23} = \frac{34.59T}{c} \times 10^{23} \text{ daltons/cm} \qquad (4)$$

where c is in mg/ml.

Hantgan and Hermans (1979) showed that:

$$\frac{44\pi Kc\lambda}{15Tn} = (1 + 184\pi^2 p^2 n^2/77\lambda^2 \ldots)/\mu \qquad (5)$$

and that for cylindrical fibres of radius r

$$p^2 = \frac{r^2}{2} \qquad (6)$$

where p is the radius of gyration and where equation (4) is derived, in part,from equations (1) and (2). Equation (5) may be expressed in the linear form y = A+Bx using equations (2) and (6) and by neglecting higher order terms:-

$$\frac{c}{\lambda^3 T}\left[\frac{88\pi^3 n(dn/dc)^2}{15N}\right] = \frac{1}{\mu} + \frac{r^2}{\mu}\,\frac{184\pi^2 n^2}{154\lambda^2} \qquad (7)$$

A more accurate determination of μ_T, therefore, for networks with thicker fibres (where turbidity is not proportional to $1/\lambda^3$) is attained from plots of $C/T\lambda^3$ as a function of $1/\lambda^2$. Five wavelengths between 600 and 800nm were used for such plots. Then, μ_T was computed from the intercept of A of the plot by the equation:-

$$\mu_T = \frac{10}{1.48A} \times 10^{12} \text{ daltons/cm} \qquad (8)$$

Refractive indices used for calculations of μ_T for plasma and fibrinogen clots were slightly different (Appendix 2).

2.3.8 Mass-length Ratio from Permeability.

Fibrin networks were formed in pre-etched glass tubes (24 hours at 1% HF) cut from 2ml glass pipettes. Each tube was 100mm in length and 3mm in internal diameter;

the individual internal dimensions of each tube were carefully measured (Carr *et al*, 1977). Clots were formed in tubes with one end covered with 2 layers of parafilm. The tube containing the clot was then attached, using plastic tubing, to a horizontally mounted 1ml pipette on a retort stand. The network was perfused with Tris HCl saline buffer of pH 7.35, ionic strength 0.153 at a pressure head of 150mm water (Figure 2.1). Care was taken to increase the pressure head by 50mm for each three-minute period. The transit time of an accurately measured volume of perfusate was then recorded. The initial 0.5ml of perfusate was collected for the determination of fibrin content of the network.

The permeability (τ) or Darcy constant of the fibrin network was calculated from:

$$\tau = \frac{Qh\eta}{FxtxP} \qquad (9)$$

Where Q is the volume flow in time t, η is the viscosity of the liquid, h is the length of the clot, F its cross-section and p the applied pressure. By assuming a value of 10 (Carr *et al*, 1977) for the fibre packing constant K, permeability is related to the mass-

length ratio, μ_P, of fibrin fibres according to the equation:

$$\mu_P = \tau k c \pi / 4 \qquad (10)$$

where c is in mg/ml

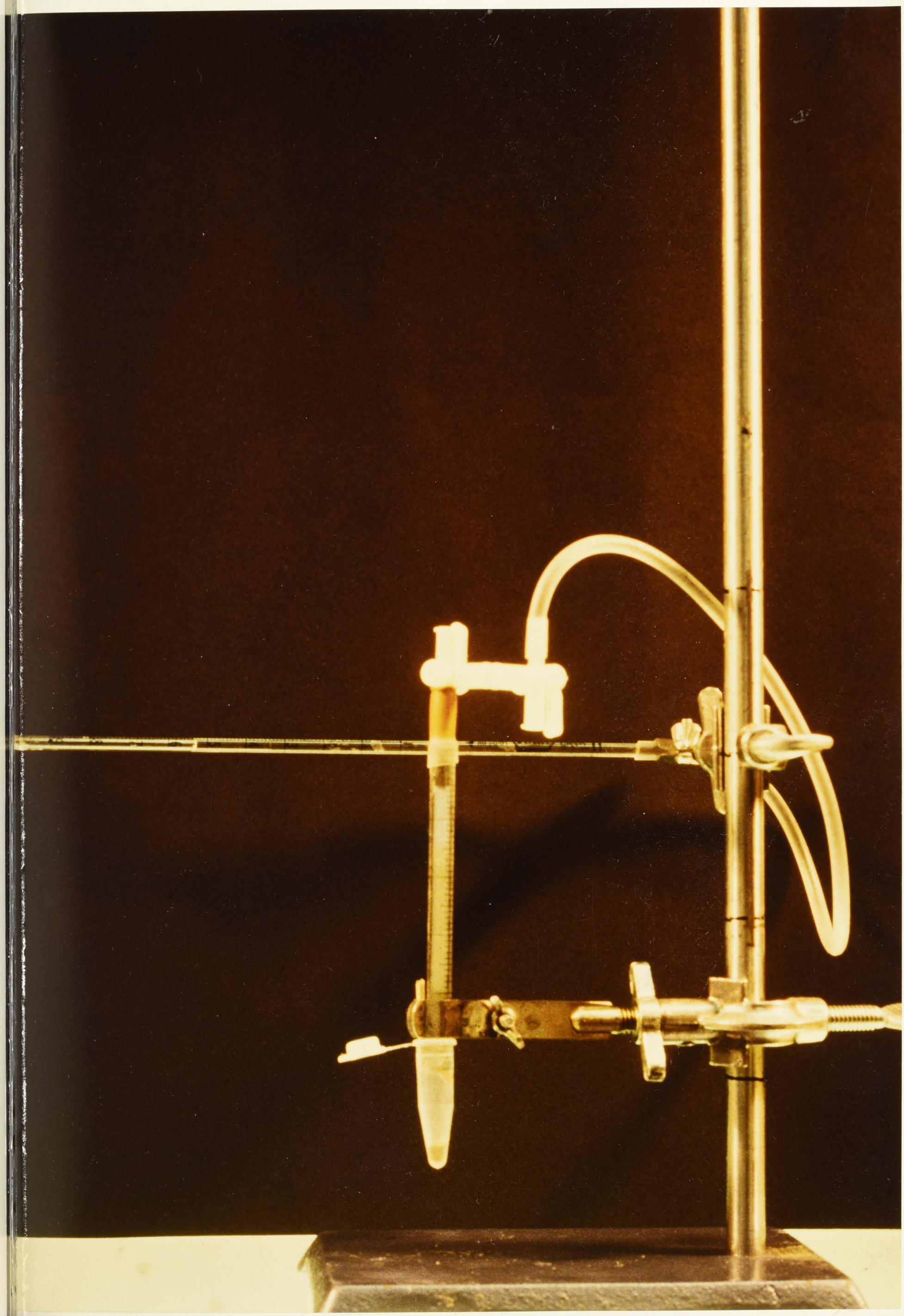
2.3.9 Compaction.

Compaction is based on the method of Dhall *et al* (1976). Fibrin network was formed in a 1.5ml Eppendorf microcentrifuge tube, pre-sprayed with lecithin-based aerosol (Spray and Cook, Woolworths) to render the surface non-adhering. The clots were centrifuged at 8000g for 45 seconds in the Eppendorf microcentrifuge, model 3200, after they had been allowed to clot for a period of time known to correspond to plateau turbidity. The volume of fluid expelled from the network was measured with a 1ml syringe, and expressed as a percentage of the initial volume. In this thesis, compaction was measured only in networks developed in plasma. This method is not applicable to fibrin networks developed in fibrinogen solutions.

2.4 Statistical Analysis.

The Hewlett Packard 9815A bench calculator and supplied statistical package was used for statistical analysis. Plots were made with a Hewlett Packard 7225A plotter attached to the calculator. Unless otherwise indicated, significance levels for all experiments were calculated using the Students' test for 2 tail distribution.

Figure 2.1 Apparatus used to calculate permeability of fibrin networks.



SECTION 1

STUDIES ON FIBRIN NETWORK STRUCTURE DEVELOPED IN FIBRINOGEN SOLUTION

CHAPTER 3

REPRODUCIBILITY AND LIMITATIONS:NETWORKS DEVELOPED IN FIBRINOGEN SOLUTION.

3.1 INTRODUCTION

In this chapter, recovery and reproducibility are examined to highlight problems associated with the methods used to characterize networks developed in fibrinogen solution.

3.2 MATERIALS AND METHODS:

Materials and methods have been fully described in Chapter 2.

Compaction was measured using thrombin concentrations between 0.19 to 1.50 units/ml.Four replicate measurements were made for each concentration of thrombin. Fibrinogen concentration was 2.43mg/ml while pH was 7.35 and ionic strength was 0.153.

3.3 RESULTS.

3.3.1 TECHNICAL LIMITATIONS AND PROBLEMS IN NETWORKS DEVELOPED IN FIBRINOGEN SOLUTIONS

3.3.1.1 Turbidity and μ_T.

Turbidity measurements were easily made and problems in measurement arose, as mentioned already in Chapter 2, only when gas bubbles appeared in the networks. The degassing procedure described in Chapter 2 ensured that networks developed were bubble-free. Care was also taken to ensure that the network was homogeneous. Inhomogeneity in the network was a problem with thrombin concentrations above 1.5NIH u/ml, and was from a very short lag period which did not allow time for adequate mixing of thrombin and fibrinogen.

Turbidity is proportional to $1/\lambda^3$ (Carr and Hermans, 1977) in dilute fibrinogen solutions. However, this relationship breaks down when small wavelengths are used. However, at large wavelengths a plot of turbidity and $1/\lambda^3$ is almost linear (see Figure 4.6,Chapter 4) and measurements were made using these wavelengths for all experiments where μ_T was derived in this thesis.

3.3.1.2 Permeation and μ_P.

The permeation technique is technically a demanding procedure. In this technique also, the highest thrombin usable was 1.50NIH u/ml. The critical variables in this method were flow rate and network protein concentration. It was always necessary to ensure that the system had stabilized before the start of any experiment. Carr *et al* (1977) and Ferguson (1983) showed that significant changes in flow rate did not occur over a period of time. Perfusion in these networks was only started after an interval which in separate experiments showed that no further increase in optical density may be expected. This ensured that measurements were made only after networks were fully developed.

3.3.2 Day to Day Variation:

Table 3.1 shows characteristics of fibrin networks developed in pure fibrinogen solution over a three day period. Three sets of experiments under identical conditions were performed over three days. Table 3.1 shows typical results. Fibrin content (C_n) of network is very reproducible, as also are permeability, μ_P and μ_T over the three day period.

3.3.3 Batch to Batch Variations.

Table 3. 2 shows results obtained from three batches of fibrinogen. Although fibrin content (C_n) is similar in the three batches, permeability, μ_P and μ_T vary according to the batch of fibrinogen used. This variation highlights the need to perform a control test using the same batch of fibrinogen as the test.

3.3.4 Recovery of Data Within Each Experiment.

An experiment was performed with four replicates of μ_T and eight replicates of μ_P and permeability. The results in Table 3.3 show that the method is highly reproducible. The standard deviation for μ_P and μ_T are small. A slightly higher variation for permeability is found. The results show that measurements can be confidently accepted.

3.3.5 Compaction

Compaction decreases as thrombin concentration increases (Table 3.4). However, the volume expelled is very small and SEM is very large making differences between low thrombin and high thrombin insignificant.

3.4 DISCUSSION

Using a single batch of fibrinogen it has been shown that day to day variation in permeability, μ_P and μ_T is small and the results are highly acceptable (Table 3.1). In any given batch fibrin content does not vary greatly either from one day to the next (Table 3.1) or from one batch to the other (Table 3.2). For μ_T and μ_P the standard error of mean was acceptably small (Table 3.3). However, permeability shows a greater variation. From the results shown it is also clear that permeation gave the greatest coefficient of variation (Tables 3.1, 3.2 and 3.3). Thus in each experiment four replicates of permeation (and therefore μ_P) were made. The recovery of turbidity measurement was such that two replicates for each observation of μ_T were considered sufficient for all future work.

Batch to batch variation in μ_T, μ_P and permeability despite relatively constant fibrin content (Table 2) is well known and has been previously examined by Becker and Waugh (1980) and Wolfe and Waugh (1981). They suggest that variation in fibrinogen solution from batch to batch may arise from thawing and other manipulations. These variations underline the need for each experiment to have its own control, and this is the manner in which methods have been used in this thesis. With these reservations,

results are highly consistent and reproducible with regards to direction of change.

Compaction relies on an accurate measurement using a 1ml syringe of fluid expelled from the network on centrifugation. Table 3.4 shows that the volumes expelled were very small and these became even smaller as the concentration of thrombin was increased. Measurement of such small volumes was inaccurate. It was decided not to use compaction measurements in networks developed in fibrinogen solution.

3.5 CONCLUSIONS.

1. Mass-length ratio derived from turbidity and permeability has been examined for its reliability.

2. Although day to day and batch to batch variations occur, concurrent control experiments minimize erroneous conclusions.

3. Compaction cannot be reliably measured in fibrin networks developed in pure fibrinogen solutions.

TABLE 3.1 DAY TO DAY VARIATION IN CHARACTERISTICS OF FIBRIN NETWORKS

The day to day variation in characteristics in fibrin networks developed in fibrinogen solution. Results are expressed as mean of three experiments. Standard error of the mean is shown in brackets.

A) Day	Fibrin Content (mg/ml)	Permeability $x10^{11}$ (cm^2)	μ_P $x10^{-12}$ daltons	μ_T $x10^{-12}$ daltons
1	2.20	173	18.0	23.0
2	2.28	149	16.1	22.1
3	2.28	156	16.8	20.8
$\bar{X}$	2.25	150	17.0	22.0
(SEM)	(0.02)	(7)	(0.5)	(0.6)

TABLE 3.2 BATCH TO BATCH VARIATION IN CHARACTERISTICS OF FIBRIN NETWORKS.

Batch to Batch variation in characteristics of fibrin network structure. Results in three different batches are shown. Each is the mean of three measurements.

Batch	Fibrin Content (mg/ml)	Permeability ($x10^{11}$ cm^2)	μ_P $x10^{-12}$	μ_T $x10^{-12}$
1	2.13	450	45.0	25.7
2	2.15	134	15.3	26.2
3	2.20	173	18.0	23.0

TABLE 3.3 REPRODUCIBILITY OF RESULTS

Reproducibility of results within one experiment on a single batch of fibrinogen on the same day. Mean of four replicates of μ_T, and 8 replicates of μ_P and permeability are shown.

μ_T	(n)	SEM	μ_P	(n)	SEM	permeability	(n)	SEM
15.75	4	0.54	13.6	8	0.6	140	8	3

TABLE 3.4 Effect of Thrombin On Compaction In Networks Developed In Fibrinogen Solution.

Results are mean of four experiments.

Thrombin Concentration u/ml	Compaction (%)	±SEM
0.19	10	1
0.30	9	1
0.70	8	2
1.50	7	1

CHAPTER 4

PHYSIOLOGICAL STUDIES ON FIBRIN NETWORK STRUCTURE.

4.1 INTRODUCTION

Since early studies by Ferry and Morrison (1947) methodology for examining fibrin networks has advanced considerably. These advances, however, have not been paralleled by comprehensive studies on fibrin networks in fibrinogen solution in the relatively narrow pathophysiological range of conditions of clotting.

As fully described in the Problems for Study in Chapter 1, Ferguson (1982) undertook comprehensive investigations on network characteristics under narrow pathophysiological conditions, but he relied on opacity ratio and mass-length ratio derived from permeability as determinants of network structure. It has already been mentioned that Ferguson's investigations were open to error for various methodological reasons: he measured turbidity at a single wavelength; his results based on opacity ratio were inaccurate; he did not ensure complete conversion of fibrinogen to fibrin and he did not critically examine the kinetics of network growth.

This chapter examines the effect of fibrinogen and thrombin concentrations on network characteristics in fibrinogen solution.

4.2 MATERIALS AND METHODS

The general methods have been described in Chapter 2.

4.2.1 Effect of Thrombin Concentration.

Networks were formed as described in Chapter 2 using thrombin concentrations of 0.15, 0.25, 0.50 and 1.00 and 1.50u/ml. Kinetics of network growth, fibrinogen conversion to fibrin, turbidity and permeability were measured, and μ_T and μ_P derived.

4.2.2 Effect of Fibrinogen Concentration.

Fibrin networks were formed in pure fibrinogen solution using fibrinogen concentrations of 1.5, 2.5, 3.5, 4.5, and 5.4mg/ml,with three different thrombin concentrations of 0.15, 0.50, and 1.50 NIH u/ml thrombin. The kinetics of network growth were followed by turbidity measurements at 800nm. When the turbidity recording had plateaued, permeability was also measured. μ_T and μ_P were derived as described in Chapter 2.

0.45ml of fibrinogen solution was clotted with 50µl of the appropriate thrombin in 1ml Ependorf centrifuge tubes. Conversion of fibrinogen to fibrin was measured by counting radioactivity in fluid expelled from the networks squeezed with wooden spatula at five minute intervals between 0 and 30 minutes, and thereafter at 1,2,3 and 4 hours after clotting had commenced. Each measurement was performed in duplicate.

4.3 RESULTS.

4.3.1 Effect of Thrombin Concentration.

The development of turbidity at 800nm when fibrinogen was clotted at different thrombin concentrations is shown in Figure 4.1. When 1.5 u/ml thrombin was used, turbidity increased rapidly and plateaued in five minutes. At progressively lower thrombin concentrations lag phase steadily increased; increase in turbidity was slower but reached higher values. These curves took longer and longer to plateau. With 0.15 u/ml thrombin, plateau was reached after 90 minutes.

The kinetics of conversion of fibrinogen to fibrin is

shown in Figure 4.2. There was maximal conversion of 91% within five minutes after clotting with 1.5 u/ml thrombin. As concentrations of thrombin were lowered, it took progressively longer for maximal conversion. With the lowest thrombin concentration of 0.15 u/ml, maximal conversion of 91% was reached within two hours. With all thrombin concentrations used, however, the maximal conversion of 91% was reached by four hours.

Figure 4.3 shows the sequential variation in μ_T (800nm) with time after the addition of different concentrations of thrombin. For both the higher thrombin concentrations used, no further increases in μ_T values were seen after ten minutes. For the lower thrombin concentrations, however, μ_T (800nm) increases even after ten minutes and continues to do so until four hours. Thus, for higher thrombin concentrations, incorporation of fibrin into the network stops after ten minutes. When lower concentrations of thrombin were used, fibrin continues to be incorporated into the network resulting in thicker fibres denoted by a higher μ_T.

Figure 4.4 shows the effect on μ_T of increasing thrombin concentrations. A log linear relationship showed that μ_T was thrombin concentration-dependent, i.e. fibrin fibres

became thinner as thrombin concentration was increased. Similar trends were seen in μ_P and τ, as shown in Figure 4.5.

4.3.2 Effect of Varying Fibrinogen Concentration.

Fibrinogen solutions containing 1.50 to 5.40mg/ml fibrinogen were clotted with 1.50u/ml thrombin and turbidity of the networks was measured at a range of wavelengths. Figure 4.6 shows the plot of turbidity against $1/\lambda^3$ in these networks. As fibrinogen concentration was increased, the relationship became increasingly curvilinear. The curve, however, extrapolated to the origin at a fibrinogen concentration of 1.50mg/ml.

$C/\tau\lambda^3$, as described in Chapter 2, was determined from a plot of $C/T\lambda^3$ as a function of $1/\lambda^2$. At higher fibrinogen concentrations, and particularly at levels which were compatible physiologically, i.e. 1.50 - 5.4 mg/ml, the curves did not extrapolate to the origin and the Y axis intercept increased with fibrinogen concentration. However, when thrombin concentration was varied at a fibrinogen concentration of 2.5mg/ml (taken in this thesis as the physiological concentration of

fibrinogen) the dependence of μ_T on thrombin concentration was given by the equation:

$$\text{Log } \mu_T = 0.40 \text{ log [Th]} + 10.0$$

where [Th] is the molar concentration of thrombin (assuming 1.00mg/ml equals 8.64×10^{-9} M thrombin). This equation compares favourably with that reported by Wolfe and Waugh for fibrinogen systems in which turbidity varies as $1/\lambda^3$, i.e:

$$\text{Log } \mu_T = 0.41 \text{ log [Th]} + 10.1$$

It is apparent from this reasoning that μ_T derivations at higher fibrinogen concentrations are not unacceptably violated and they may be used confidently as highly acceptable values for comparative purposes.

Figure 4.7(a) shows the effect of increasing fibrinogen concentrations on μ_T. Three thrombin concentrations were used. At low thrombin concentrations μ_T increased, showing thicker fibres were formed as fibrinogen concentration (C) increased. At the highest thrombin concentration, however, C does not have a significant effect on μ_T, as in all cases conversion of fibrinogen to fibrin was 91% by four hours.

Figure 4.7(c) shows the effect on permeability of networks of increasing fibrinogen concentration clotted with three different thrombin concentrations. At all three concentrations, the permeability of the network decreased as fibrinogen concentration was increased. The results are in agreement with Blomback and Okada (1982), where curves of τ against 1/C were found to be linear and extrapolated towards the origin (see Figure 4.8).

Although τ decreased with increasing fibrinogen, μ_P remained unchanged under similar conditions (Figure 4.7(b). Thus, at lower thrombin concentrations the relationship between μ_T and μ_P broke down. The invariance of μ_P can be ascribed to the relationship that it is proportional to the product $C\tau$ (equation 6, Chapter 2) which remained unchanged as C was increased. At higher thrombin concentrations, however, μ_T and μ_P were unchanged at different fibrinogen concentrations whilst τ decreased.

4.4 DISCUSSION

Accurate determinations of mass-length ratios can be made

from turbidity measurements, where μ_T can be derived from plots of $c/T\lambda^3$ as a function of $1/\lambda^2$. Such observations can be made even at high fibrinogen concentrations where, as shown in Figure 4.6, plots of turbidity against $1/\lambda^3$ became increasingly curvilinear. The higher thrombin levels are compatible with those in the physiological range. It has been shown that μ_T values are highly acceptable albeit approximate values. Thus, μ_T measured at physiological ranges of fibrinogen and thrombin do not unacceptably violate assumptions made in its derivation.

The above observations are very important in that they show that the methodology described can be employed to examine network characteristics in physiological studies. Many studies in the past four decades on fibrin network structure have used purified fibrinogen as the medium (Ferry and Morrison, 1947; Carr *et al*, 1977; Carr and Hermans, 1978; Wolfe and Waugh, 1981; Blomback and Okada, 1982; Okada and Blomback, 1983). Further, these studies have investigated characteristics of fibrin networks developed in fibrinogen solution under non-physiological conditions of clotting, such as very high ionic strength, pH and fibrinogen and thrombin concentrations. The

present study has shown that fibrinogen and thrombin, when varied in the physiological range may induce significant alterations in fibrin network characteristics.

Ferguson (1983) attempted a similar study when he examined the effect of thrombin and fibrinogen concentrations on network structure . He allowed networks to develop for various time periods; e.g. using thrombin concentrations of 0.05, 0.10, 0.15, 0.25 and 0.50u/ml, he allowed networks to develop for 90, 45, 30, 18 and 9 minutes respectively. As shown in the present study (Figures 4.1 and 4.2), with a thrombin concentration of 0.15u/ml although the minimum time required for full conversion was 45 minutes, full network development (taken as the plateau phase of the turbidity curve) took about two hours. Thus, in his study Ferguson had compared networks with varying amounts of fibrin content and this was incorrect. Although fibrin conversion itself was accounted for in his study, measurements of turbidity - required for μ_T or opacity ratio derivation - were taken when the network was still developing. This deficiency is further underlined when the permeation studies are

considered. Here, it is apparent from his studies that permeation experiments may have commenced when network development was incomplete. Therefore, permeating buffer through the network may have dislodged and washed away fibrin monomers which were still in the process of incorporating into the network.

Furthermore, Ferguson relied on measurements of opacity ratio and mass-length ratio derived from turbidity measured at one single wavelength. In the opacity ratio technique, turbidity of clots is measured at two different wavelengths (350nm and 608nm in Ferguson's study) and expressed as a ratio as a semiquantitative measure of fibre thickness. Shah *et al* (1984) have shown that at certain wavelengths of incident light, measured turbidity in very turbid clots is an underestimate. Furthermore, the discrepancy between measured and theoretical turbidities increases proportional to an increase in the concentration of fibrinogen. Thus, Ferguson's conclusions were unreliable, based as they were on techniques the theoretical and experimental bases of which are now open to question. Mass-length ratio derived from turbidity gives a quantitative description of fibrin fibre thickness, and when μ_T is derived from

turbidity measured at several wavelengths, it becomes a more precise method which does not suffer from the experimental and theoretical limitations imposed on opacity ratio or μ_T derived from turbidity at one single wavelength.

Furthermore, Ferguson used 0.15u/ml thrombin for clotting fibrinogen solution because Schuman and Majerus (1976) showed that thrombin concentration at the instant of clotting in freshly-drawn native blood is 0.15u/ml. It is shown in this chapter that this concentration of thrombin led to the formation of thicker fibres and was very sensitive to variation in fibrinogen concentration. In all later experiments with fibrinogen solution, however, 0.5u/ml thrombin was used. This concentration was selected because the maximal conversion and the plateau (or equilibrium phase) of network growth was quickly achieved. Furthermore, this was an intermediate concentration of thrombin which provided ample room to discern changes caused to network structure by systematic changes in the environment in a pathophysiological range. Finally, it may be argued that although 0.15u/ml thrombin may be present at the instant of clotting, in the microenvironment of the thrombus the concentration may be many times higher, especially since in the physiological system thrombin is continually generated throughout the clotting process.

Blomback and Okada (1982) and Wolfe and Waugh (1981) suggested that the thickness of fibrin fibres was determined by the kinetics of its growth. The results of this study support this view. Their premise was that fibrin network develops initially as a mesh of protofibrils whose lengths were determined by the speed at which fibrinogen was converted to fibrin monomer, as well as other environmental conditions like ionic strength, pH and temperature. Fibrin monomer generated subsequently was incorporated into the existing network making fibres thicker. Thus, when mass-length ratio was determined sequentially from turbidity at 800nm, it was found to be similar at ten minutes for all thrombin concentrations (Figure 4.3). After ten minutes μ_T (800nm) remained unchanged at high thrombin concentrations because the substrate, i.e. fibrinogen, had been fully consumed. However, at low thrombin

concentrations, because of slow fibrinogen conversion fibre growth continued beyond ten minutes (Figure 4.2) leading to the formation of thicker fibres. In keeping with this scheme, the network at four hours showed that both μ_T (whether derived from measurements at 800nm or from a range of wavelengths) and μ_P decreased in a log-linear fashion with increase in thrombin concentration (Figures 4.4 and 4.5). Permeability of the networks as a whole was seen to decrease in a similar fashion (Figure 4.6).

The effect of fibrinogen concentration (C) on μ_T (Figure 4.7) is thrombin concentration-dependent. At low thrombin concentration increasing C increases μ_T. Increasing C slows down fibrinogen conversion because of an abundance of substrate and a limited concentration of the enzyme. This slowing down of reaction led to the formation of thicker fibres. At 1.5u/ml thrombin (highest thrombin concentration examined) fibrinogen conversion was not influenced by an increase in fibrinogen concentration and neither was μ_T.

In accordance with the findings of Blomback and Okada

(1982) τ against $^1/C$ (Figure 4.8) were found to be linear and extrapolated towards the origin at most thrombin concentrations tested. (However, at 1.50u/ml thrombin τ against 1/C did not extrapolate towards the origin). Consequently , the product CT remained unchanged as C was increased. Since μ_P is proportional to C (equation 6, Chapter 2) it showed no dependence on the concentration of fibrinogen even at the lowest thrombin concentration (Figure 4.7(b)). It is clear from this data that not only are the mass-length ratios derived independently, they are also dissimilar in values and may, at times, follow dissimilar patterns when conditions under which networks are developed are altered systematically. This peculiarity can be explained if one considers the two-network model of fibrin network structure enunciated by Shah *et al* (1982). In that study it was shown that fibres in a network are polydisperse in diameter , varying by a factor of 10 . These fibres can, therefore, be divided into a major network of thicker fibres and a minor network of thinner fibres. The minor network contributes very little to turbidity (and therefore μ_T) while it can markedly affect the permeability of the network. Therefore, according to this

model, a proportional increase in the protein content of the network ensues when C increases, i.e. there is an increase in the numerical density of minor network fibres. Thus, although major network fibres become thicker as C increases, the expected rise in permeability following this effect is masked by a decrease in permeability due to an increase in the numerical density of major and minor network fibres, in such a way that μ_P remains invariant.

As mentioned earlier in this discussion, this study agrees with the suggestion that fibrin network structure is determined by the kinetics of fibrin fibre growth. However, Okada and Blomback (1982) have also further suggested that thrombin clotting time could be used as a determinant of network structure. This premise is questioned. Abildgaard (1965) had shown, using N-terminal analysis, that the bulk of fibrinogen conversion occurs beyond the gel point. Caution should be exercised with over-reliance on gel point, especially in plasma or whole blood systems where antithrombin may influence enzyme kinetics beyond the gel point.

This study is critical to the interpretation of results of experiments described fully later in this thesis.

4.5 CONCLUSIONS

1. Theoretical assumptions in methodology are not unacceptably violated when applied to networks developed in fibrinogen solution within the pathophysiological range of fibrinogen concentrations.

2. Fibrin networks developed in purified fibrinogen solutions were sensitive to changes in fibrinogen concentrations in a pathophysiological range.

3. Kinetics of network development showed that thickness of fibrin fibres were determined by the kinetics of growth.

4. At high thrombin concentrations fibrinogen variation had no effect on μ_T or μ_P but decreased permeability. At low thrombin, μ_T was increased with increasing fibrinogen but μ_P was unaffected.

5. μ_T and μ_P need not necessarily mirror each other, nor were they experimentally found so under all conditions.

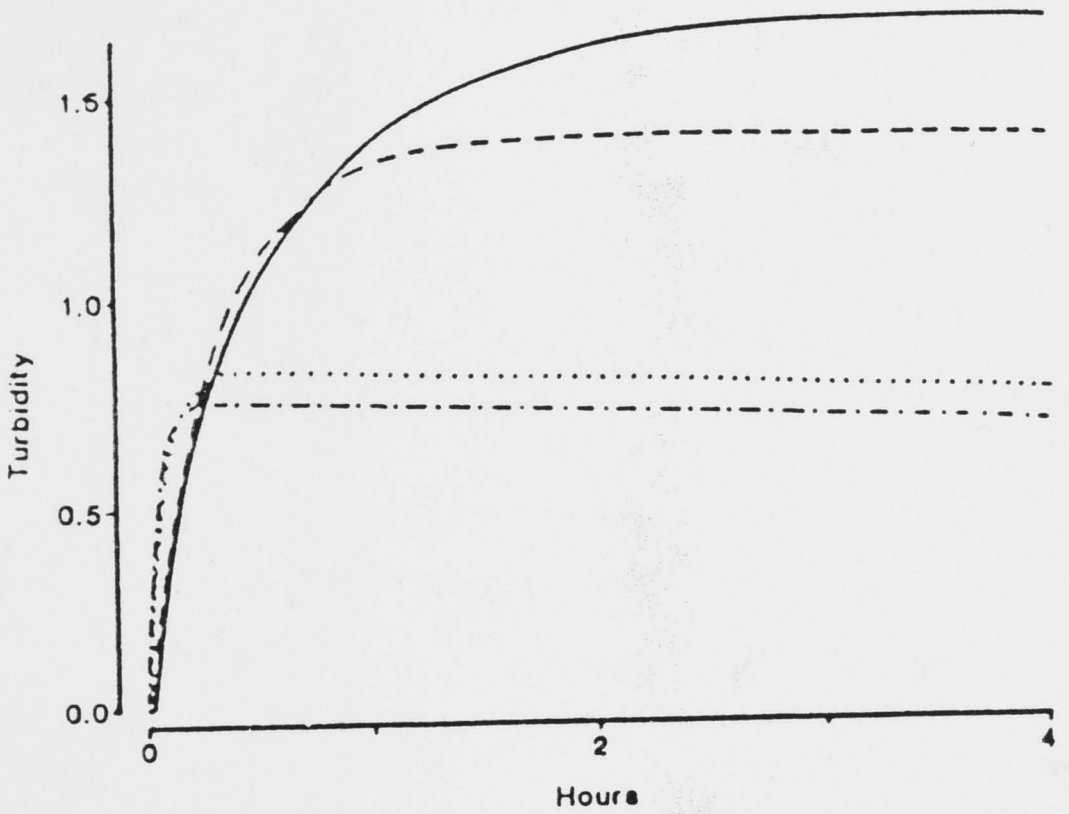
6. The view that clotting time could be used as a determinant of network structure is questioned.

Figure 4.1. Development of Network Growth with Varying Concentrations of Thrombin.

Turbidity at 800nm of fibrinogen solution clotted with 1.50 (-·-·), 1.00 (...), 0.25 (---), and 0.15 (-) u/ml thrombin. The fibrinogen concentration was 2.5mg/ml.

Figure 4.2 The Effect of Varying Thrombin Concentrations on the Conversion of Fibrinogen to Fibrin.

Conversion of fibrinogen to fibrin using a range of thrombin concentrations at a final concentration varying between 0.15 and 1.50 NIHu/ml.
Final fibrinogen concentration was 2.5mg/ml.
% conversion with 1.50 (-·-·), 1.00 (...), 0.25 (---) and 0.15 (-) u/ml of thrombin.


1.5
1.0
0.5
0.0
Turbidity
0
2
4
Hours

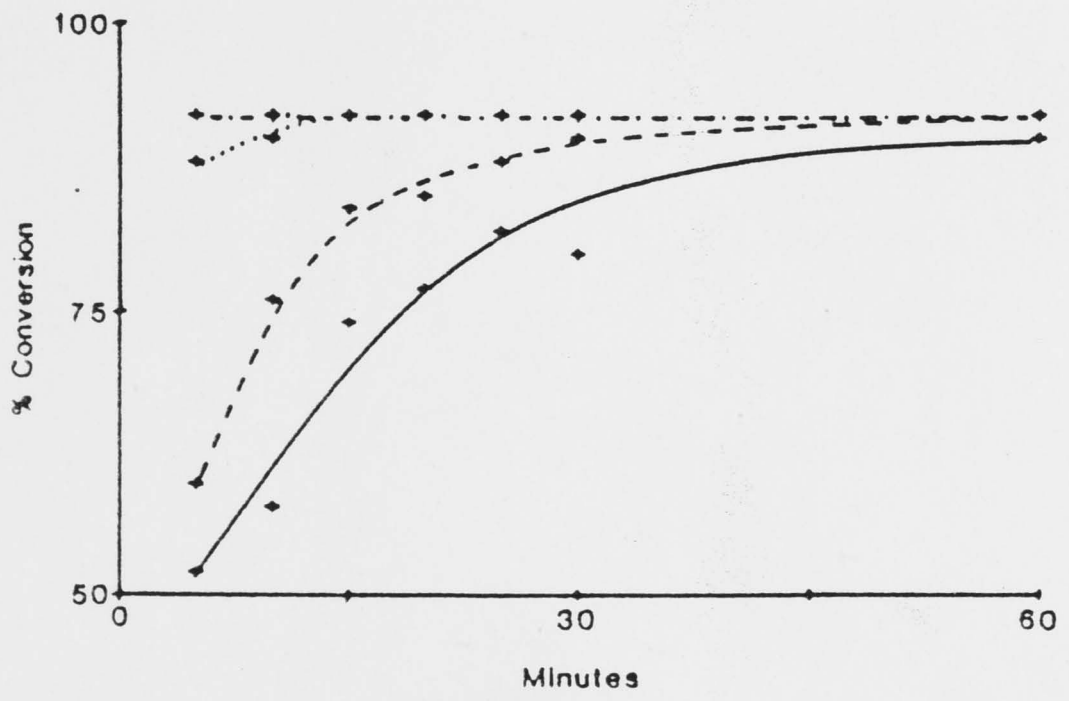

100
75
50
% Conversion
0
30
60
Minutes

Figure 4.3 Variation in μ_T(800nm) with Varying Thrombin Concentrations.

Sequential variation in μ_T(800nm) with time using a range of thrombin concentrations.
1.50 (-·-·), 1.00 (...), 0.25 (- - -) and 0.15 (-) u/ml of thrombin. Final fibrinogen concentration was 2.5mg/ml.

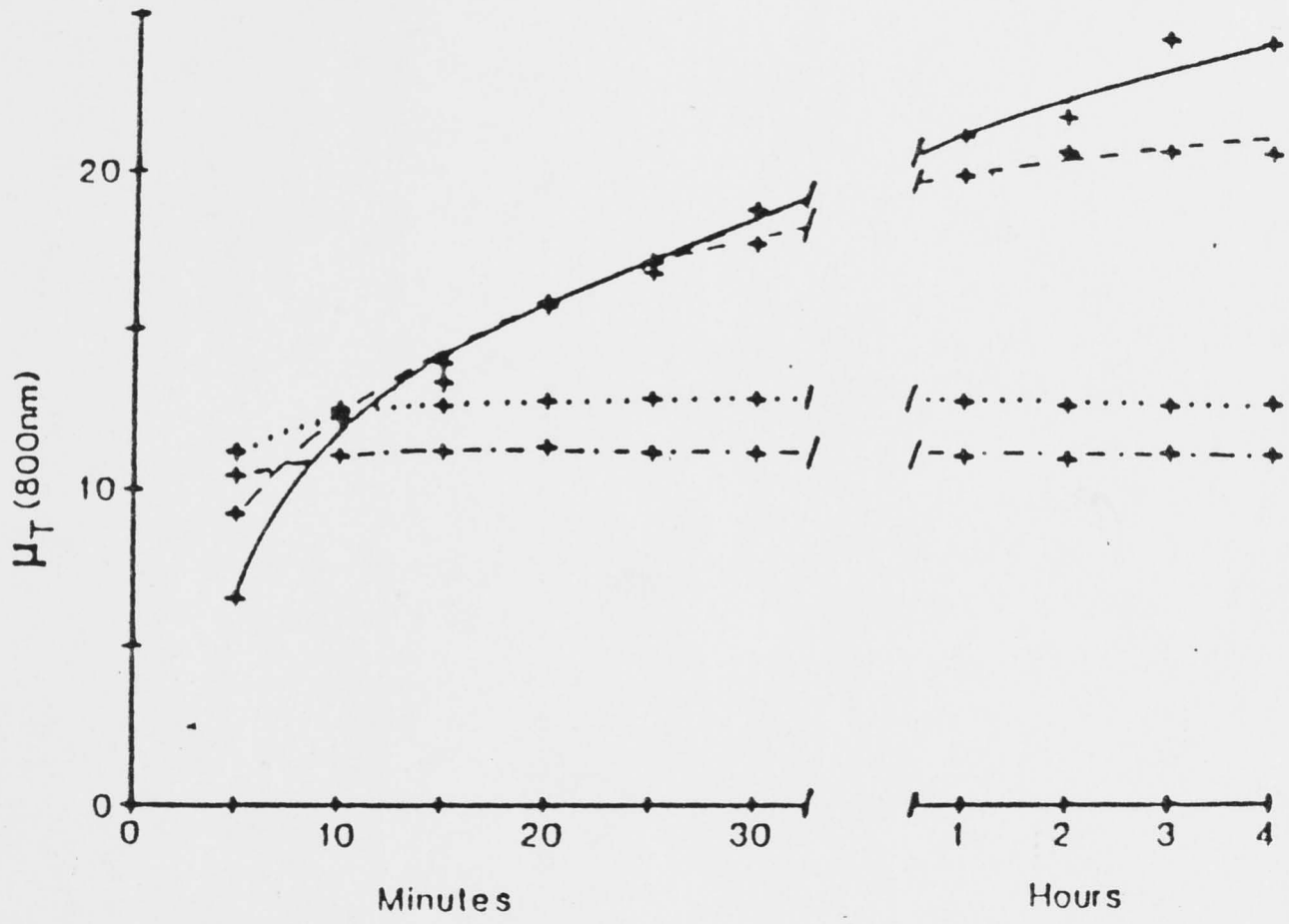

μ_T (800nm)
20
10
0
0
10
20
30
1
2
3
4
Minutes
Hours

Figure 4.4 Effect on μ_T of Varying Thrombin Concentration.

Thrombin concentration was varied in a range between 0.15 and 1.5 NIHu/ml. Final fibrinogen concentration was 2.5mg/ml. Bars are SEM.

Figure 4.5 Effect on μ_P and τ of Varying Thrombin Concentration.

Thrombin concentration was varied in a range between 0.15 and 1.50 NIHu/ml. Final fibrinogen concentration was 2.5mg/ml. The X axis is in a logarithmic scale. μ_T is shown as a solid line whilst μ_P is shown as a dotted line. Bars are SEM.

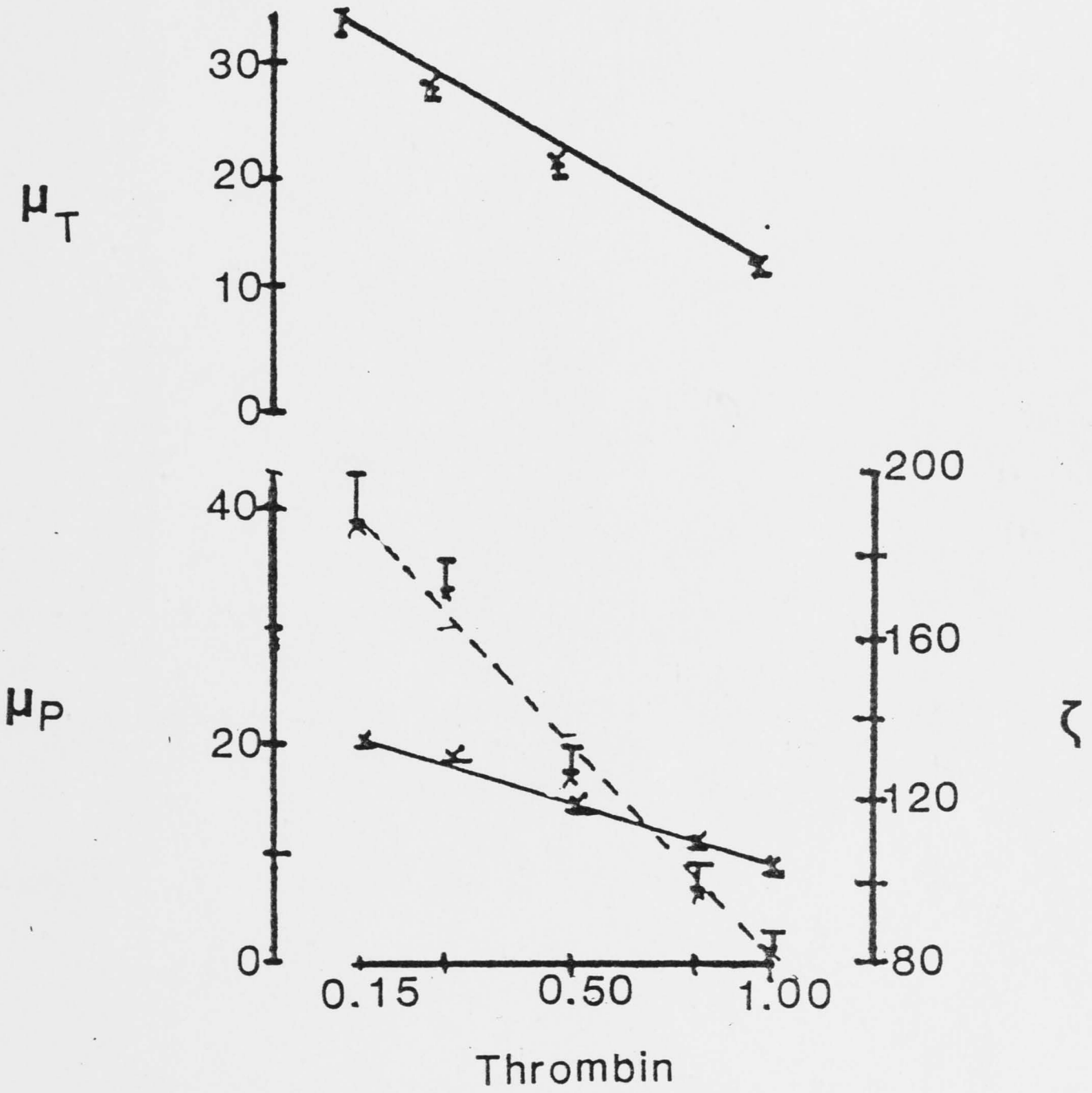

μ_T
30
20
10
0
μ_P
40
20
0
200
160
120
80
ζ
0.15
0.50
1.00
Thrombin

Figure 4.6 Plots of Turbidity and $1/\lambda^3$ in Fibrinogen Solutions.

The final thrombin concentration was 1.50u/ml. The final fibrinogen concentration was varied between 1.5 and 5.4mg/ml.

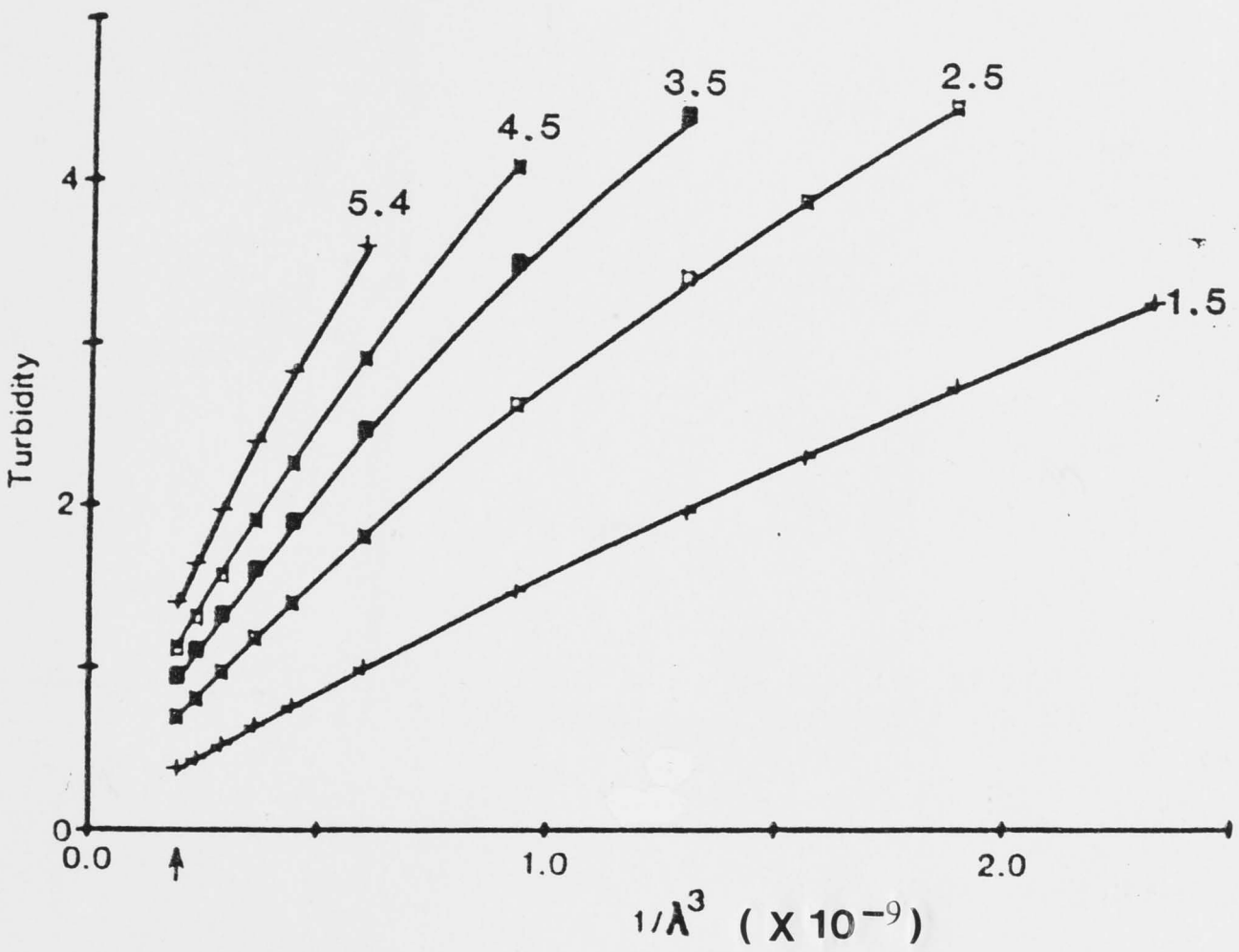
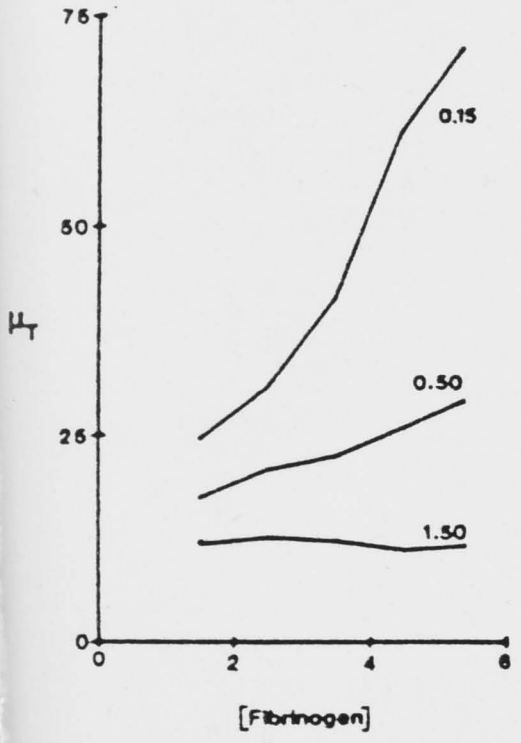
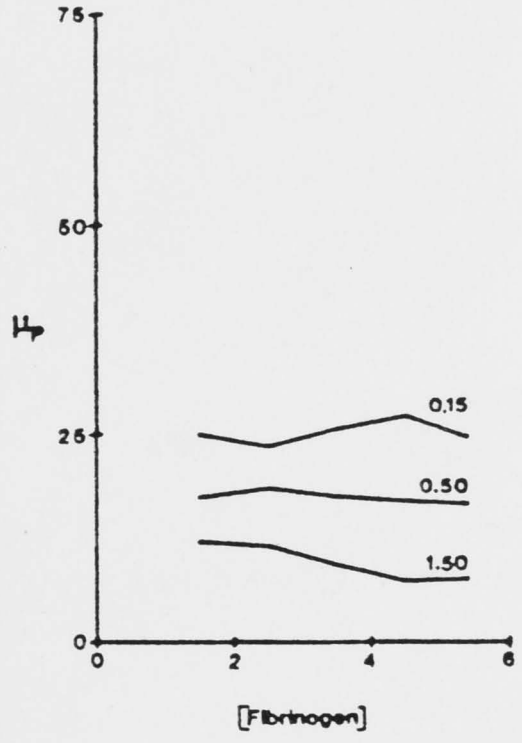

Turbidity
4
2
0
0.0
1.0
2.0
5.4
4.5
3.5
2.5
1.5
1/λ³ (X 10⁻⁹)

Figure 4.7 Effect on μ_T, μ_P and τ with Varying Fibrinogen Concentration.

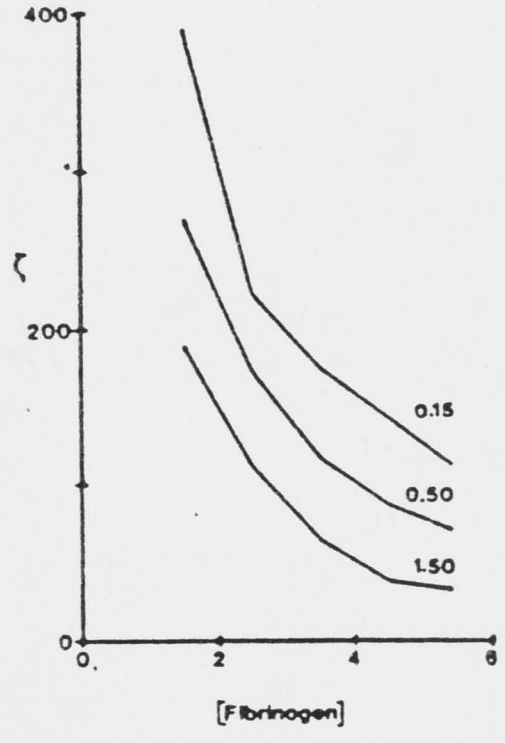
Fibrinogen concentration was varied between 1.5 to 5.4mg/ml fibrinogen whcih was clotted using three final concentrations of thrombin: 0.15, 0.50 and 1.50 NIHu/ml.


75
50
25
0
0
2
4
6
μ_T
0.15
0.50
1.50
[Fibrinogen]

(a)


75
50
25
0
0
2
4
6
μ_P
0.15
0.50
1.50
[Fibrinogen]

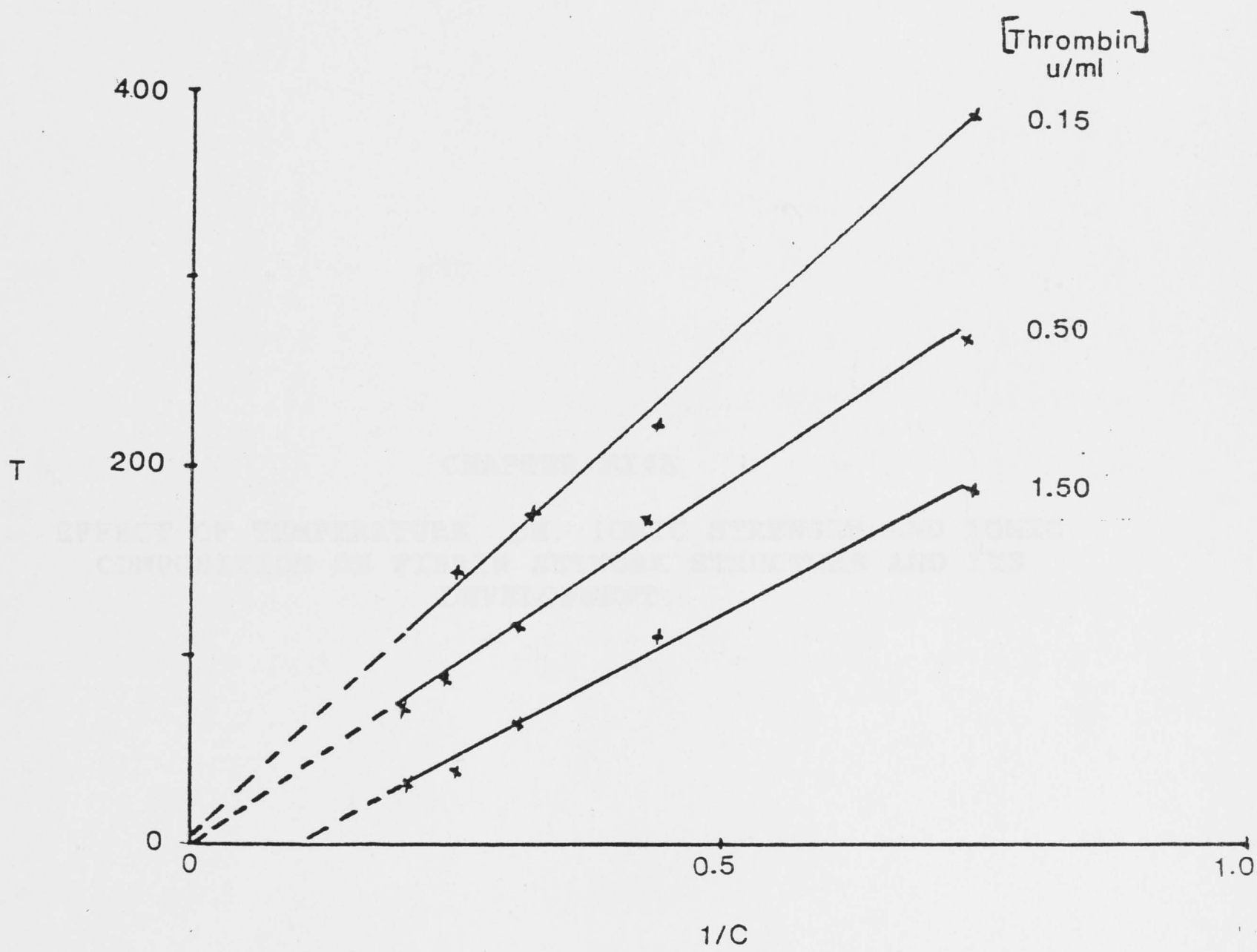
(b)


400
200
0
0
2
4
6
ζ
0.15
0.50
1.50
[Fibrinogen]

(c)

Figure 4.8 Plots of τ against 1/c.

Fibrinogen concentration was varied between 1.50 and 5.4mg/ml and clotted using three different final thrombin concentrations of 0.15, 0.5 and 1.50u/ml. Dotted lines show extrapolation to the origin.


[Thrombin]
u/ml
0.15
0.50
1.50
400
200
0
T
0
0.5
1.0
1/C

CHAPTER FIVE

EFFECT OF TEMPERATURE, pH, IONIC STRENGTH AND IONIC COMPOSITION ON FIBRIN NETWORK STRUCTURE AND ITS DEVELOPMENT.

5.1 INTRODUCTION.

Ferry and Morrison (1947) investigated the effect of pH, temperature and ionic strength on fibrin networks developed in Cohn Fraction I. In their studies, as already reviewed, they found these factors could modify network structure. However, the range of pH, temperature and ionic strength examined was very wide. They did not investigate changes in networks structure in response to minor variations in these factors which may be expected to occur in the pathophysiological range. Ferguson's (1983) study could be faulted on account of several methodological deficiencies fully discussed in Chapter 1. In this chapter effects of changes in pH, temperature, ionic strength and composition have been examined fully.

5.2 Materials and Methods.

General methods have been described in Chapter 2. In experiments described in this chapter, fibrinogen concentration used was 2.5 mg/ml. Networks were developed using 0.5 NIH u/ml thrombin, final concentration, for reasons fully elaborated in the previous chapter. Unless otherwise stated, conversion of fibrinogen to fibrin was maximal, i.e. around 90%.

5.2.1 Effect of Ionic Strength and pH:

Networks were formed in fibrinogen solution at ionic strengths varying from 0.133 to 0.173 using NaCl. This range was used to evaluate the effect of small changes in ionic strength compatible with physiological variation. The effect of pH was examined in networks developed in fibrinogen solution at pH of 7.15, 7.25, 7.35, 7.45 and 7.55; the pH was maintained at the desired level by adjusting it with the appropriate amounts of Tris-HCl buffer. For both pH and ionic strength studies kinetics of development of the network was continuously followed.

5.2.2. Effect of Ionic Composition:

Ca^{++}, Mg^{++}, Na^{+}, K^{+}, HCO_3^{-}, and HPO_4^{-}:

Networks were developed in the presence of final concentrations of one of the following:
2.02mM $CaCl_2$; 0.82mM $MgCl_2$; 2.02mM $CaCl_2$ plus 0.82mM $MgCl_2$; 4.30mM KCl; 0.35mM Na_2SO_4; 1.11mM NaH_2PO_4; and 29.00mM $NaHCO_3$. Final concentrations of components used were equal to normal physiological values.

Ionic strength was carefully held constant at 0.153 by adjusting the concentration of NaCl. In experiments using NaH_2PO_4, it was ensured that the pH of the fibrinogen

solution was 7.35 when thrombin was added. This was necessary as pH changes occurred if solutions were left standing for a long period of time.

5.2.3. Effect of Temperature:

Networks were formed at four different temperatures: 15°C, 22°C (room temperature), 30°C and 37°C. Technical limitations did not allow continuous turbidimetric measurements during network formation. Thus, pilot studies were made at each temperature to determine the time required for turbidimetric tracings to plateau for at least ten minutes. This was then taken as the time required for the complete formation of network at that specified temperature. Keeping all clotting conditions similar to those in pilot experiments, networks were then developed at the appropriate temperature for a period corresponding to that taken to reach the plateau on the turbidity curve at 800nm. For each temperature, water baths or ovens with thermostats ensured that at all times the temperature was within ±0.5°C of the desired temperature. Final turbidity measurements were then made at room temperature.

5.3 RESULTS

5.3.1 Effect of Ionic Strength:

Figure 5.1 shows turbidity curves at 800nm obtained when ionic strength was varied between 0.133 to 0.173. The lag phase of the curve increased as ionic strength was increased. Plateau or equilibrium was reached in all curves within one hour. The concentration of fibrin in the network was similar (around 90%) in all cases and the conversion of fibrinogen to fibrin was also similar. Final turbidity at 800nm decreased as ionic strength was increased. The family of curves did not cross.

Figure 5.2 shows the effect of variation in ionic strength on μ_T. Ionic strength and μ_T are inverse-linearly related, i.e. as ionic strength is increased, μ_T is reduced. This result confirms turbidity results shown in Figure 5.1.

The effect of variation in ionic strength on permeability and μ_P are shown in Figure 5.3 and 5.4. Both permeability (τ) of the networks and μ_P decrease as the ionic strength is increased. Thus, both μ_T and μ_P measurements indicate that fibrin fibres become thinner when ionic strength is increased.

5.3.2. Effect of Variation in pH.

Increasing pH retards kinetics of network growth (Figure 5.5) in a fashion similar to that found with ionic strength (Figure 5.1). The lag phase increased and equilibrium turbidity decreased as the pH was raised from 7.15 to 7.55. Equilibrium turbidity was always reached within 1½ hours. Figure 5.6 shows the effect of pH on μ_T: increase in the pH caused a decrease in μ_T, indicating that fibrin fibres were rendered thinner. Reduction in μ_T was greatest when pH increased from 7.25 to 7.35.

Both permeability (Figure 5.7) and μ_P (Figure 5.8) also decrease as pH is progressively increased.
pH increments decreased network permeability and fibrin fibre thickness.

5.3.3. Effect of Common Ions on Network structure:

5.3.3.1 Calcium and Magnesium.

Figure 5.9 shows typical turbidity curves obtained when Ca^{++} or Mg^{++}, or Ca^{++} and Mg^{++} together were added to fibrinogen solution before it was clotted. The lag phase without divalent cations was the longest. At thrombin

concentration of 0.5 u/ml, equilibrium turbidity in networks without divalent cations was lowest. Although the equilibrium turbidity in networks with Ca^{++} or Mg^{++} or both was similar, the rate of rise of turbidity was most marked in networks with both Ca^{++} and Mg^{++} , showing that the initial aggregation of fibrin monomer to protofibrils and the growth of protofibrils to opaque networks is accelerated.

Table 5.1 shows the effect of physiological concentrations of divalent cations on network structure in networks developed in fibrinogen solution. μ_T was significantly increased in networks with divalent cations, indicating that fibres were rendered thicker. However, neither permeability nor μ_P was significantly altered by divalent cations, singly or in combination. Thus, increase in thickness shown in μ_T was not expressed in permeability or μ_P .

When network characteristics were examined with and without divalent cations using a higher fibrinogen and a lower thrombin concentration (Table 5.2), significant alterations in μ_T were not induced at low thrombin concentrations (0.15u/ml). μ_P and τ, however, were not

affected. This effect was similar to that observed when high thrombin was used. Divalent cations singly and in combination increase fibrin content in networks. Greater fibrin incorporation was observed when Ca^{++} was used singly.

The effect of increasing Mg^{++} on network structure using high fibrinogen and low thrombin are shown in Table 5.3. Increase in μ_T was found dependent on the final Mg^{++} concentration. μ_P and permeability also increased, with the greatest increase when 1mM $MgCl_2$ was added. Cn also increased significantly when Mg^{++} was added, but was independent of final Mg^{++} concentration.

5.3.3.2 Physiologically Common Ions in Plasma and Network Structure.

Figure 5.10 shows the effect of common ions on turbidity curves developed in the presence of K^+, Na^+, HCO_3^- and HPO_4^-, in physiological concentrations. Of those investigated, only HCO_3^- had any effect - it increased the lag phase, slowed the network growth and lowered the equilibrium turbidity.

Table 5.4 shows the results of investigations using KCl, Na_2SO_4, NaH_2PO_4 and $NaHCO_3$. Only 29.00mM $NaHCO_3$ altered fibrin network structure. μ_T, μ_P and permeability were significantly decreased, confirming the observations already described and shown in Figure 5.10.

Thus, of the ions examined, only HCO_3^- influences fibre thickness and network permeability.

5.3.4 Variation of Temperature.

Turbidity curves of a complex nature are shown in Figure 5.11 when temperature was varied. The lag phase increased as temperature was lowered from 37°C to 15°C (Figure 5.11(a)), whilst the rate of development of turbidity was inversely proportional to the temperature. The family of curves crossed over (Figure 5.11(b)).

The effect of temperature on μ_T, μ_P and permeability are shown in Figure 5.12. The values of μ_T, μ_P and permeability were significantly greater at 15°C than at room temperature. Significant differences, however, were not induced by variation in temperature above 24°C.

5.4 DISCUSSION.

Ferry and Morrison (1947) showed by studies on clot opacity that fine clots were developed in a solution of Cohn Fraction I as the pH and the ionic strength were increased from 6.3 to 7.5 and from 0.15 to 1.30 respectively. Since then the effect of pH, ionic strength and temperature on fibrin network structure has been investigated by several workers (Ferry and Schulman, 1949; Latallo *et al*, 1961; Carr *et al*, 1977; Carr and Hermans, 1978; Schulman and Ferry, 1980; Blomback and Okada, 1982; Okada and Blomback, 1983; and Ferguson, 1983). However, most of these studies employed semiquantitative techniques and examined these variables over a larger and non-physiological range. Ferguson used techniques similar to those used in this chapter but, as fully described in Chapter 4, he used inadequate incubation times and measured opacity ratio, rather than mass-length ratios derived from turbidity measurements at many wavelengths. These serious criticisms of his observations have made his investigations unsatisfactory. The present study on the effects of varying pH, ionic

strength and temperature overcomes previous deficiencies and examines fibrin network structure over a pathophysiological range. Further, the influence of kinetics of network development and its contribution to final network structure has been fully explored.

As described in this chapter, both pH and ionic strength induce detectable and significant alteration in mass-length ratio in fibrin fibres over a pathophysiological range (Figures 5.2, 5.4, 5.6, 5.8). Increase in both the pH and ionic strength within the pathophysiological ranges render fibrin networks less permeable and the fibres increasingly thinner . The amount of protein in the network is not altered. Ferry and Morrison attributed changes in opacity ratio to solubility of fibrin(ogen), which they found was greater with increasing pH or ionic strength. An increase on solubility leads to a lessened tendency for fibrin monomers to aggregate. However, as shown in this chapter, the amount of fibrinogen converted to fibrin is not changed by alterations in pH and ionic strength. More recently, Schulman and Ferry (1980) have suggested that an increase in pH above neutrality increases net charge on proteins and,because of electrical repulsion, thinner fibres are formed. Increase

in ionic strength also promotes formation of thinner fibres because of enhanced interaction of electrolytes with dipole and multipole moments arising from charge configuration of the protein. Latallo *et al* (1962) showed by using fibrin monomer that these effects were independent of thrombin action.

Figures 5.1 and 5.5 show the development of turbidity when ionic strength or pH was varied between 0.133 to 0.173 and 7.15 to 7.55. These curves have three phases: the lag phase, phase of increasing turbidity and the equilibrium phase, and collectively represent the breakdown of fibrinogen to fibrin monomer, the initial aggregation of monomer to protofibrils and the growth of protofibrils to an opaque network. When pH or ionic strength was increased, the lag phase was increased corresponding to an increase in the time required for the overall action of thrombin and fibrinogen, until the appearance of turbidimetrically detectable fibrin. It also includes the enzymatic breakdown of fibrinogen and the initial aggregation to protofibrin. Wolfe and Waugh (1981) have proposed (see Chapter 4) that the main increase in turbidity was due to the growth in the thickness of the initial network of protofibrils. A

longer lag phase, caused by increase in pH or ionic strength therefore, allowed a greater proportion of available fibrin to be incorporated into the initial protofibril network. The remaining reduced amount of fibrin is incorporated subsequently into the initial protofibril network leading to decreased μ_T. The enzyme kinetics in this system remained unaltered and, therefore, there was no crossover of curves. In summary, as ionic strength and pH were increased, the lag phase was progressively prolonged corresponding to less and less available fibrin for subsequent incorporation, and therefore reduced μ_T.

When temperature was lowered from 37°C to 15°C, significant alteration in mass-length ratio or permeability were not detected (Figure 5.12). Ferry and Morrison (1947) showed that increasing temperature caused clots to become steadily "finer", but only observed this phenomenon at a pH of 6.8. At pH 6.3, the effect was slight. Experiments reported in this chapter were performed at a pH of 7.35, and fibre thickness increased only at 15°C. Generally, networks formed above 15°C have thinner fibres. These results, therefore,in general confirm observations by Ferry and Morrison.

However, a decrease in fibre thickness was not observed at higher temperatures at a pH of 7.35,

The kinetics of network development (Figure 5.11) were altered as temperature was increased. Decreasing temperature to 15°C seems kinetically to lead to similar effects as decreasing thrombin concentration (see Chapter 4). In both cases the lag phase was prolonged and it took longer to reach equilibrium turbidity. Also in both, the equilibrium turbidity inversely related to temperature and thrombin concentration. As shown in Figure 5.11(b), and Figure 4.1, these conditions promoted the crossover of turbidity curves. In both cases, the amount of fibrin in the protofibril networks was diminished leaving a larger amount available for subsequent incorporation. Further incorporation occurred mainly by growth in thickness of the initial protofibril. Therefore, as temperature and thrombin were lowered, a larger amount of fibrin was available for incorporation into thicker fibres, resulting in an increase in μ_T. Thus, ionic strength and temperature influence the final network structure by affecting kinetics of thrombin-induced conversion of fibrinogen to fibrin.

The present observations on the effects of divalent cations Ca^{++} and Mg^{++} confirm previous studies (Ferry and Morrison,1947; Shen *et al*, 1975; Brass *et al*, 1978; Shah *et al*, 1982; Blomback and Okada, 1982; and Okada and Blomback, 1983) in that μ_T was significantly increased by their addition (Table 5.1), lag phase was shortened and the rate of rise of turbidity (which represents the initial aggregation of monomer to protofibrils) also increased. Thus, as Brass *et al* (1978) suggested, Ca^{++} decreased the time required for fibrin formation from fibrinogen by markedly accelerating the phase of fibrin monomer polymerization.

It was shown in Figure 5.9 that although equilibrium turbidity, and therefore μ_T, and network protein content (Cn) were increased to a similar value whether Ca^{++} or Mg^{++} or a combination of Ca^{++} and Mg^{++} was added, the rate of rise of the turbidity was more marked after the addition of a combination of Ca^{++} and Mg^{++}. It is probable that there was synergistic action on monomer aggregation to protofibrils and growth of protofibrils to the opaque network under conditions which approximate those found physiologically.

Although μ_T is increased by Ca^{++} and Mg^{++}, μ_P and τ were invariant (Table 5.1). The effect of Ca^{++} on μ_P depends upon the concomittant presence in fibrinogen solution of Factor XIIIa. Shah *et al* (1982) have shown, using SDS polyacrylamide gel electrophoresis, that in the presence of small amounts of Factor XIIIa μ_P may be markedly decreased by Ca^{++} even while μ_T is significantly increased. This breakdown in the correlation between μ_P and μ_T arises from the dual effects of Ca^{++}. Major network fibre diameter is increased by Ca^{++} as reflected by the turbidimetric measurements. Minor network fibres contribute very little to turbidity, but when strengthened by cross-linking, markedly reduce permeability and hence its derivate, μ_P. Therefore, any increases that may have been brought about by Ca^{++} and Mg^{++} could have been masked.

5.5 CONCLUSIONS.

1. Variation of pH and ionic strength within pathophysiological range significantly altered fibrin network structure.

2. Fibrin network structure was altered at low temperature of 15°C

3. Kinetics of network development explain the effects of temperature, pH and ionic strength on fibrin network.

4. Ca^{++} and Mg^{++} increased fibre thickness whether added singly or in combination.

5. Of the cations examined, only HCO_3^- had significant effect on network structure. However, whether this effect was from modification of pH requires to be further examined.

TABLE 5.1 Effect of Physiological Concentrations of Divalent Ions on Fibrin Network Structure.

Fibrinogen Concentration was 2.5mg/ml and thrombin concentration was 0.5 u/ml. Results are the mean of three experiments.

	T	μ_P	μ_T	Cn
	10^{11} (cm^2)	$\mu x10^{12}$ (daltons/cm)		mg/ml
Control	300	29.8	18.0	2.20
2.02mM $CaCl_2$	283	30.0	24.4**	2.23
0.82mM $MgCl_2$	281	31.0	24.3**	2.21
2.02mM $CaCl_2$ + 0.82mM $MgCl_2$	303	27.4	22.1**	2.17

(** denotes P<0.05).

TABLE 5.2 Effect of Physiological Concentrations of Ca^{++} and Mg^{++} on Network Structure at Higher Fibrinogen and Lower Thrombin Concentrations.

Fibrinogen concentration was 3.3 mg/ml. Thrombin concentration was 0.15 u/ml. Results are mean of three experiments.

	μ_T	μ_P	T	Cn
	[$x10^{12}$ daltons/cm]		10^{11} (cm^2)	(mg/ml)
Control	39.2	23.4	198	2.55
$Ca^{++}+Mg^{++}$ (2.02+0.82mM)	48.5*	25.4	198	2.70*
Ca^{++} (2.02mM)	45.8**	22.5	176	2.78**

(* denotes P < 0.025, ** P <0.005)

TABLE 5.3 The Effect Of Magnesium Concentrations On Network Structure.
Fibrinogen and thrombin concentrations were 3.3mg/ml and 0.15u/ml. Results are a mean of three experiments.

	μ_T	μ_P	T	Cn
	[10^{12} daltons/cm^2]		10^{11} (cm^2)	mg/ml
Control	39.1	15.4	140	2.31
$MgCl_2$ (1mM)	41.4	44.1***	354***	2.63
$MgCl_2$ (2mM)	43.9*	25.3***	198**	2.69
$MgCl_2$ (3mM)	47.6**	26.4***	207***	2.69

(* denotes $P < 0.025$, ** $P < 0.005$,
*** $P < 0.001$)

TABLE 5.4 Effect of Physiological Concentrations of Various Ions on Network Structure.

Fibrinogen and thrombin concentrations were 2.5mg/ml and 0.5u/ml.
Results are the mean of three experiments.

	T	μ_P	μ_T
	10^{11} cm^2	[10^{12} daltons/cm^2]	
Control	450	45.0	25.7
4.30mM KCl	413	41.3	24.4
0.35mM Na_2SO_4	385	38.3	24.7
1.11mM NaH_2PO_4	420	41.3	25.9
29.00mM $NaHCO_3$	273**	24.71**	19.30**

(** denotes P< 0.0001).

Figure 5.1 Effect of Ionic Strength on Turbidity Curves.

Turbidity curves at 800nm when ionic strengths were 0.133, 0.143, 0.16 and 0.173.
Final fibrinogen and thrombin concentration 2.5mg/ml and 0.5u/ml respectively.

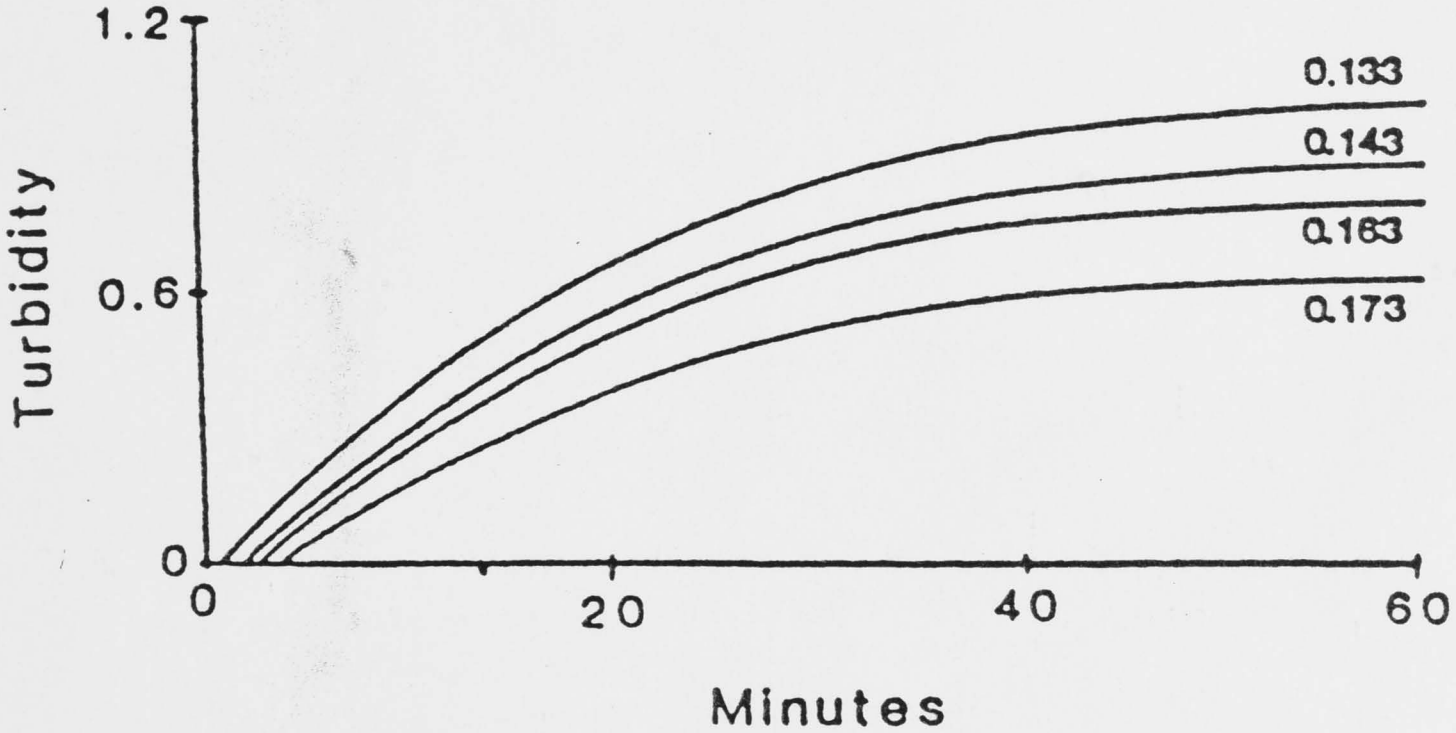

1.2
0.6
0
Turbidity
0.133
0.143
0.163
0.173
0
20
40
60
Minutes

Figure 5.2 Effect of Ionic Strength on μ_T

Final fibrinogen concentration was 2.5 mg/ml and final thrombin concentration was 0.5 u/ml.
Ionic strength was varied between 0.133 and 0.173.

Figure 5.3 Effect of Ionic Strength on Permeability (τ).

Final fibrinogen concentration was 2.5 mg/ml and thrombin concentration was 0.5 u/ml.
Ionic strength was varied between 0.133 and 0.173.

Figure 5.4 Effect of Ionic Strength on μ_P

Final fibrinogen concentration was 2.5 mg/ml and thrombin concentration was 0.5 u/ml.
Ionic strength was varied between 0.133 and 0.173.

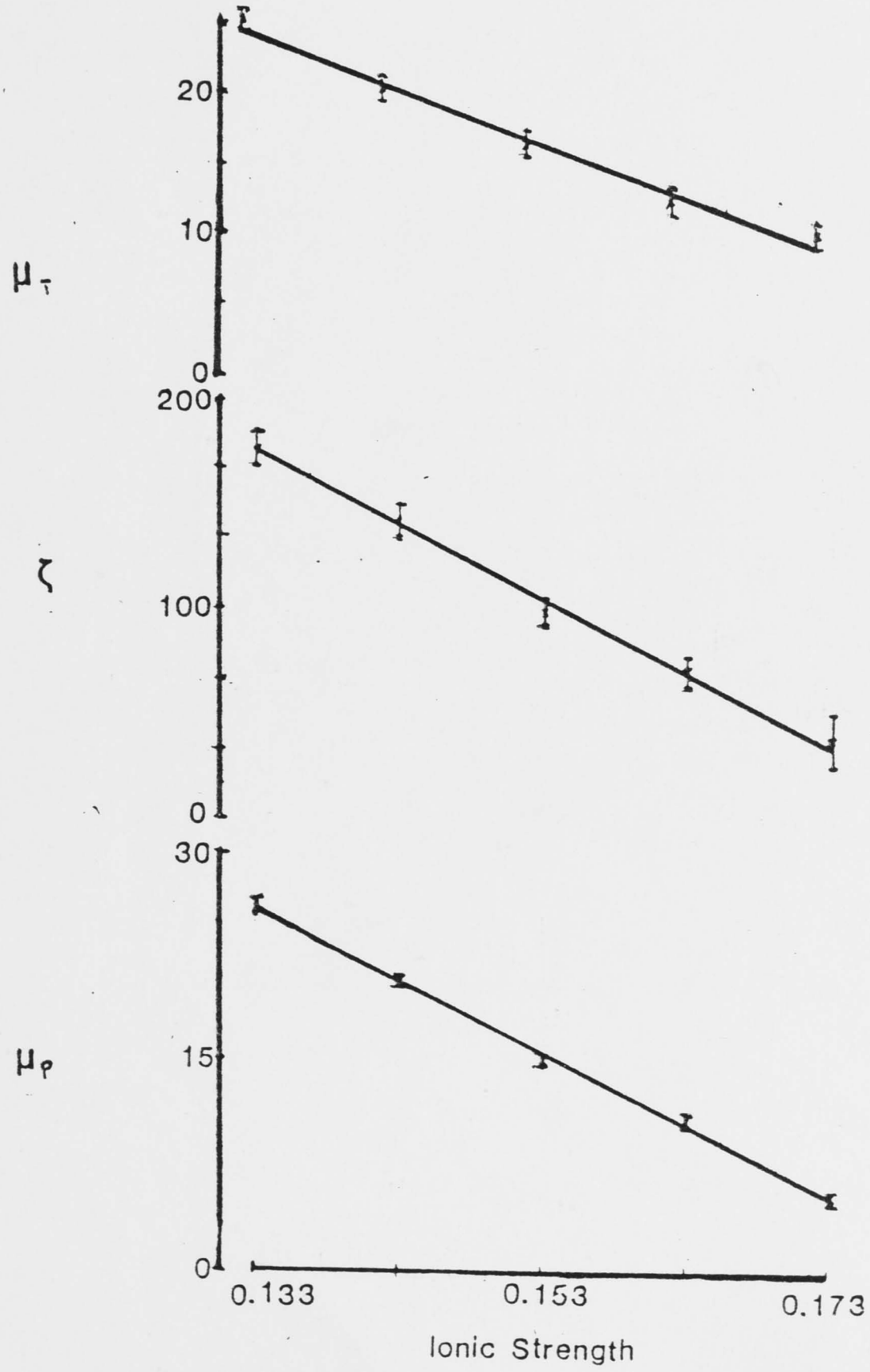

20
10
0
μ_T
200
100
0
ζ
30
15
0
μ_P
0.133
0.153
0.173
Ionic Strength

Figure 5.5 Effect of pH on Turbidity Curves.

Turbidity at 800nm to show the effect of pH which was varied between 7.15 and 7.55.
Final fibrinogen concentration was 2.5mg/ml and final thrombin concentration was 0.5u/ml.
Ionic strength was 0.153.

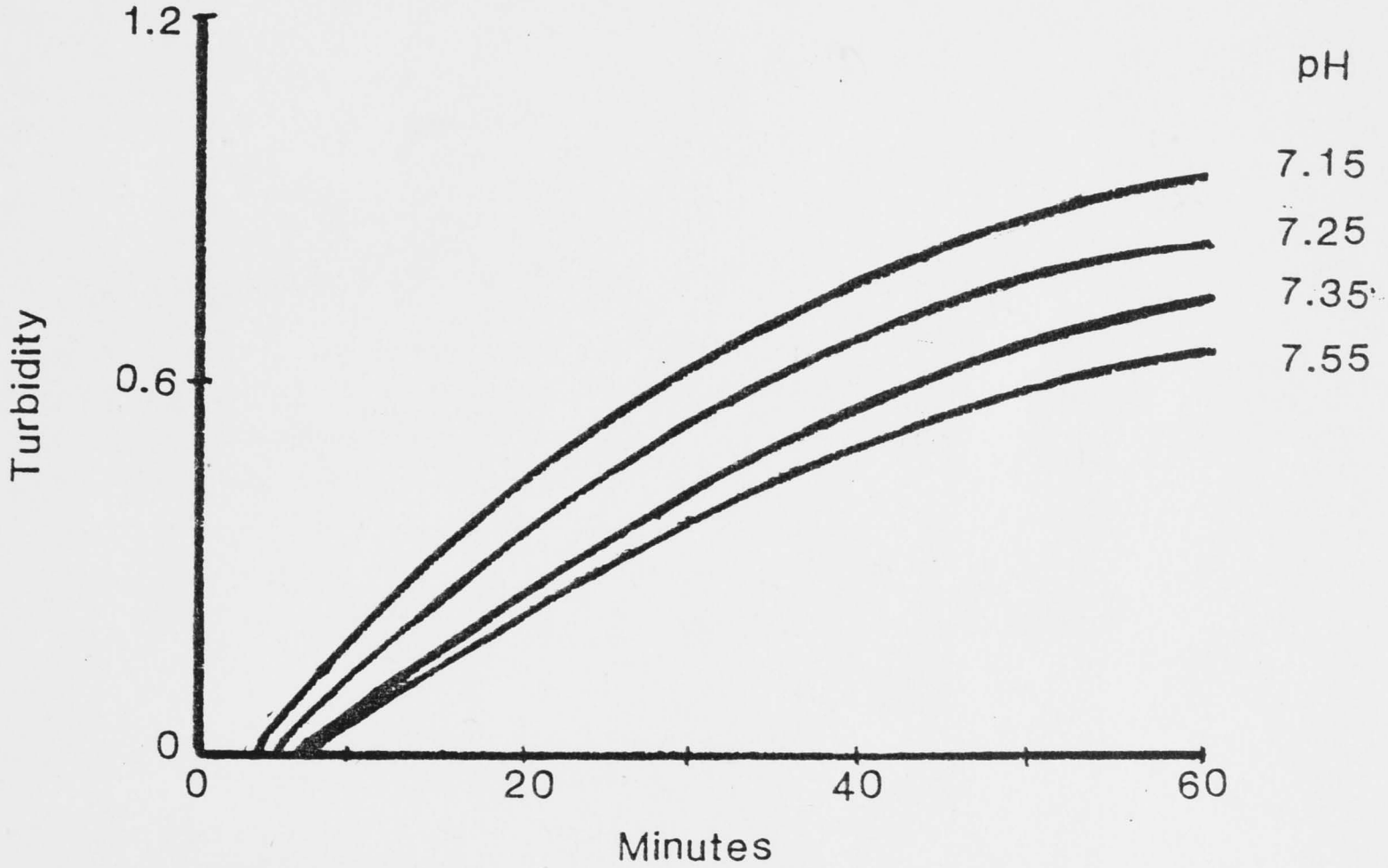

1.2
0.6
0
Turbidity
0
20
40
60
Minutes
pH
7.15
7.25
7.35
7.55

Figure 5.6 Effect of pH on μ_T

pH was varied between 7.15 and 7.55
Ionic strength was 0.153 and final
fibrinogen and thrombin concentrations were
2.5 mg/ml and 0.5 u/ml respectively.

Figure 5.7 Effect of pH on Permeability (τ)

pH was varied between 7.15 and 7.55.
Ionic strength was 0.153 and final
fibrinogen and thrombin concentrations were
2.5 mg/ml and 0.5 u/ml respectively.

Figure 5.8 Effect of pH on μ_P

pH was varied between 7.15 and 7.55.
Ionic strength was 0.153.
Final fibrinogen concentration was 2.5
mg/ml and final thrombin concentration
was 0.5 u/ml. pH was 7.35 and ionic
strength was 0.153.

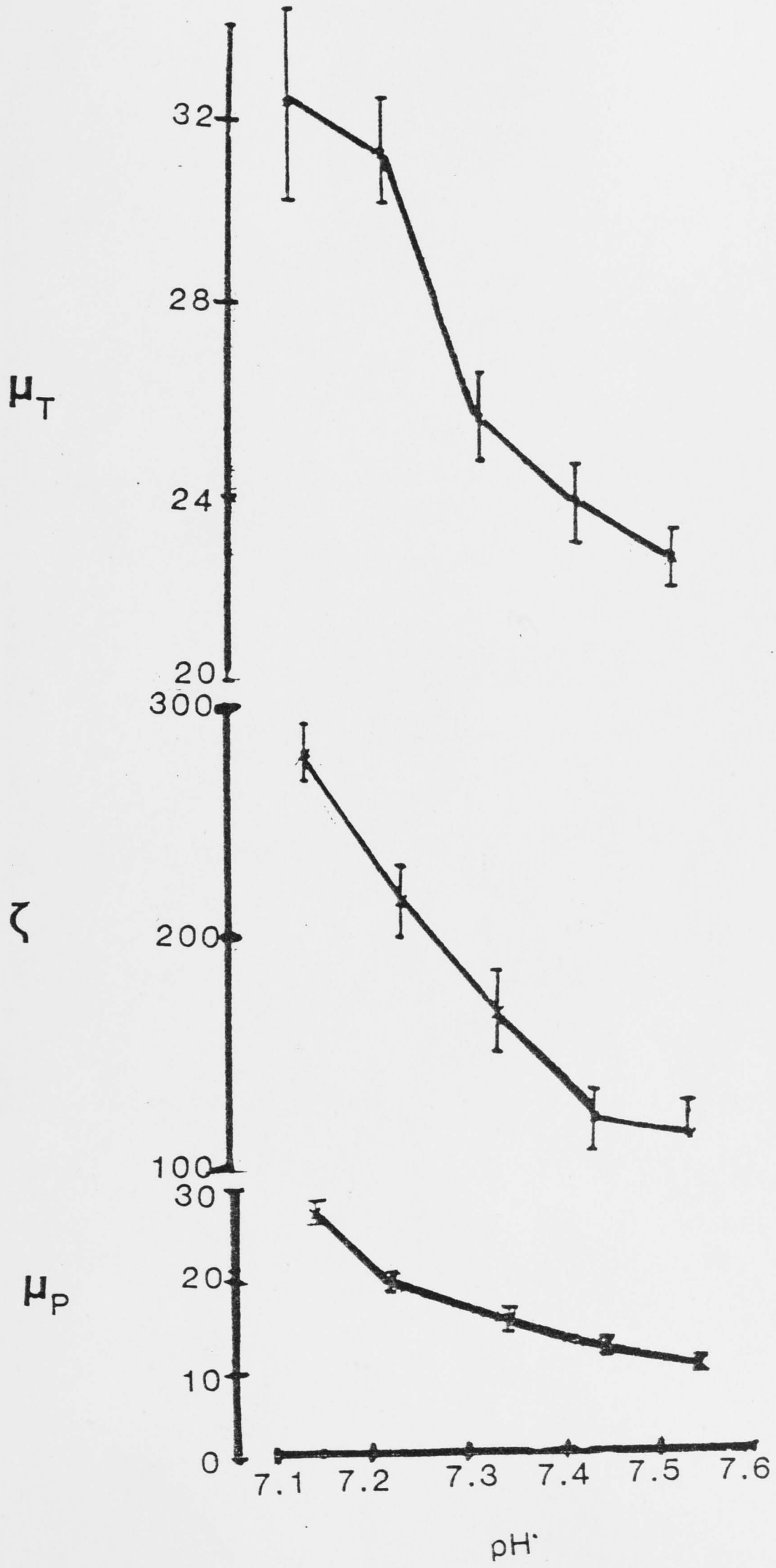

μ_T
32
28
24
20
300
200
100
ζ
30
20
10
0
μ_P
7.1
7.2
7.3
7.4
7.5
7.6
pH

Figure 5.9 Effect on Divalent Cations on Turbidity Curves.

Turbidity at 800nm.
Effect of calcium (2.02 mM), calcium and magnesium (2.02 mM and 0.82 mM) in networks.

Figure 5.10 Effect of Common Ions Found in Plasma on Turbidity.

Turbidity at 608nm when
4.30mM KCl or 0.35mM Na_2SO_4, or
1.11mM NaH_2PO_4, or 29mM $NaHCO_3$ were added.
Final fibrinogen concentration was
2.5mg/ml and final thrombin concentration was 0.5u/ml. pH was 7.35 and ionic strength 0.153.

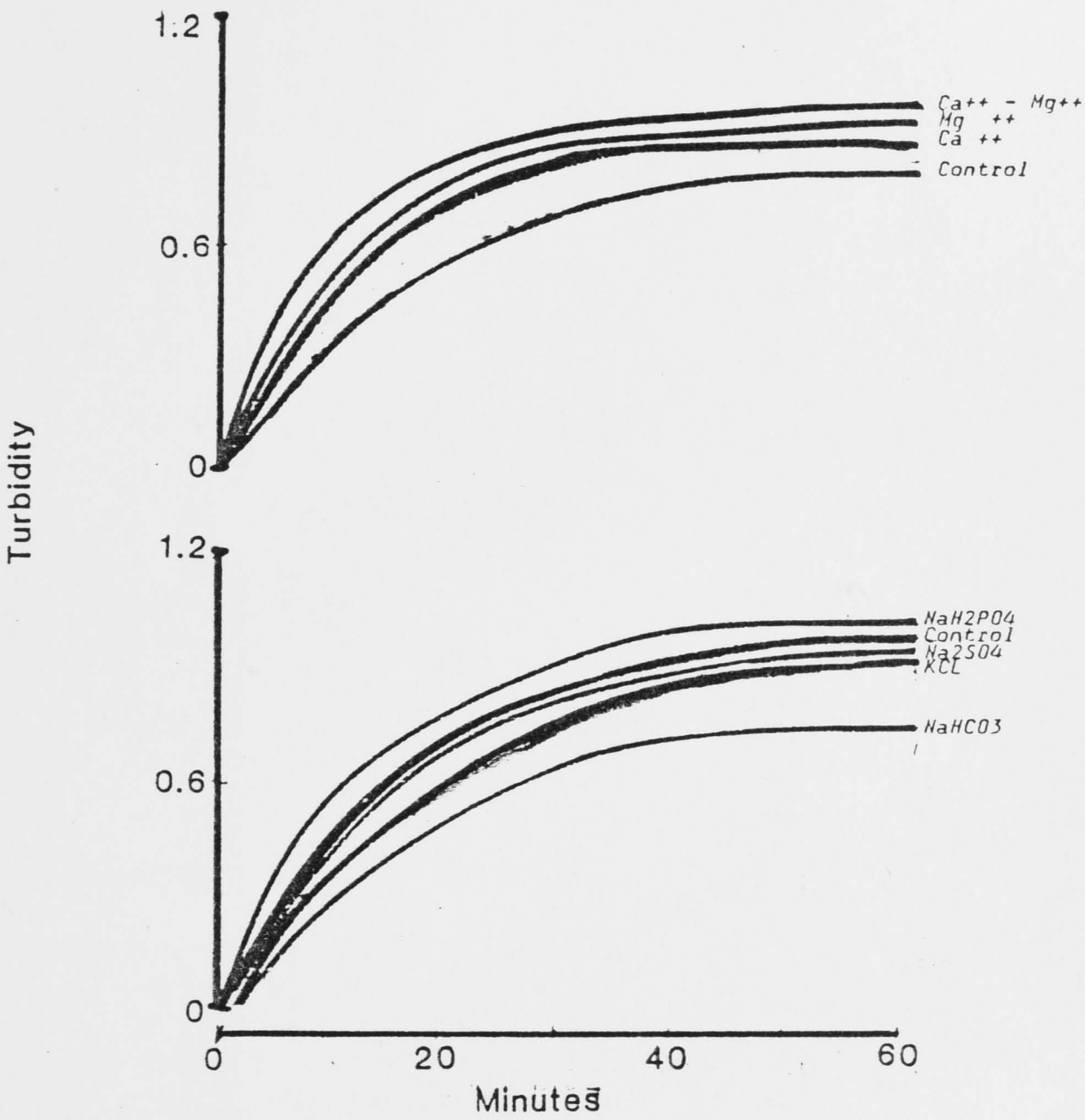

1.2
0.6
0
Turbidity
Ca++ - Mg++
Mg ++
Ca ++
Control
1.2
0.6
0
NaH2P04
Control
Na2S04
KCL
NaHC03
0
20
40
60
Minutes

Figure 5.11 Effect of Temperature on Turbidity Curves.

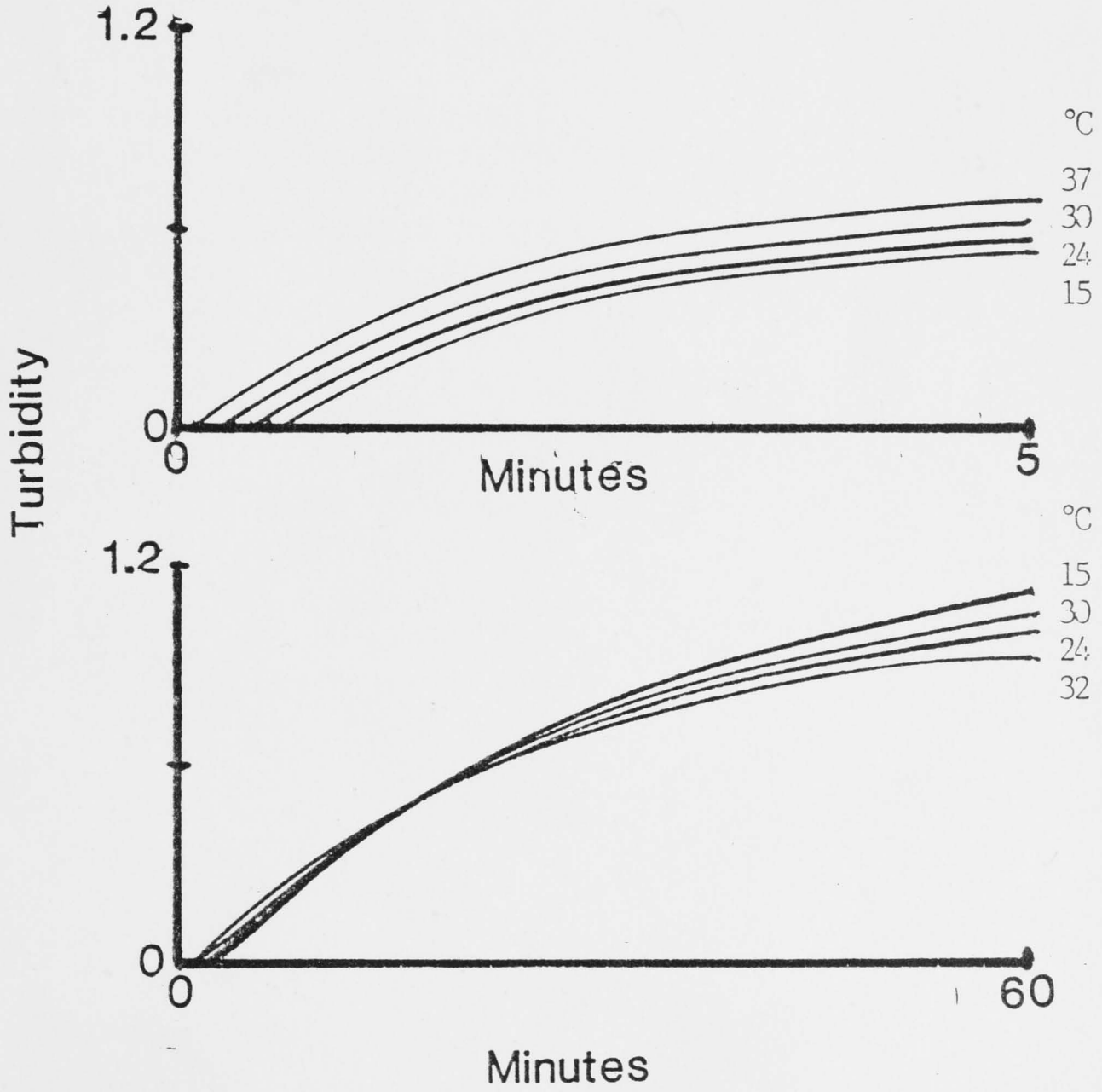
Turbidity at 800nm. Effect of varying temperatures between 15°C and 37°C.

(a) Turbidity curves for the first five minutes after clotting showing lag phase.

(b) Turbidity curves for sixty minutes showing crossover.

Fibrinogen concentration used was 2.5mg/ml and final thrombin concentration was 0.5u/ml. pH was 7.35 and ionic strength was 0.153.

(a)

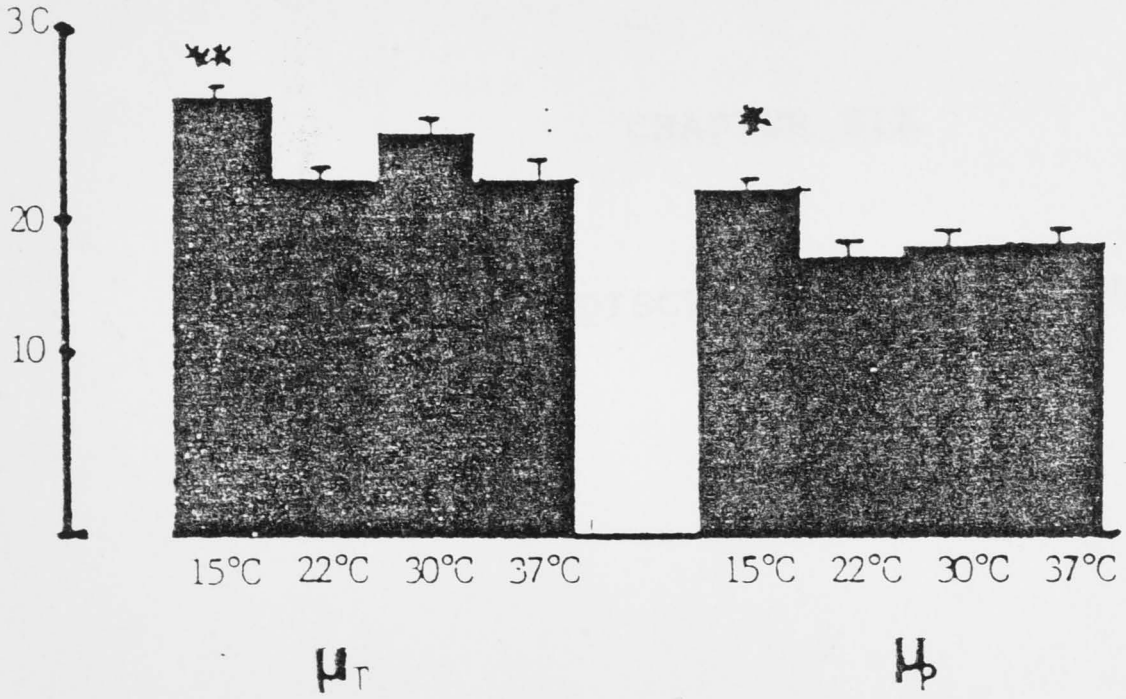

1.2
Turbidity
0
0
Minutes
5
°C
37
30
24
15

(b)

1.2
0
0
60
Minutes
°C
15
30
24
32

Figure 5.12 Effect of Temperature on Network Characteristics.

Effect of temperature when varied between 15°C and 37°C on μ_T and μ_P.
Results are the means of three experiments ± SEM.
Permeability results have been omitted as μ_P mirrors permeability.
Final fibrinogen concentration was 2.5mg/ml and final thrombin was 0.5u/ml.
(* P<0.01; ** P< 0.001)


30
μ
20
10
15°C 22°C 30°C 37°C
15°C 22°C 30°C 37°C
μr
μp

CHAPTER SIX

GENERAL DISCUSSION ON SECTION 1

Several points in the preceding section warrant further comment and are elaborated in the following discussion under five major sub-headings.

6.1 Major and Minor Network.

Morphometric analysis of electronmicrographs has shown that diameters of fibrin strands in a network are polydisperse and that the distribution of fibre diameter is bimodal (Shah et al, 1982). Fibrin strands can, therefore, be separated into two networks according to their fibre diameter and these have been termed major and minor networks. Fibrin strands in the minor network are thinner than in the major network. On the basis of experiments described in Chapter 4 and 5 is is clear that the relationship between these networks is neither fixed nor invariant. It would seem that the two network system is highly responsive to conditions of clotting and perhaps to the biological demands placed on the system. For example, when, at low thrombin, fibrinogen concentration is increased fibre thickness derived from turbidity (μ_T) is found to increase by 300%. Fibre thickness derived from permeability (μ_P) as well as the

permeability of the network as a whole, on the other hand, remain unchanged. Network fibrin content also remains unchanged. There is thus an apparent paradox: μ_T and μ_P, both of which are purported to measure fibre thickness, do not alter in parallel under certain experimental conditions. At higher thrombin concentrations μ_T and μ_P do not alter whilst permeability shows a marked decrease. Clearly, permeability can be reduced without affecting network fibre diameter through an increase in the numerical density of fibrin fibres in the minor network. How fibre thickness in the major network correlates with the quality and numerical density of the minor network is uncertain. But studies (Shah *et al*, 1982; Ferguson, 1982) have shown that the minor network is composed of about 3% of the total protein content. Thus, physiologically relevant regulation of network permeability can be achieved very economically by minute changes in the amount of protein in the minor network.

6.2 μ_P and μ_T As Twin Tools in Network Characterization.

Since correlation between μ_P and μ_T has been shown to

break down under certain experimental conditions because of polydispersity in the diameters of fibrin strands, it follows that if a single method is used for mass-length ratio it could be erroneous. The magnitude of the error will depend on the range of diameters and the shape of their distribution. To avoid an error from this source it is suggested that at least two techniques be used to characterize network structure. Further, it is to be noted that μ_T and μ_P are mean measurements of a range of fibre diameters and if used singly (and also when used in combination) do not give any idea of the distribution of fibre thickness.

Finally, μ_P is derived from permeability. Permeability of the network is an important characteristic and highlighted in studies by Shah and Dhall (1983) who have shown that penetration of circulating plasminogen may be important in thrombolysis and maybe be governed by the permeability characteristics of the fibrin network. Thus, the derivation of μ_P employs the measurement of τ which is another tool which can be used to characterize network structure.

6.3 Physiological Factors.

Although it has been known for some time that many factors, such as extreme changes in pH, fibrinogen or thrombin concentration are capable of modifying fibrin network characteristics, the influence of these factors in the pathophysiological range had remained unexamined. Studies in Section 1 have shown that variation in pH, ionic strength, temperature, thrombin and fibrinogen concentration in the narrow pathophysiological range can also alter network structure. Whether network structure is altered *in vivo* in response to the varying conditions under which it forms remains unknown and is an area for future research. Likewise, any alterations in network structure in pathological conditions (for instance when pH and ionic strength of plasma are abnormal in diabetic ketoacidosis), and any role such alterations might have in haemostatic and thrombotic disorders is unknown and requires full examination.

6.4 Influence of Ca^{++} and Mg^{++}.

Of the various cations tested only Ca^{++} and Mg^{++} were found to exert significant effects on network structure. It was shown that the presence of Ca^{++} or Mg^{++} led to higher μ_T without a corresponding change in permeability or μ_P, and reasons for this paradox were fully discussed in Chapter 4. It is to be noted that Marguerie *et al* (1977) showed that at pH 7.5 fibrinogen has three high-affinity binding sites and several low-affinity binding sites. The low-affinity sites are eliminated on the addition of $MgCl_2$. Marguerie *et al* (1979) concluded that Ca^{++} catalyses proteolysis of fibrinogen to fibrin through the formation of a calcium-dependent dimer. In experiments described in Chapter 5, both Ca^{++} and Mg^{++} were added at physiological concentrations at a pH of 7.35. Turbidity curves showed a greater rate of rise when these cations were added together, than when added singly. It is possible that Mg^{++} in combination with Ca^{++} eliminates low-affinity binding sites and makes high-affinity sites more responsive to Ca^{++} which further enhances the proteolytic breakdown of fibrinogen.

However, the most interesting aspect of Ca^{++} effects on network structure came from studies by Ferguson (1983). He showed that in networks developed in the presence of Ca^{++}, Factor XIIIa influenced cross-linkages such that the network was reinforced and stabilized. Thus Ca^{++} causes the formation of thicker fibres in the major network and a reinforced minor network. Such a network would be expected to be more resistant to stresses of vascular flow and to fibrinolysis (McDonagh, 1971).

6.5 Role of Kinetics.

From observations described in Section 1 it would appear that kinetics of network development determine the final structure through influence of events which even precede the appearance of visible fibrin. As has been shown there are three clearly separated phases in a turbidity curve which develops when thrombin is added to fibrinogen solution: a lag phase, a phase of rapidly increasing turbidity, and an equilibrium phase. The lag phase corresponds to the time required for fibrinogen to breakdown to fibrin monomer and the initial aggregation

of the monomers to a network of protofibrils. Once the thickness of the fibres exceeds the wavelength of incident light, turbidity begins to rise. The main increase in turbidity during the phase of rapidly increasing turbidity is from the growth in the thickness of fibres (Wolfe and Waugh, 1979) in the initial network of protofibrils. If the experimental system is manipulated to decrease the amount of fibrin in the protofibril network at the end of the lag phase (and given that the net conversion of fibrinogen to fibrin remains constant) a larger amount of fibrin is available for subsequent incorporation, leading to thicker fibres and higher μ_T. Such a set of circumstances occurs when enzyme kinetics are altered such that fibrin monomer release is slowed. This type of alteration is induced by lowering thrombin concentration or by reducing ambient temperature. Under such conditions, final fibre thickness is directly related to the length of the lag phase.

On the other hand, when enzyme kinetics are not affected (as when pH and ionic strength are altered) but the lag phase is increased,a greater amount of fibrin is

incorporated into the protofibril network during this phase. Since the total fibrinogen converted to fibrin is unaltered, a smaller amount of fibrin is left for subsequent incorporation. As a result, fibre thickness is reduced.

Thus, the final network is determined by several factors under pathophysiological conditions, and these factors modify network structure either through influencing kinetics of fibrin generation or those of fibrin assembly.

6.6 General Comment.

From the foregoing discussion it is clear that several factors are capable of modifying network characteristics in purified fibrinogen systems. Whether similar alterations are induced in plasma by minor variations in pH, ionic strength, temperature, fibrinogen and thrombin concentration was not known when these studies were commenced. Indeed, whether techniques developed for measuring fibre thickness in dilute fibrinogen solutions were applicable to plasma was also unknown. These and related areas have been examined in the following chapters.

SECTION II

STUDIES ON FIBRIN NETWORK STRUCTURE DEVELOPED IN HUMAN PLASMA

CHAPTER 7

ADAPTATION OF METHODS FOR APPLICATION TO PLASMA

7.1 INTRODUCTION

Methods based on turbidity and permeability, to allow measurement of mass-length ratio of fibrin fibres developed in pure fibrinogen solution have been shown in preceding chapters to be highly reproducible and capable of detecting changes induced by minor variations in clotting conditions. It is now necessary to investigate these techniques in respect of their applicability to human plasma. Theoretical assumptions made in calculating mass-length ratios need critical examination. Method of handling plasma, reproducibility of the technique, storage of plasma and other factors such as age of subjects also need to be investigated. Compaction, a semiquantitative technique based on the tensile behaviour and shown not to be acceptably applicable to networks developed in fibrinogen solution, also needs to be systematically examined. These and related areas are fully explored in this chapter.

7.2 THEORETICAL CONSIDERATIONS.

7.2.1 Turbidity and Mass-Length Ratio Derived from Varying Wavelengths

Theoretical basis for the derivation of mass-length ratio from turbidity (μ_T) has been fully described in Chapter 2.

7.2.2 Application to Plasma.

Turbidity of fibrin gel is proportional to the inverse of the third power of the wavelengths (λ^{-3}) over a considerable wavelength range (Carr and Hermans, 1978). Turbidities were measured in the range from 600 to 800nm and at different thrombin concentrations, and plotted as shown in Figure 7.1. The predicted proportionality which was applicable to fibrinogen systems (Figure 3.1, Chapter 3) did not, however, hold for plasma networks. If the lines are extrapolated to the origin the relationship is in fact a curvilinear one. It is to be noted that the intercept of the graph was similar in each case whether low or high thrombin concentrations were used. Since the relationship was curvilinear the slopes

of these plots cannot be confidently used to calculate mass-length ratio. Following the suggestion of Carr and Hermans (1978) $C/T\lambda^3$ was plotted as a function of λ^{-2} where C is the fibrin content(Figure 7.2). The intercept of such a plot can be used validly to calculate mass-length ratio (μ_T). Since such plots for the various thrombin concentrations were similar, data for 1.5u/ml thrombin concentration only is shown.

Refractive index values for plasma and fibrinogen are not significantly different as shown in Appendix 1. However, μ_T derivations were based on the refractive index of plasma which was found 1.347 ± 0.0046. This value was used in all future calculations of μ_T from plasma.

7.3 METHODS.

7.3.1 Mass-Length Ratio Derived from Turbidity (μ_T).

Mass-length ratio from turbidity (μ_T) was derived using a range of incident wavelengths between 600 and 800nm as described in Chapter 2.

7.3.2 Permeability and Mass-Length Ratio Derivation from Permeability (μ_P).

This method and the derivation of μ_P is fully described in Chapter 2. In preliminary experiments 10 of 12 networks developed with plasma collapsed on perfusion. To overcome this difficulty the tubes were coated with a thin layer of fibrinogen solution which was then clotted with 0.5u/ml thrombin. Fibrinogen concentration was 2.5mg/ml, pH 7.35 and ionic strength 0.153. These tubes were left to stand for one hour before use.

7.3.3 Compaction.

The method for compaction was fully described in Chapter 2. Compaction was measured in networks developed with thrombin concentrations ranging from 0.19 to 1.50u/ml.

7.3.4 Recovery and Reproducibility Measurements.

Reproducibility and recovery measurements (three μ_T, five compaction and five permeation and μ_P) were made in plasma pooled from five donors.

7.3.4.1 Day to Day Variation.

60ml of blood collected from each of six healthy donors between the ages of 25 to 44 years was divided into two aliquots in 50ml test tubes. The first 30ml were centrifuged to obtain platelet free plasma (PFP) as described in Chapter 2. μ_T, permeability, μ_P and compaction were then determined. The second test tube was left to stand at room temperature for one hour before the same procedure was repeated. Turbidity measurements were in duplicates; five compaction tubes and four permeation tubes were used. Blood was then collected from the same donors 24 hours later and the whole procedure repeated to investigate day to day variation.

7.3.5 Storage of Plasma at -70°C.

Pooled platelet free plasma obtained from 20 donors was stored in 30ml aliquots in four 50ml test tubes. Measurements of μ_T, permeability, μ_P and compaction were made on fresh pooled plasma and then after storage at -70°C for 24 hours and 120 hours.

7.4 RESULTS

7.4.1 Compaction.

It has already been discussed that this technique has low reliability when applied to networks developed in fibrinogen solution: the main problem being the low expelled volume (Chapter 3). However, when applied to plasma the expelled volume is larger (Table 7.1). At the highest thrombin concentration used the compaction was 31 ± 2%. These results show that measurement of compaction in networks in plasma does not suffer from inaccuracies inherent in the measurement of small volumes of expelled fluid.

7.4.2 Recovery.

Recoveries are shown in Table 7.2 in a typical experiment. The SEM is acceptably small. For all future experiments turbidity measurements were made in duplicates. Because expelled volume in compaction experiments had a large variation from one tube to the next it was decided to make future compaction studies in

quadruplicates. For similar reasons all future studies on permeability were also made at least in quadruplicates.

7.4.3 Reproducibility.

μ_T, τ, μ_P and compaction measured immediately were not significantly different from that measured one hour after venepuncture (Table 7.3). Fibrinogen concentration and fibrin conversion also did not vary significantly when blood had been kept at room temperature for one hour. Furthermore,all these measurements were reproducible when made on the same subjects 24 hours later. It was concluded that network structure does not alter when blood is left to stand at room temperature for one hour. There was highly acceptable day to day reproducibility of measurements as shown in Table 7.3.

Figure 7.4 is a scattergram of μ_T against age. In 21 subjects tested, there was no correlation between age of subjects and μ_T. The mean μ_T in 2 subjects was 20.00 ± 1.48 (Table 7.4). Figures 7.5 and 7.6 show scattergrams in which μ_T and τ and compaction are plotted against age of subjects. There was no statistical correlation with

age, although it was noticed that older individuals tended to have less permeable networks, and were less subject to variations than younger subjects. Firm conclusions could not be drawn as number of subjects was insufficient. The mean permeability in the population studied was 429 ± 38; the mean μ_P was 45.7 ± 3.8, and the mean compaction was 25 ± 1% (see Table 7.2).

7.4.4. Storage of Plasma at -70°C.

The effect of storage of plasma at -70°C on fibrin network structure is shown in Table 7.5 in two experiments. μ_T seemed to decrease as did τ, μ_P and compaction in one experiment, while they seemed to increase in the other experiment. It would seem from this preliminary work that reproducible measurements could be made only within one hour after blood was freshly collected into citrated plasma.

7.5 DISCUSSION.

The methods to characterize networks developed in fibrinogen can also be confidently applied to plasma. In networks developed in plasma, the relationship between

turbidity and $1/\lambda^3$ is not linear, just as has been shown to be the case when thrombin concentration is lowered or fibrinogen concentration is increased in fibrinogen solution (Carr and Hermans, 1978). In the latter case it is possible nevertheless to derive a measurement of mass-length ratio from an intercept obtained from a plot of $C/T\lambda^3$ and $1/\lambda^2$ (Carr and Hermans, 1978). In the investigations described in this thesis it is shown that in plasma networks also plots of $C/T\lambda^3$ and $1/\lambda^2$ may be used to derive a reliable measure of μ_T.

Permeability (τ) and μ_P can also be confidently derived in a fashion similar to that for networks developed in fibrinogen solution. However, the method requires modification to ensure that permeation tubes are coated with a thin layer of fibrin so that clots do not collapse during perfusion.

The use of compaction in addition to μ_P and μ_T for characterization of fibrin networks in plasma provides three independent methods. Compaction is a semiquantitative technique based on the tensile behaviour of fibrin fibres. It is highly reproducible and can be used in any clinical laboratory.

Recovery experiments (Table 7.2) have shown all the three methods are highly reliable when used to examine fibrin networks in plasma. Turbidity measurements showed very little variation and it was considered adequate to make observations in duplicate. Permeation, on the other hand, was subject to a greater degree of variation (see Table 7.2 and 7.3). As a rule, at least four permeation tubes were used for each experimental condition. Compaction showed very little variation within a set of measurements. However, because the expelled fluid was measured with the aid of a syringe it was considered open to greater degree of observer error. To minimize observer error at least four compaction tubes were always used.

It was found that blood need not be immediately processed after venepuncture to measure τ, μ_P and μ_T and could be left to stand for at least one hour without significant change in fibrin network characteristics (Table 7.4). Nor was a significant variation found in network characteristics, plasma fibrinogen or network protein content from one day to the next (Table 7.4). This result facilitates the use of these techniques in clinical situations in which it is not always convenient

to make observations immediately after venepuncture. Furthermore, it was found that the blood does not have to be centrifuged immediately after collection, and plasma does not have to be frozen.

The data described has shown no correlation between network characteristics and age. It must be mentioned, however, that in subjects over the age of sixty networks tended to be less permeable although greater numbers are needed to establish this with certainty.

In the small population tested mean μ_T was 20.00 ± 1.48, μ_P was 45.7 ± 3.8, τ was 429 ± 38 and compaction was 25 ± 1%. These measurements can be used to compare data from patients with certain clinical disorders.

7.6 CONCLUSIONS.

1. Methods can be confidently used in plasma to derive μ_T, μ_P and τ compaction.

2. All three methods are reliable.

3. Blood may be processed for up to one hour after venepuncture without sacrificing accuracy of reproducibility.

4. There seems to be little correlation between age and network characteristics.

TABLE 7.1 Effect Of Thrombin Concentration On Compaction Of Networks Developed in Human Plasma.

Results are mean of four experiments.

Thrombin Concentration (u/ml)	Compaction (%)	±SEM
0.19	65	1
0.38	51	1
0.70	40	1
1.50	31	2

Table 7.2 Recoveries In Plasma: μ_T, μ_P, τ and Compaction.

Results are mean of number of experiments as shown in the table.

μ_T (SEM) [x 10^{12} daltons/cm]	μ_P (SEM)	τ (SEM) x10^{11} cm^2	Compaction (SEM) (%)
14.08 (0.42)	42.60 (2.62)	417 (27)	50 (0.9)
n = 3	n = 5	n = 5	n = 5

Table 7.3 Reproducibility Of Data In Networks Developed In Human Plasma.

Measurements were made at the start of the experiment (0 hrs), after one hour (1 h) and 24 hours (24 h).

μ_T and μ_P are presented as $x10^{12}$ daltons/cm, τ is in $x10^{11}$ cm^2. Fibrinogen concentration (Fib.Conc.) and network fibrin content (Cn) are in mg/ml.

Sex	Age	Time of measurement	Fib.Conc.	Cn	μ_T	τ	μ_P
M	29	0	2.71	2.30	15.30	571	62.4
		1h	2.71	2.30	13.80	572	64.5
		24h	2.71	2.30	13.30	601	65.6
F	25	0	4.75	3.36	15.50	398	63.4
		1h	4.15	3.36	13.70	401	63.8
		24h	4.00	3.36	15.10	372	54.5
F	30	0	5.48	4.44	11.70	321	67.4
		1h	5.48	4.44	11.00	368	77.3
		24h	5.51	4.46	12.80	419	77.8
M	34	0	2.20	1.85	15.73	-	-
		1h	2.20	1.85	16.50	441	38.5
		24h	2.10	1.76	18.63	360	30.0
F	41	0	3.80	3.19	14.6	-	-
		1h	3.80	3.19	15.1	355	53.6
		24h	3.75	3.15	15.4	354	52.8
F	44	0	2.18	1.83	14.80	928	81.2
		1h	2.18	1.83	15.50	871	76.2
		24h	2.00	1.72	14.76	946	76.8

F (female), M (male).

Table 7.4 μ_T, μ_P, τ and Compaction As Measured In A Population Of 21 Normal Subjects.

μ_T and μ_P are presented as $x10^{12}$ daltons/cm, τ is in $x10^{11}$ cm^2. Network fibrin content (Cn) is in mg/ml.

No.	Age (yrs)	Sex	μ_T	μ_P	τ	Comp. (%)	Cn
1	29	M	15.37	62.4	571	22	2.30
2	25	F	15.52	63.4	398	23	3.36
3	30	F	11.74	67.4	321	29	4.44
4	38	M	16.50	38.5	441	21	1.85
5	41	F	15.10	53.6	355	24	3.19
6	44	F	14.80	81.2	928	38	1.83
7	64	F	23.13	30.2	263	21	2.43
8	75	F	21.60	29.0	372	23	1.68
9	32	F	11.50	32.0	311	23	2.18
10	64	F	25.46	19.6	321	29	1.29
11	28	F	29.60	26.2	474	22	1.17
12	52	F	13.01	53.4	403	23	2.74
13	63	M	20.44	-	-	-	-
14	70	M	13.61	-	-	-	-
15	48	M	17.72	37.2	270	-	3.41
16	84	F	24.12	33.6	208	-	2.20
17	34	F	24.19	60.3	579	-	2.26
18	76	F	22.26	49.9	467	-	1.60
19	45	M	37.71	46.1	609	-	1.67
20	33	M	32.82	53.5	677	-	2.71
21	34	M	16.28	31.2	351	-	1.90
X			20.00	45.7	429	25	2.33
SEM			1.48	3.8	38	1	0.19
n			22	19	20	12	1.9

M = Male, F = Female.

Table 7.5 Storage Of Plasma At -70°C.

A and B represent two individual experiments.

	μ_T x 10^{12} daltons/cm		T x10^{11} cm^2		μ_P x10^{12} daltons/cm		Compaction (%)	
	A	B	A	B	A	B	A	B
Day 0	10.61	22.20	358	334	38.7	34.6	21	25
Day 1	9.66	18.90	338	349	33.8	35.9	21	26
Day 5	9.22	23.90	270	376	28.2	35.9	20	29

Figure 7.1 Graph Of Turbidity As A Function Of λ^{-3} ($1/\lambda^{3}$).

Turbidity is proportional to λ^{-3} in networks developed in plasma. Wavelength range: 600 to 800nm. Thrombin concentrations used are given next to each plot.

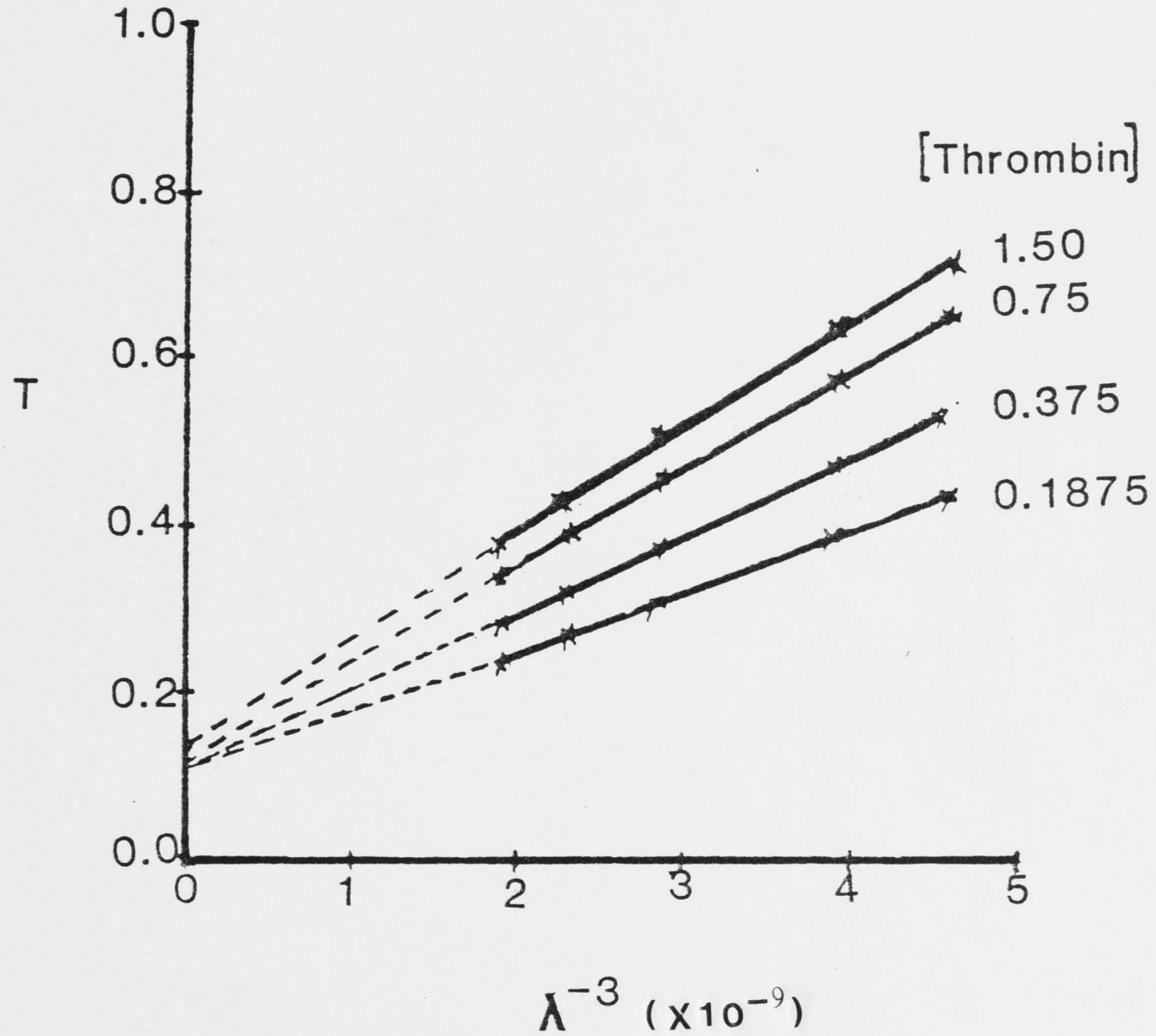

[Thrombin] u/ml
1.50
0.75
0.375
0.1875
T
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
λ^{-3} ($\times 10^{-9}$)

Figure 7.2 Graph $C/T\lambda^3$ As A Function of λ^{-2}.

Turbidity was measured at number of wavelengths between 600 and 800nm. Thrombin concentration was 1.5u/ml.

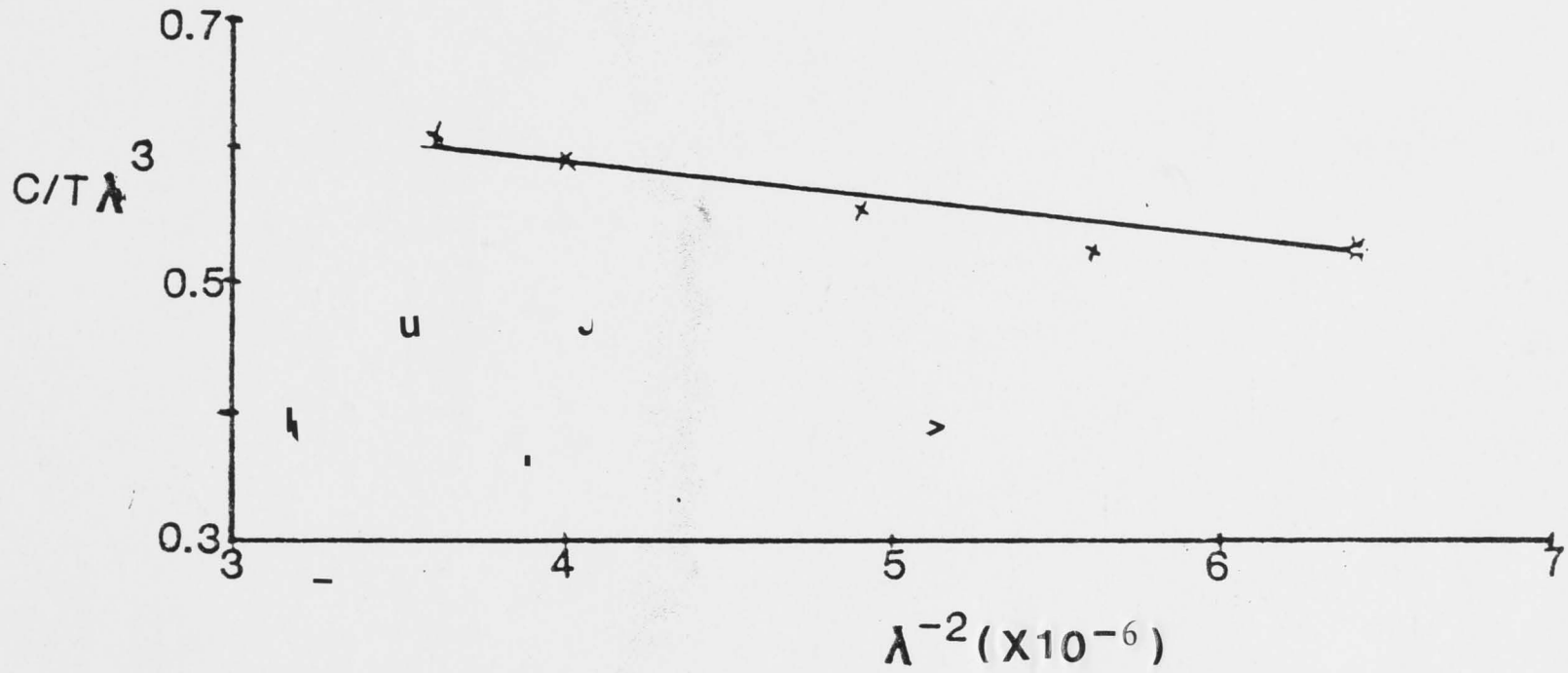

0.7
C/T λ 3
0.5
0.3
3
4
5
6
7
λ-2 (X10-6)

Figure 7.3 μ_T As A Function Of Age.

Data was plotted from Table 7.4.
(r^2 = .020)

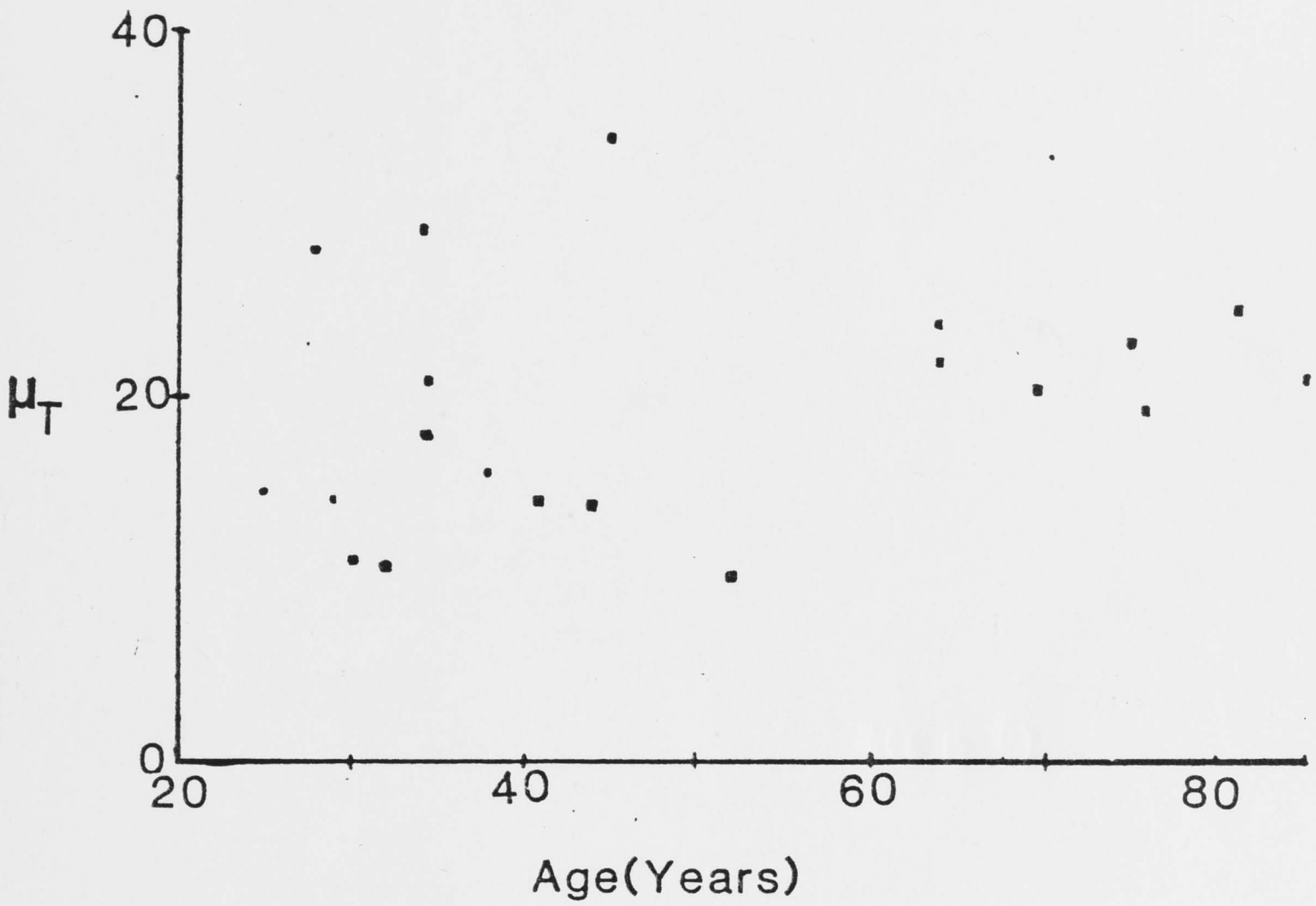

40
μ_T 20
0
20
40
60
80
Age(Years)

Figure 7.4 Graph Of μ_P and τ As A Function Of Age.

Data was plotted from Table 7.4
Crosses are data representing τ,
whilst squares represent μ_P.
(r^2 for μ_P = 0.016,
r^2 for τ = 0.110)

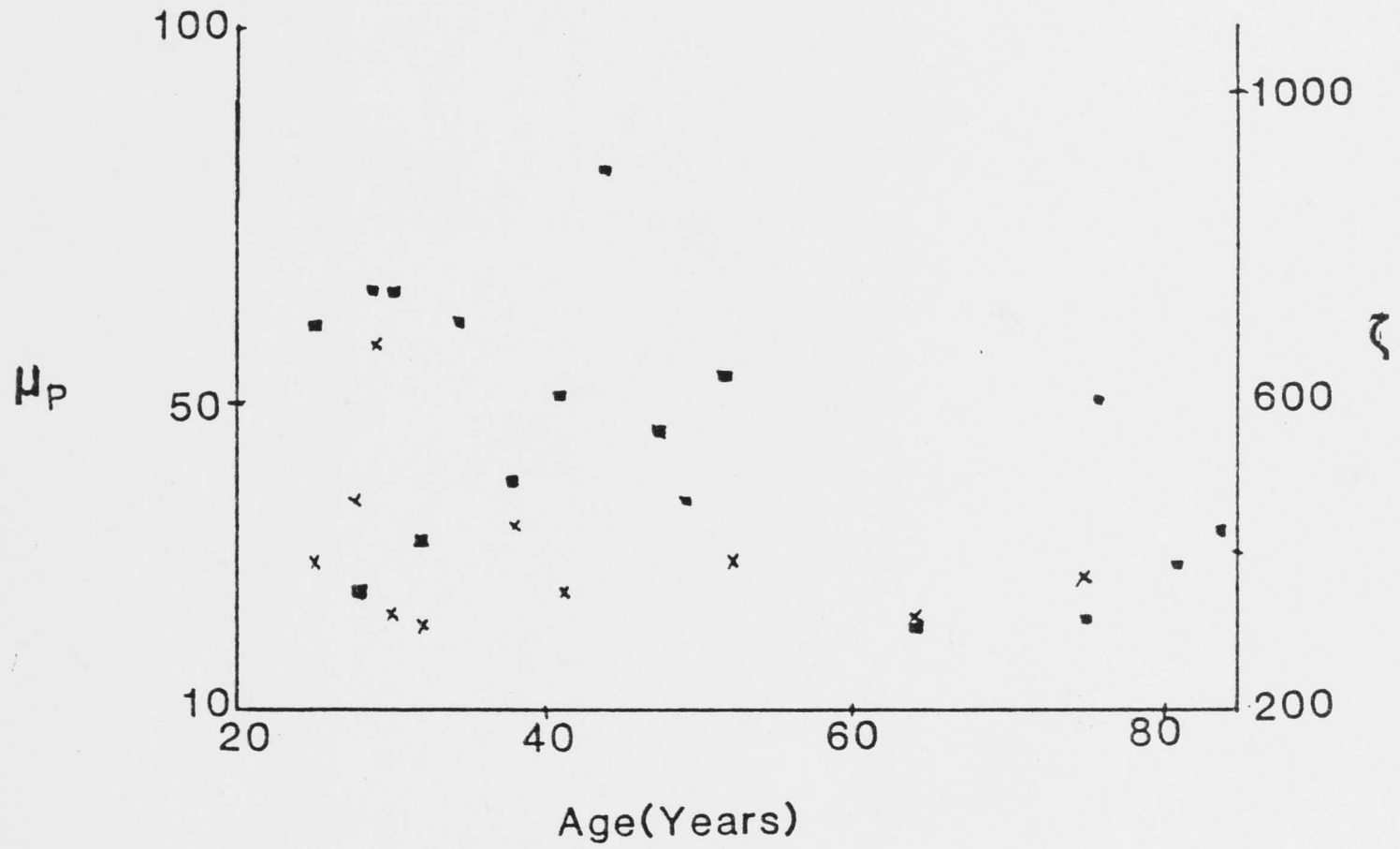
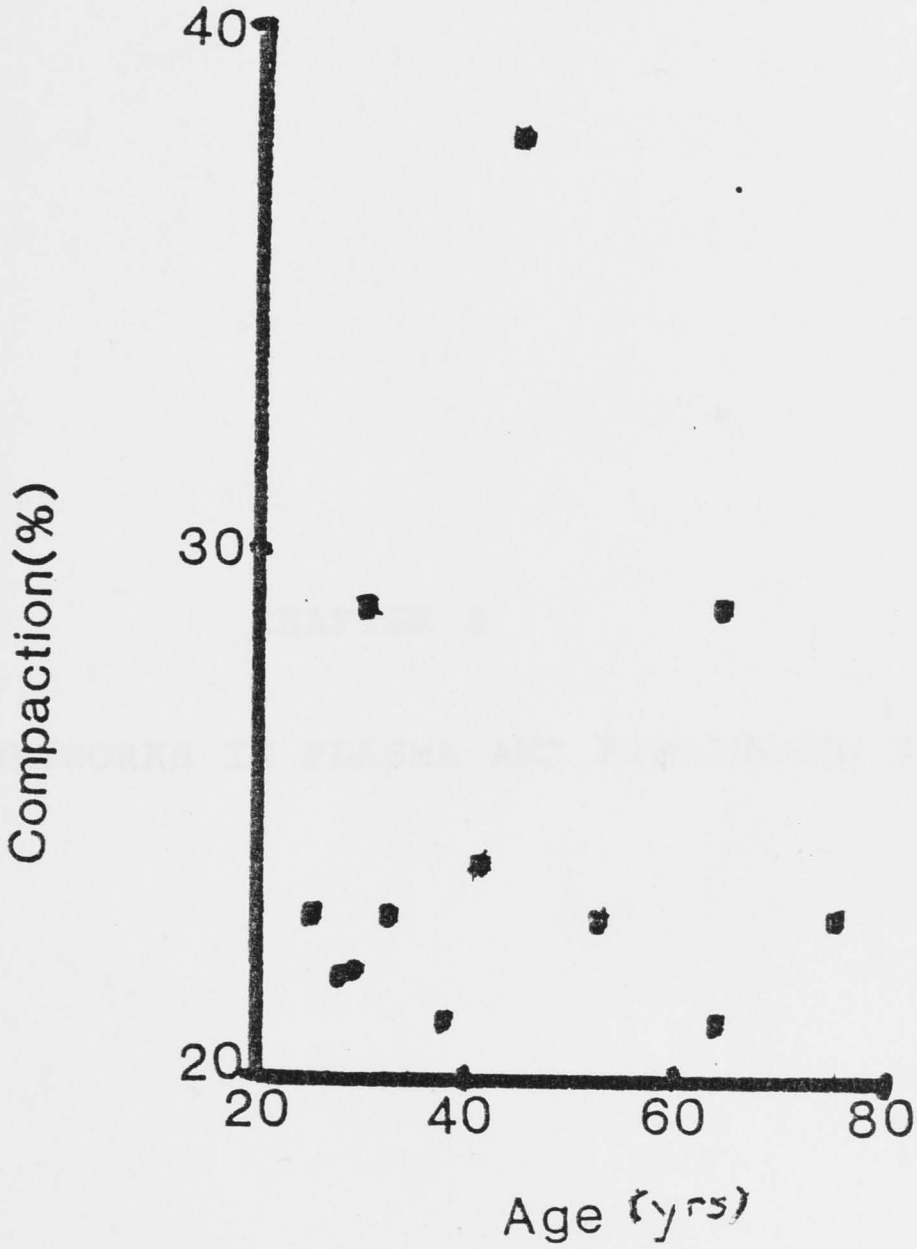

100
50
10
μ_P
1000
600
200
ζ
20
40
60
80
Age(Years)

Figure 7.5 Compaction As A Function Of Age.

Data was plotted from Table 7.4
(r^2 = 0.00)


40
30
20
Compaction(%)
20
40
60
80
Age (yrs)

CHAPTER 8

COMPARISON OF NETWORKS IN PLASMA AND FIBRINOGEN SOLUTION.

8.1 INTRODUCTION

Plasma is a complex solution containing several ions and non-ionised inorganic and organic substances,including plasma proteins, albumin, globulin and fibrinogen,and in this regard differs greatly from pure fibrinogen solution in NaCl. It seemed possible that networks formed in plasma may not be similar to those in fibrinogen solution. Whether conclusions drawn from studies described in Chapters 4 and 5 could be directly applied to plasma require clarification. In this chapter, investigations undertaken to dissect out similarities and differences between the networks in the two systems are described.

8.2 MATERIALS AND METHODS.

General materials and methods have been described in Chapter 2.

8.2.1 Effect of Thrombin Concentration on Plasma Fibrin Network Structure.

Networks were developed in plasma pooled from at least

ten healthy donors. Final thrombin concentration used for clotting was varied between 0.1875 u/ml and 1.5u/ml thrombin. In each of three sets of experiments, μ_T, τ and compaction were measured. Fibrin conversion to fibrinogen was also measured every five minutes for the first 30 minutes, and thereafter every 60 minutes. 0.45ml of citrated plasma with trace amounts of I^{125}-labelled fibrinogen was added to 0.05ml of solution containing varying amounts of thrombin. Networks were squeezed using wooden spatulas and the extracted serum measured for radioactivity. In kinetic studies turbidity was followed at 800nm.

Networks in pure fibrinogen solution were developed (pH 7.35, ionic strength 0.153) at a final fibrinogen concentration equal to that of the mean of concentrations of three plasma pools. In both fibrinogen solution and pooled plasma, observations were made at room temperature (22°C ± 2°C). In preliminary studies it was found that thrombin concentration of 1.5u/ml, allowed near maximal conversion of fibrinogen to fibrin (see Results and Discussion). This was chosen as the final thrombin concentration for further experiments.

8.2.2 Effect of 25mM Calcium on Networks Developed in Plasma.

In two experiments networks were developed in plasma with a final concentration of 25mM $CaCl_2$, using two thrombin concentrations of 0.25u/ml and 1.5u/ml. In both, 0.45ml of plasma was clotted with thrombin, and fibrin conversion measured at five minute intervals until the 30th minute, and thereafter at one hour, in a fashion similar to that described earlier. A similar experiment was also carried out without the addition of calcium chloride.

8.2.3 Enrichment of Fibrinogen and its Effect on Networks Developed in Plasma.

μ_T , μ_P, τ and compaction were determined in two plasma pools defibrinated by heating at 56°C for three minutes. To ascertain that defibrination had been adequate, fibrinogen concentration was measured by heat estimation (Watson, 1961) as described in Chapter 2. Fibrinogen, purified as described in Chapter 2 (pH 7.35, ionic strength 0.153), was then added to the defibrinated serum to give final concentration of 2, 3, 4 and 5 mg/ml and clotted with 1.5u/ml of thrombin. In a separate

experiment, a plasma pool from 5 donors was analysed for fibrinogen concentration (Ratnoff and Menzies, 1951), and divided into two aliquots. One aliquot was defibrinated and the serum subsequently enriched to 2.4mg/ml of fibrinogen. The other aliquot was known to have the same fibrinogen concentration. 0.25 and 1.5u/ml thrombin was added to samples from both aliquots and turbidity followed at 800nm.

8.3 RESULTS.

8.3.1 Effect of Thrombin Concentration.

Effect of thrombin concentration on networks developed in plasma and in purified fibrinogen solutions are shown in Table 8.1. In both systems, fibrinogen concentration was 2.43 mg/ml and thrombin was 1.5u/ml. Fibrin content of the network at one hour was similar in both. Networks developed in plasma had significantly higher permeability (τ) and higher mass-length ratios, as shown by μ_P and μ_T, than those developed in fibrinogen solution.

Figures 8.1 (a) - (d) show the effect of increasing thrombin concentration on μ_T, μ_P τ and conversion. Fibrin

conversion one hour after thrombin addition (Figure 8.1a) increased log-linearly in plasma while it was invariant at around 90% in the fibrinogen system. 90% conversion in plasma was only observed when 1.50u/ml thrombin was added, and it is for this reason 1.5u/ml thrombin was used when networks developed in plasma and fibrinogen were compared.

Figure 8.1b shows the effect of thrombin concentration on μ_T which decreases log-linearly in fibrinogen solution indicating that major fibres become thinner. This effect,however,was spectacularly marked in fibrinogen,an observation similar to that discussed in Chapter 4. μ_T was nine-fold greater in plasma networks than in fibrinogen solution at the lowest thrombin concentration. Permeability (τ) in plasma networks was 15-fold greater than in networks in fibrinogen solution (Figure 8.1d) at the lowest thrombin concentration. It decreased more markedly in networks developed in fibrinogen than in those developed in plasma when thrombin concentration was increased.

Figure 8.1c shows the effect of thrombin concentrations on μ_P. These results are similar to those for permeability. Compaction also decreases as thrombin

concentration is increased (Figure 8.2), indicating that networks had higher tensile strength. Similar experiments could not be performed in fibrinogen solution for reasons elaborated in Chapter 3.

Changes in turbidity at 800nm, and fibrinogen conversion in plasma, upon addition of 0.25u/ml and 1.50u/ml thrombin are shown in Figure 8.3. The concentrations of thrombin were chosen to highlight the differences in turbidity and conversion kinetics in the two systems. Fibrinogen conversion plateaus within ten minutes with both thrombin concentrations but, as expected from observations shown in Figure 8.1a, conversion is dependent on thrombin concentration. With 0.25u/ml thrombin, the lower plateau is consistent with less fibrin in the network.

Figure 8.4 shows fibrinogen conversion and turbidity changes at 800nm over 60 minutes in fibrinogen solution clotted with thrombin. With 1.50u/ml of thrombin, fibrinogen conversion is maximal and plateaus quickly. Turbidity increase follows a similar time course. When 0.25u/ml thrombin is used, fibrinogen conversion shows a time-dependent increase and reaches near maximal value at

60 minutes. Initial increase in turbidity is slow but continues for up to 60 minutes. This increase corresponds to the incorporation of fibrin into the network. Although conversion at 60 minutes is similar, conversion kinetics are different with the two thrombin concentrations. The latter underlies the formation of thicker fibres with lower thrombin concentrations.

In contrast to the findings in fibrinogen solution, conversion with low thrombin concentration does not proceed to completion in plasma but stops within ten minutes of thrombin addition. Conversion kinetics at highest thrombin concentration used , however, is not dissimilar in the two systems.

8.3.2 Networks Developed in Plasma: Effect Of Ca^{++} and Conversion Kinetics.

The effect of 25mM $CaCl_2$ on conversion kinetics with two concentrations of thrombin is shown in Figure 8.5. Conversion is dramatically accelerated and is quantitatively increased at equilibrium. With low thrombin concentration, 25mM $CaCl_2$ causes conversion to

increase from 58% to 76% at five minutes. Maximal value of 92% is reached in ten minutes. Without Ca^{++} only 63% of fibrinogen is converted at equilibrium. With high thrombin concentrations only the rate of conversion is accelerated and the maximal value of 90% is reached within the first five minutes. Without Ca^{++} conversion was maximal after ten minutes.

Maximal conversion of fibrinogen to fibrin was thrombin concentration-dependent without Ca^{++} but independent of the two concentrations examined when Ca^{++} was added.

8.3.3 Networks Developed in Defibrinated Plasma: Effect of Fibrinogen Concentration.

μ_T in defibrinated plasma did not change significantly with fibrinogen concentration (Figure 8.6) above 2mg/ml. On the other hand, and surprisingly, μ_P (Figure 8.7) and τ (Figure 8.8) decreased dramatically as fibrinogen concentration was increased. Compaction also decreased when fibrinogen concentration was increased, showing that the tensile strength of fibre in the network decreased (Figure 8.9). Although the net amount of fibrin in the network increased, the percentage converted decreased (Figure 8.10).

Figure 8.11 shows turbidity changes in plasma and in defibrinated plasma both containing 2.4mg/ml fibrinogen in response to thrombin at final concentrations of 0.25 and 1.5u/ml. Untreated plasma showed higher turbidity at both thrombin concentrations. Also, there was a perceptible lag phase when treated plasma was clotted with 0.25 U/ml thrombin. The lag phase in untreated plasma at a similar concentration of thrombin was smaller.

8.4 DISCUSSION.

The bulk of the work on fibrin network structure has concentrated on networks developed in fibrinogen solutions (Ferry and Morrison, 1947; Carr *et al*, 1977; Carr and Hermans, 1978; Blomback and Okada, 1982; Shah *et al*, 1982). Human plasma is 90 to 91% water by weight, 6.5 to 8% protein and the rest is composed of low molecular weight substances (Schmidt and Thews, 1983). Some of the proteins are known to influence the kinetics of fibrinogen conversion (for example, the anti-thrombins) or to bind with fibrin as, for example, fibronectin

(Ruoslahti and Vaheri, 1975; Stathakis *et al*, 1978; Stemberger and Hörmann, 1976; Holm and Brosstad, 1982), and α-antiplasmin (Bennett *et al*, 1984). A thorough study to compare networks developed in plasma and fibrinogen systems has hitherto not been undertaken.

This study has shown that for similar fibrin content, networks in plasma differ from those developed in purified fibrinogen systems in a number of ways (Table 8.1). Networks developed in plasma using high thrombin concentrations have markedly higher permeability and mass-length ratios, indicating that fibrin fibres are thicker than in fibrinogen solution under similar clotting conditions. Since fibre thickness is determined by the kinetics of its growth (Blomback and Okada, 1980; Wolfe and Waugh, 1981: Chapters 4 and 5 in this thesis) the differences in the fibre thickness in the two systems arise from differences in the kinetics of fibrinogen breakdown and fibrin assembly in plasma and in fibrinogen solution.

In both systems, fibrinogen is converted by thrombin to fibrin monomer which then forms intermediate polymers called protofibrils. As discussed before, Wolfe and Waugh (1981) suggested that fibrin networks develop initially

as a network of protofibrils whose length is determined by the rate at which fibrin monomer is generated, as well as certain experimental conditions like pH, temperature and ionic strength (see Chapters 4and 5). Fibrin monomer generated subsequently is incorporated into the existing network making individual fibres thicker in diameter. A further increment in the protofibril network does not occur. Thus, for networks with similar fibrin content, thicker fibres will develop if initial protofibril network is made up of a smaller proportion of protein leaving a greater proportion to incorporate subsequently. In plasma, it would seem that this is the case. In fibrinogen solution, however, the initial protofibril network is made up of a much greater proportion of protein, and this limits the subsequent growth in protofibril thickness. This reasoning is supported by Abildgaard (1965) who showed that at gel point, 40% of the fibrinogen is converted to monomer in fibrinogen solutions, while at gel point only 6% fibrinogen is so converted in plasma.

Interesting variation in μ_T was found in the two systems when both low and high thrombin concentrations were used. In fibrinogen solution with low thrombin concentrations,

the rate of fibrinogen conversion is slow and continues well beyond ten minutes (Figure 8.4). Fibrin fibres, therefore, continue to increase in thickness leading to higher μ_T at equilibrium. When high thrombin concentration is used, conversion ceases within twenty minutes and μ_T correspondingly reaches maximal value at this time. In plasma, however, conversion ceases within twenty minutes (Figure 8.3) regardless of thrombin concentration used, and correspondingly μ_T does not vary with time at lower thrombin concentration (Figures 8.1a and 8.1b). It must, nevertheless, be noted that conversion within the first ten minutes increases as thrombin concentration is increased. This results in an increase in protein incorporation into the network, which should be interpreted as an increase in the number of fibres, rather than increase in the thickness of existing fibres, as shown by the invariant μ_T (Figure 8.1b). This receives support from studies with compaction which show (Figure 8.2) an inverse relationship with thrombin concentration. The decreased tensile strength in fibres in networks developed at low thrombin is attributed to the low conversion of fibrinogen. With increase in thrombin concentration more protein is incorporated into the network resulting in an increase in numerical density of fibres and the strength of network structure .

Even though μ_T remains relatively unchanged in plasma with thrombin concentration, μ_P and τ decrease log-linearly . This contrasts with the invariant μ_P and τ in fibrinogen solution (Figures 8.1c and 8.1d). The question arises whether μ_T and μ_P are measuring the same aspect of fibrin network structure. A previously noted breakdown in correlation between μ_P and μ_T in fibrinogen systems was attributed to dissimilar changes in major and minor components of the network. However, this observation cannot be so explained. Below 0.375u/ml thrombin there is a significant rise in μ_P and τ. Below 0.375u/ml thrombin conversion is around 50%. It is probable that under conditions of such low fibrinogen conversion the network is not able to withstand mechanical disturbances caused by permeation and is washed out during perfusion. This results in a falsely greater τ and μ_P. This suggestion is supported by observations on network compaction which show that such networks are 100% more compactible. At higher thrombin, μ_P and τ remain relatively unchanged. Thus, if thrombin concentrations lower than 0.375 U/ml are used to develop networks in plasma, μ_T should be used as the preferred technique to characterize network structure. Turbidity measurements

and derivations of fibre thickness are not subject to mechanical disturbances.

There are, therefore, systematic differences between fibrinogen solutions and plasma. As described before, plasma is composed of many proteins and the suggestion that some of the proteins underlie the differences is fully examined in the next chapter. For the present it should be noted that these differences arise from conversion kinetics - in fibrinogen solution conversion stops only when the substrate has been consumed, whereas in plasma conversion ceases ten minutes after the addition of thrombin, regardless of the amount of remaining substrate. However, when Ca^{++} is added to thrombin and plasma, regardless of thrombin concentration, fibrinogen conversion is maximal (Figure 8.5). Calcium accelerates the reaction between thrombin and fibrinogen and protects the reaction from the inhibitory effects of other substances which may interfere with the reaction. Experiments on thrombin concentration show that maximal conversion of fibrinogen to fibrin occurs with 1.5u/ml thrombin. This thrombin concentration was used in all further experiments in plasma.

Using defibrinated plasma the effect of fibrinogen concentration could be studied in plasma and compared with parallel studies in fibrinogen solution (Chapter 4). Percentage of fibrinogen converted to fibrin fell as concentration of fibrinogen was increased. However, Cn (i.e. fibrin content of networks) rose due to increased total amount of fibrinogen converted to fibrin. μ_T, however, remained unchanged. This result is similar to that found in purified fibrinogen system when high thrombin was used (Chapter 4). In plasma, however, μ_P and τ decreased as fibrinogen concentration increased, and this result contrasted to those in fibrinogen solution in which, although τ decreased with increasing fibrinogen, μ_P was invariant. This was because it was proportional to the product $C\tau$ (equation 10, Chapter 2). The results obtained from networks developed in plasma, however, must be viewed cautiously because of the method used for defibrination. It is probable that at 56°C many other proteins besides fibrinogen may have been denatured. This may have caused a decrease in fibrinogen conversion. At comparable fibrinogen concentration maximal turbidity was lower in defibrinated plasma than in undefibrinated plasma (Figure 8.11). This effect was seen at both low

and high thrombin concentrations. At low thrombin, the lag phase is in fact increased. These results suggest that heating may have denatured proteins necessary for the integrity of networks developed in plasma. Further work in this area is required.

CONCLUSIONS.

1. Networks developed in plasma had significantly thicker fibres and were more permeable than those developed in fibrinogen solution.

2. The differences arise from differing kinetics of assembly in the two systems.

3. Lower thrombin renders fibres of networks developed in fibrinogen solution thicker while Cn is unaltered.

4. In plasma lower thrombin leaves μ_T relatively unaltered while increasing μ_P.

5. Maximal conversion of fibrinogen to fibrin was thrombin concentration-dependent without Ca^{++} but independent when Ca^{++} was added.

6. μ_T in networks developed in defibrinated plasma remained unaltered with increasing fibrinogen. However, μ_P and τ decreased dramatically

TABLE 8.1

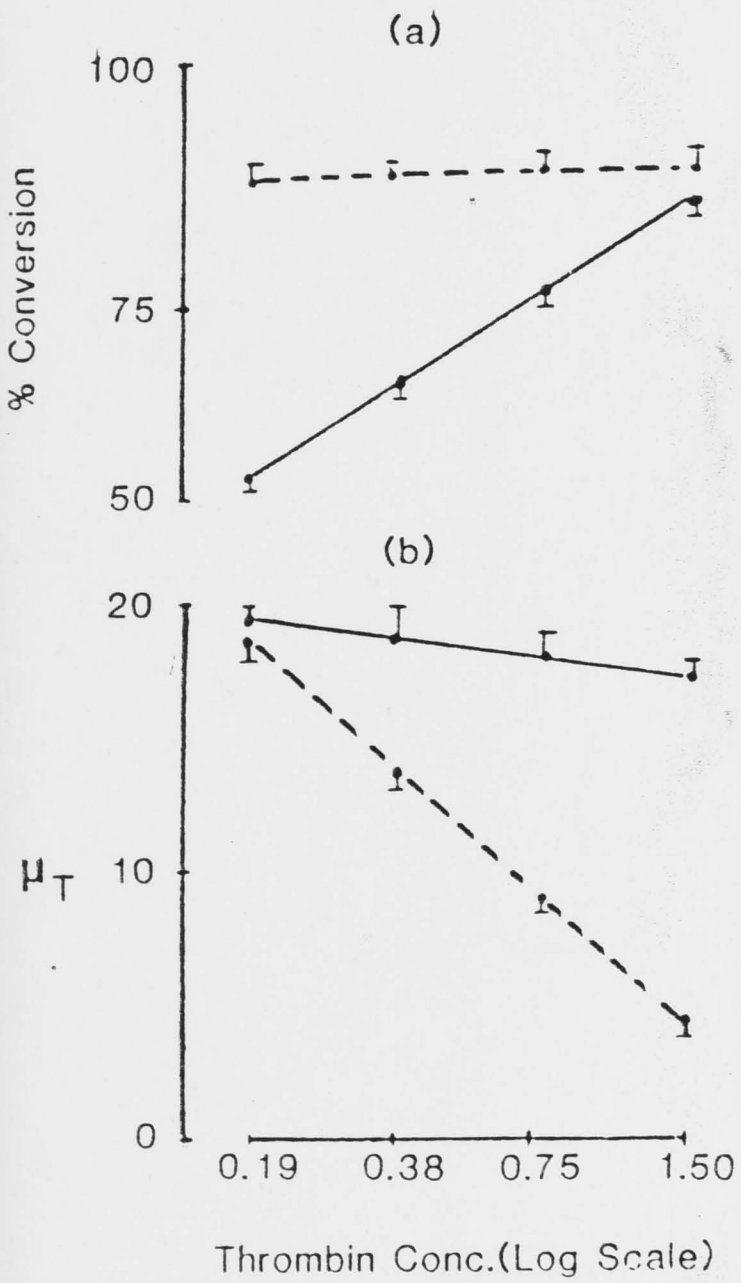
COMPARISON OF NETWORKS DEVELOPED IN FIBRINOGEN SOLUTION AND IN PLATELET-FREE PLASMA.

Results are mean of three experiments.

	Platelet Free Plasma	Fibrinogen Solution	P (two tailed t test)
Permeability ($x10^{11}$ cm^2)	36.1	20	0.002
μ_P ($x10^{12}$ daltons/cm)	35.2	2.0	0.002
μ_T ($x10^{12}$ daltons/cm)	18.89	5.65	0.001
Fibrinogen Conversion(%)	90	90	NS
Fibrinogen Concentration (mg/ml)	2.43	2.43	
Thrombin Concentration used (u/ml)	1.50	1.50	

Figure 8.1 Comparison Of Effect Of Thrombin in Networks Developed in Plasma and Fibrinogen Solution.

Results are mean ± SEM. Plasma (solid lines) and fibrinogen solution (dashed lines) are shown.
Fibrinogen concentration in plasma and fibrinogen solution was 2.43mg/ml.


(a)
100
75
50
% Conversion
(b)
20
10
0
μ_T
0.19
0.38
0.75
1.50
Thrombin Conc.(Log Scale)

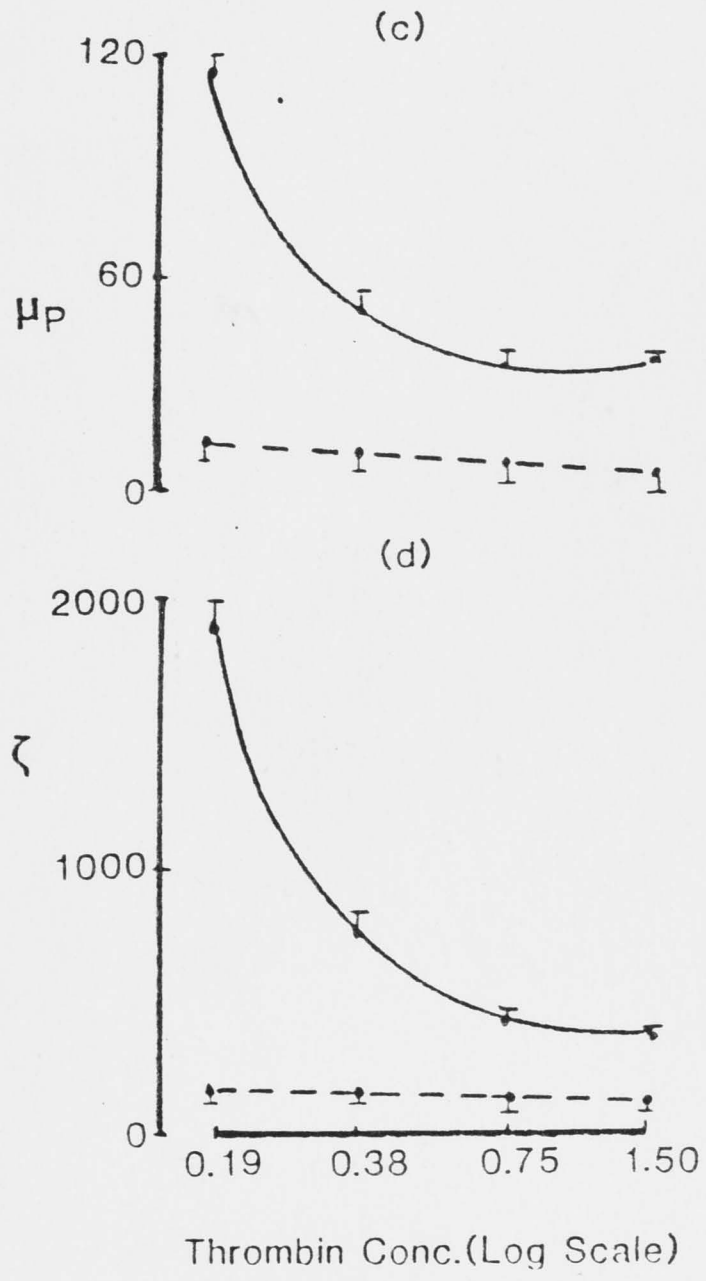

(c)
120
60
0
μ_P
(d)
2000
1000
0
ζ
0.19
0.38
0.75
1.50
Thrombin Conc.(Log Scale)

Figure 8.2 Effects Of Thrombin On Compaction Of Networks Developed In Plasma.

Results are mean of three experiments ± SEM.

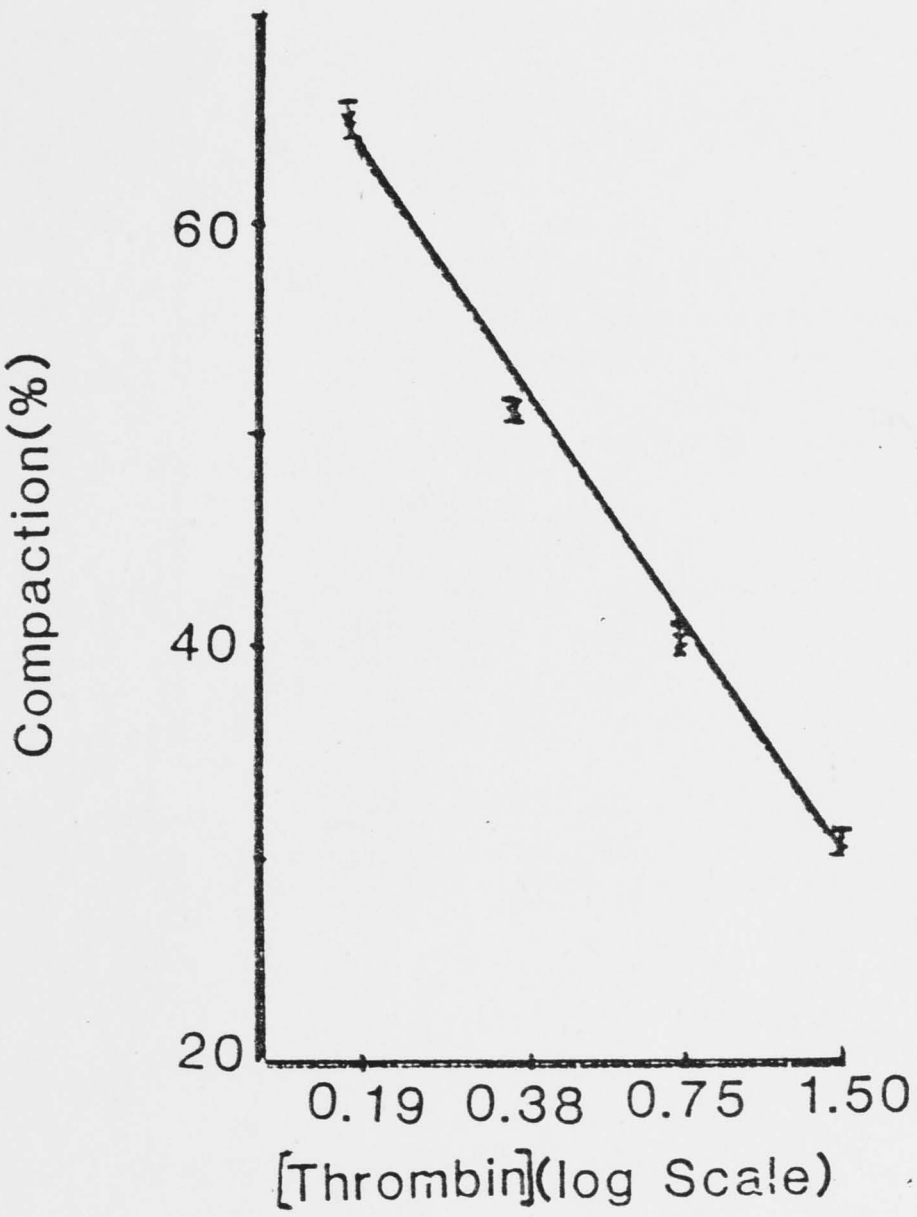
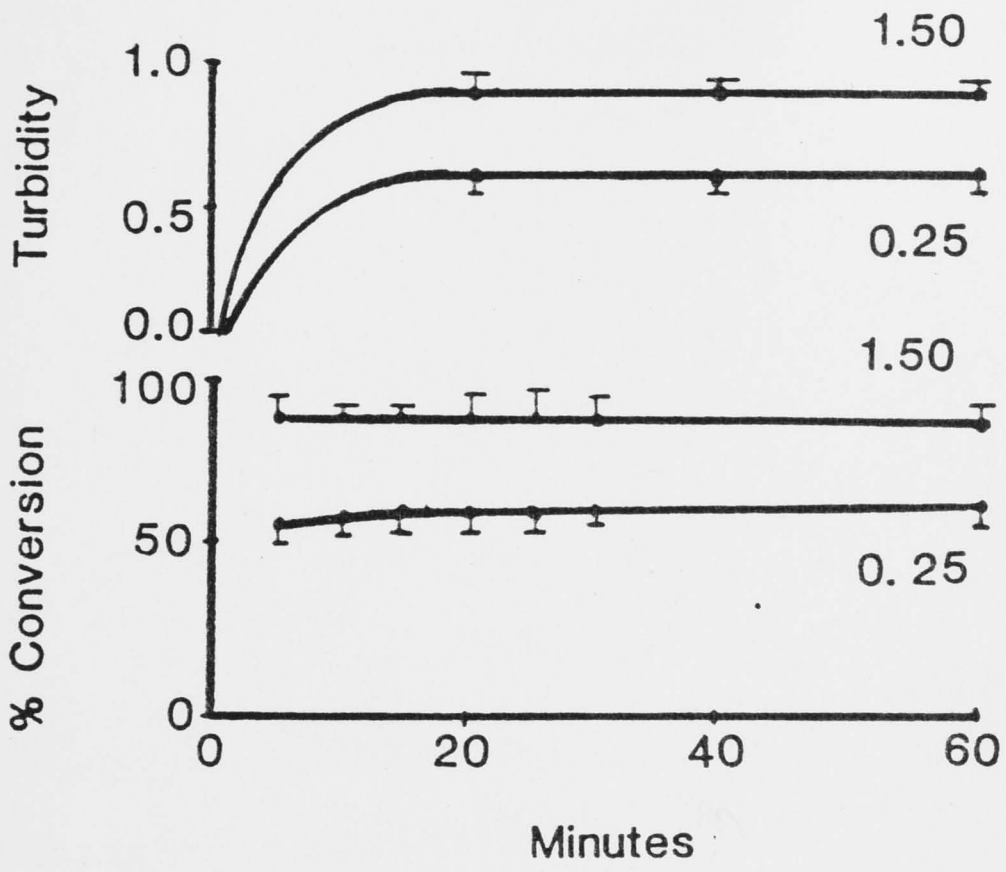

Compaction(%)
60
40
20
0.19 0.38 0.75 1.50
[Thrombin](log Scale)

Figure 8.3 Changes In Turbidity at 800nm and Fibrinogen Conversion In Plasma.

Plasma was clotted using either 0.25 or 1.50u/ml thrombin.
Results are mean of three experiments ± SEM.
Fibrinogen concentration was 2.43mg/ml.

Figure 8.4 Changes In Turbidity At 800nm and Fibrinogen Conversion In Purified Fibrinogen Solution.

Fibrinogen was clotted using 0.25 or 1.50u/ml thrombin.
Results are mean of three experiments ± SEM.
Fibrinogen concentration was 2.43mg/ml.


1.50
1.0
Turbidity
0.5
0.25
0.0
1.50
100
50
0. 25
% Conversion
0
0
20
40
60
Minutes

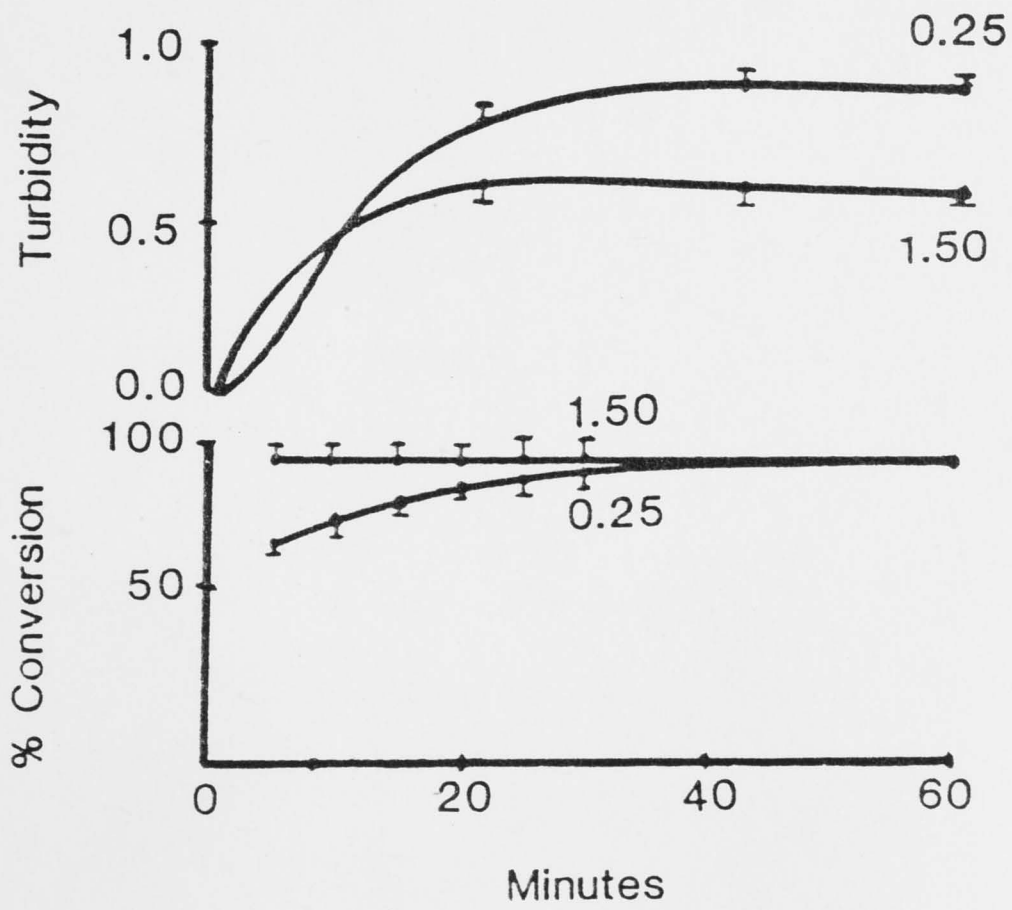

0.25
1.0
Turbidity
0.5
1.50
0.0
1.50
100
0.25
50
% Conversion
0
20
40
60
Minutes

Figure 8.5 Effect Of Addition Of 2.5mM $CaCl_2$ On Conversion Kinetics.

Networks were developed in plasma with two concentrations of thrombin. (0.25 and 1.50u/ml).

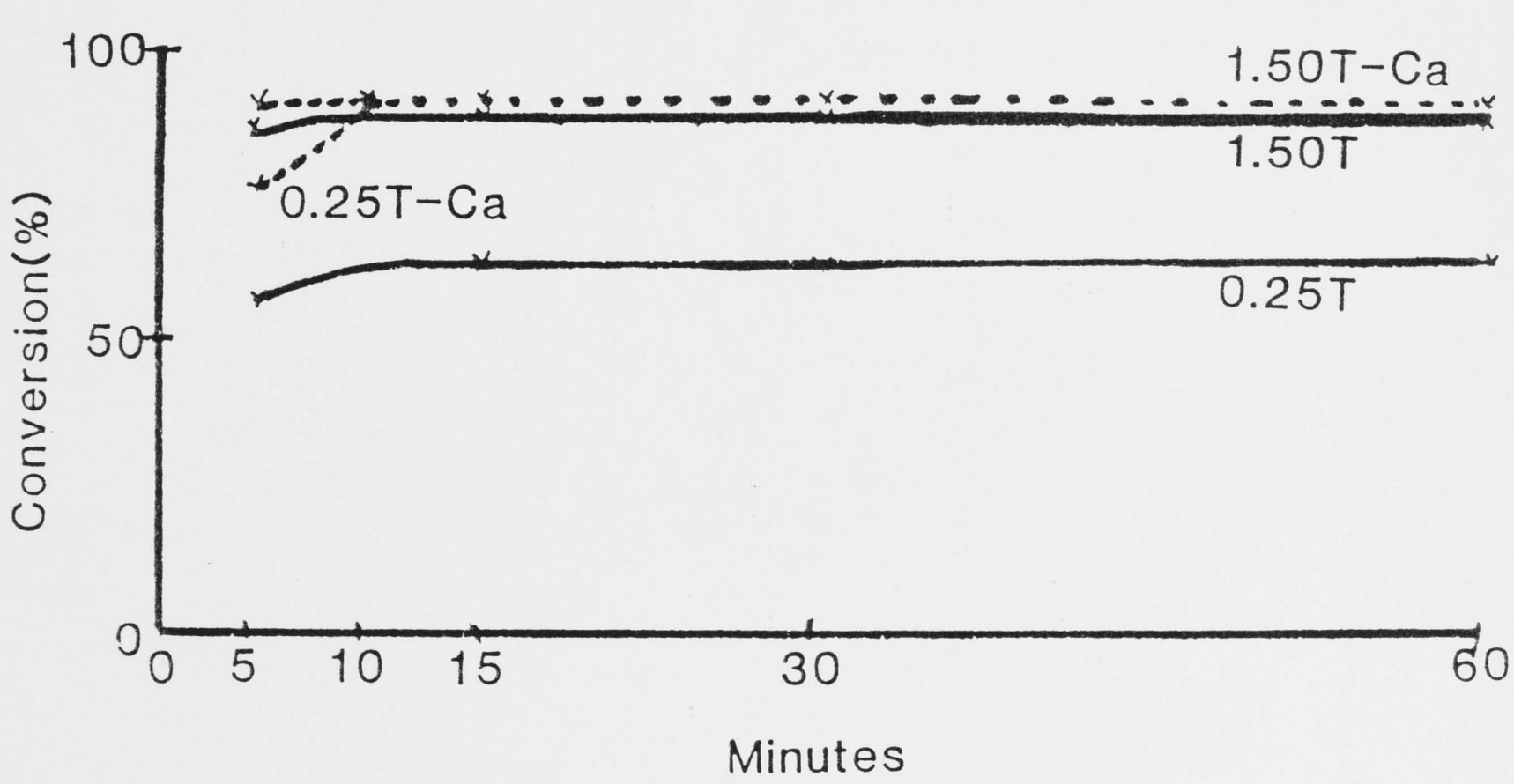

100
50
0
Conversion(%)
1.50T-Ca
1.50T
0.25T-Ca
0.25T
0
5
10
15
30
60
Minutes

Figure 8.6 Effect Of Fibrinogen On μ_T In Networks Developed In Defibrinated Plasma.

Results are a mean of three experiments ± SEM.

Figure 8.7 Effect Of Fibrinogen On μ_P In Networks Developed In Defibrinated Plasma.

Results are a mean of three experiments ± SEM.

Figure 8.8 Effect Of Fibrinogen On Permeability (τ) In Networks Developed In Defibrinated Plasma.

Results are a mean of three experiments ± SEM.

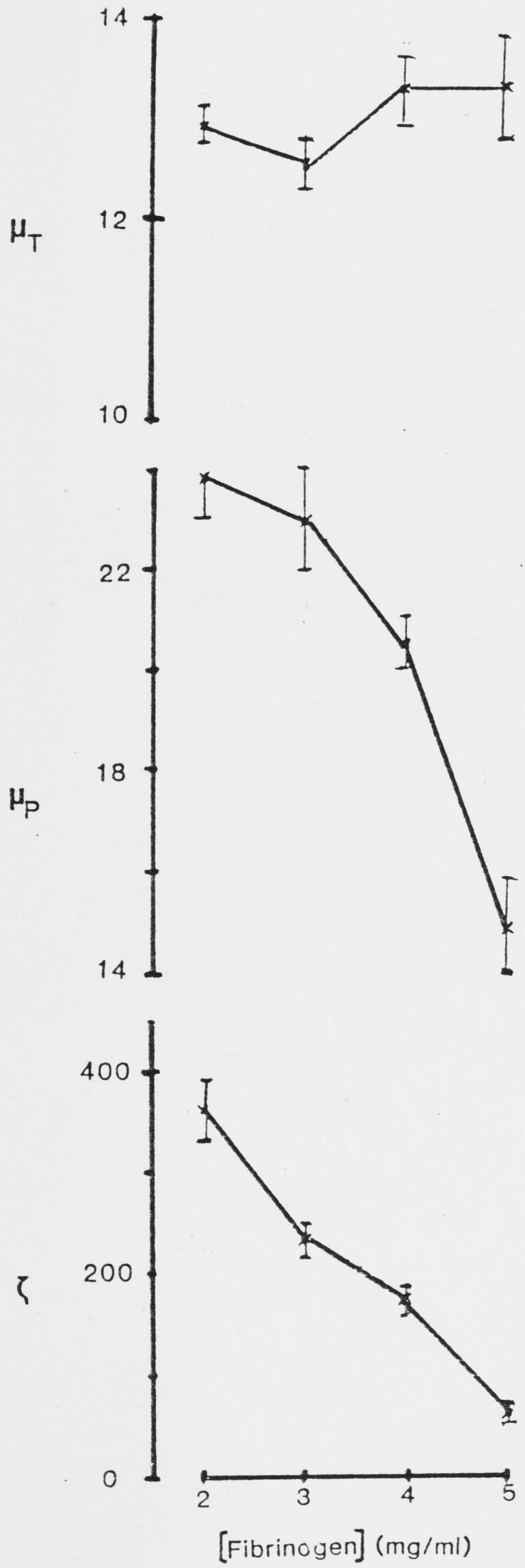

14
12
10
μ_T
22
18
14
μ_P
400
200
0
ζ
2
3
4
5
[Fibrinogen] (mg/ml)

Figure 8.9 Effect Of Concentration Fibrinogen On Compaction Of Networks Developed In Defibrinated Plasma.

Results are a mean of three experiments ± SEM.

Figure 8.10 Effect Of Fibrinogen On Conversion In Networks Developed In Defibrinated Plasma.

Results are a mean of three experiments ± SEM.

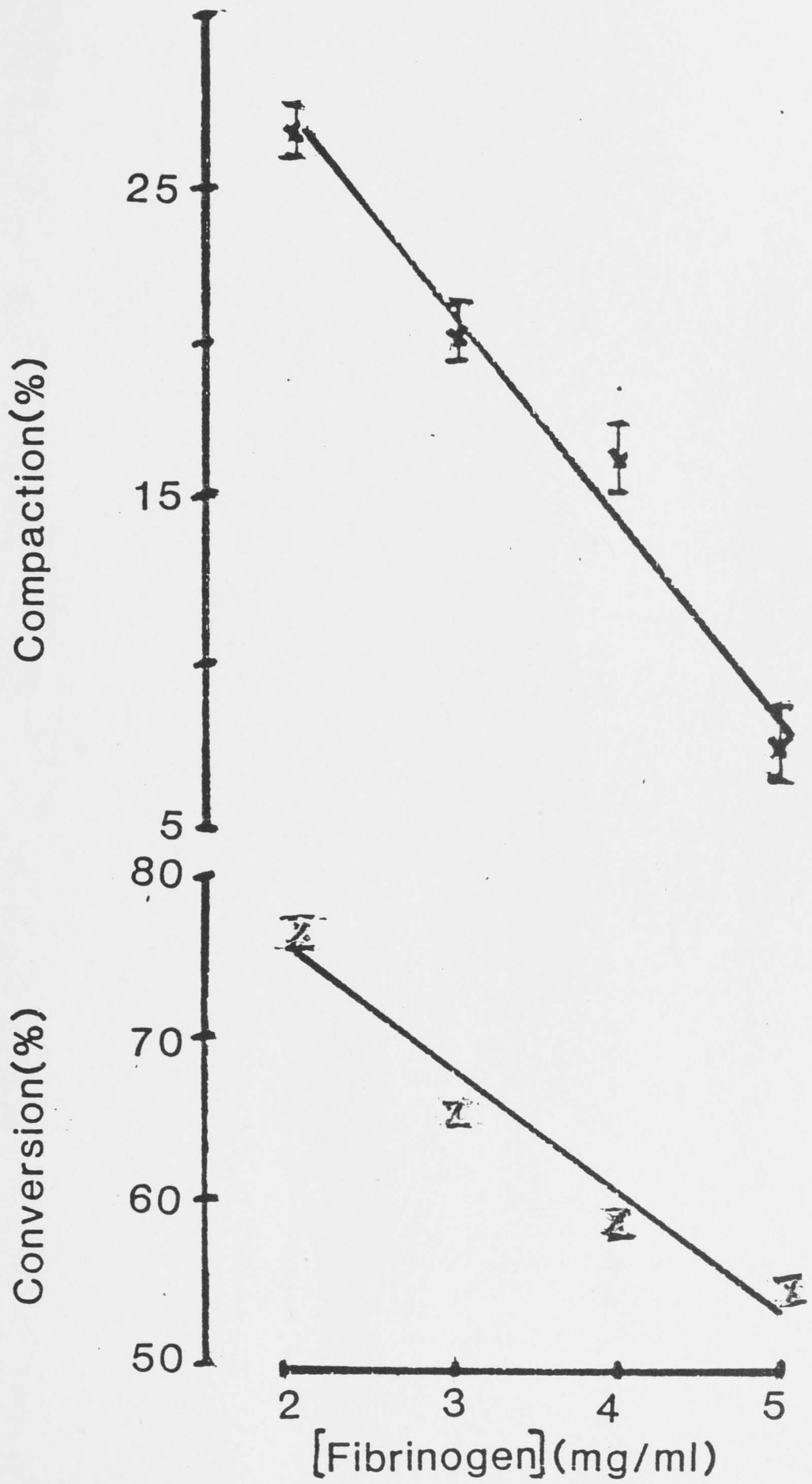
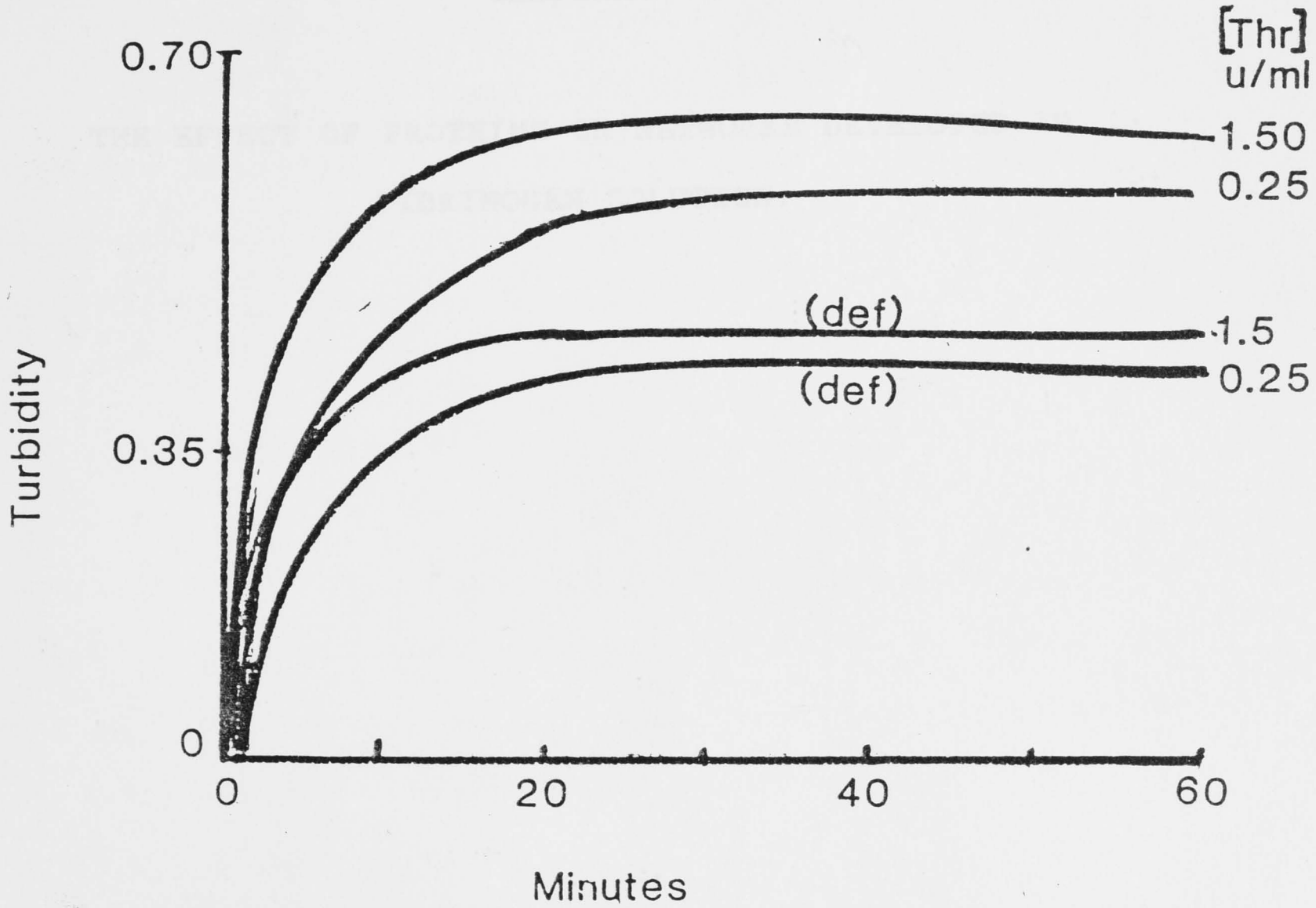

Compaction(%)
25
15
5
Conversion(%)
80
70
60
50
2
3
4
5
[Fibrinogen](mg/ml)

Figure 8.11 Turbidity At 800nm In Plasma and In Defibrinated Enriched Plasma During Network Formation.

Fibrinogen concentration in plasma was 2.4mg/ml. Fibrinogen concentration in defibrinated enriched plasma was 2.4mg/ml. Concentrations of thrombin were 0.25 and 1.50u/ml.


[Thr]
u/ml
0.70
1.50
0.25
(def)
1.5
(def)
0.25
0.35
Turbidity
0
0
20
40
60
Minutes

CHAPTER 9

THE EFFECT OF PROTEINS ON NETWORKS DEVELOPED IN FIBRINOGEN SOLUTION.

9.1 INTRODUCTION

In the previous chapter it was shown that networks developed in plasma have thicker fibres and are more permeable than those developed in fibrinogen solution under similar conditions. Since some of the proteins in plasma, for example fibronectin and α_2-antiplasmin (Rouslati and Vaheri, 1975; Bennett *et al*, 1984) can bind to fibrin(ogen), it seems possible that these substances might modulate network structure. The following investigations were undertaken to explore the effect of plasma proteins like albumin, γ-globulin, fibronectin and ATIII on network structure.

9.2 Materials and Methods.

General methods have been described in Chapter 2. Unless otherwise stated, conversion of fibrinogen to fibrin was maximal at 85% ± 5% In all experiments fibrinogen concentration was 2.5mg/ml, pH 7.35 and ionic strength 0.153. pH was always tested after the addition of proteins to ensure that it remained at pH 7.35. Final thrombin concentration was 0.5u/ml.

9.2.1 Effect of Albumin on Fibrin Network Structure.

μ_T, μ_P and τ were determined in networks developed in fibrinogen solution with and without physiological concentrations of Bovine albumin (Calbiochem, Analar) of 4mg/ml. Turbidity was followed at 800nm as fully described in Chapter 2.

9.2.2 Effect of γ-globulins on Fibrin Network Structure.

γ-globulin (Sigma, St.Louis, USA) in lyophylized form was dissolved in saline and dialysed against 0.9% NaCl for 12 hours, to minimize contamination. In preliminary investigations it was found that addition of γ-globulin to fibrinogen solution caused the solution to gel (paracoagulation). To overcome this, thrombin (0.5u/ml) and γ-globulin (0.6mg/ml) were added to fibrinogen solution together and turbidity followed at 800nm. Control networks were made without γ-globulin and similarly examined.

9.2.3 Effect of Antithrombin III (ATIII) On Fibrin Network Structure.

5u/ml antithrombin III (ATIII) was added to fibrinogen solution which was clotted with 0.5u/ml of thrombin. ATIII was studied at half the concentration because physiological concentrations altered the networks so much that determinations of μ_T, μ_P and τ were not possible. Networks were rendered very impermeable, and even with the highest head of pressure used (30cm H_2O), 24 hours were required for the transit of 0.1ml of buffer. During this period the network integrity was suspect as it began to dry out. Turbidity of these networks was unacceptably low for μ_T determination. Alterations induced with 5u/ml ATIII were not as spectacular and this was the final concentration investigated using 0.25u/ml and 1.5u/ml of thrombin. Turbidity was followed at 800nm.

9.2.4 Effect of Fibronectin on Fibrin Network Structure.

Effect of fibronectin on network structure was examined using a range of concentrations between 150ug/ml - 450ug/ml (300ug/ml is the physiological concentration of

fibronectin) in fibrinogen solution which was clotted with 0.50u/ml thrombin). Turbidity was followed at 800nm.

9.3 RESULTS.

In the bar charts shown test results are expressed as a percentage of control (i.e. of networks without the addition of plasma constituents).

9.3.1 Effect of Albumin.

Figure 9.1 shows μ_T, μ_P and τ in networks with and without albumin. μ_T, μ_P and τ were all decreased when 4mg/ml albumin was added to fibrinogen solution. Albumin induces fibrin fibres to become thinner and network permeability to decrease significantly .

Figure 9.2 shows turbidity development at 800nm in networks with and without albumin. The lag phase in the test experiments was increased from 14 seconds to 34 seconds, the rate of rise of turbidity was slowed and the equilibrium turbidity was significantly decreased.

9.3.2 Effect of γ-globulin.

The effect of γ-globulin on networks developed in fibrinogen is shown in Figure 9.3. Whilst μ_T was slightly higher in networks with γ-globulin, μ_P and τ were significantly raised ($p < 0.001$). Figure 9.4 shows turbidity growth in networks with and without γ-globulin, The lag phase was decreased. The rate of increase in turbidity was accelerated and the equilibrium turbidity was enhanced.

9.3.3 Effect of Antithrombin III (ATIII).

Physiological concentrations of ATIII (10u/ml) dramatically reduce fibre thickness and network permeability as a whole. However, μ_T was significantly decreased while μ_P and τ remained unchanged when 5u/ml ATIII was added to networks developed in fibrinogen solution (Figure 9.5). Thus, although μ_T indicated thinner fibres, μ_P was at variance with this observation.

Figure 9.6 shows that the development of turbidity was arrested in the presence of 5 u/ml ATIII. At low

thrombin concentration, the turbidity curves in the fibrinogen system with added ATIII are similar in character to those in plasma, shown in Chapter 8.

9.3.4 Effect of Fibronectin.

Although fibrinogen conversion was similar when physiological concentration of fibronectin was added to fibrinogen solution, networks had significantly higher μ_T, μ_P and τ (Figure 9.7). Fibres were rendered thicker and networks rendered more permeable than corresponding controls. Turbidity curves (Figure 9.8) showed that control networks had a longer lag phase and slower rate of increase in turbidity than test networks. Figures 9.9 and 9.10 show dose-response curves for fibronectin. μ_T increases directly with fibronectin concentration (Figure 9.9). μ_P and τ (Figure 9.10) both increase as fibronectin concentration was increased to 300ug/ml. However, above this concentration μ_P and τ decrease.

9.4 DISCUSSION

Plasma is an aqueous solution of proteins, lipids, carbohydrates, amino acids, salts and other substances. Proteins contribute a major part to the soluble material in plasma. There are probably more than 150 different types of protein (Blomback and Hanson, 1979). It is not surprising that components of such a complex nature should exert some influence on fibrin network structure. Experiments described in this chapter show that several proteins examined have effects on fibrin networks developed in fibrinogen solution, but the direction of alteration of network characteristics is not the same.

9.4.1 Albumin.

Albumin occupies a special position among plasma proteins in that it contributes 50 - 60% of total protein in blood. Its most significant function, probably, is that it is responsible for 80% of colloid oncotic pressure in blood although it also has other functions, for example, it acts as an easily accessible storage protein and also as a transport protein (Anderson, and Lunden, 1979). As shown in Figure 9.1, albumin reduced fibre diameter

and also the permeability of the network as a whole. Since albumin maintains a reputation for binding virtually every substance it encounters (Finlayson, 1980), it is tempting to ascribe these effects on network structure to its binding of thrombin. However, the conversion of fibrinogen to fibrin with and without albumin is similar. Therefore binding to albumin is unlikely to underlie the effect of albumin. Further, prolongation of the lag phase (Figure 9.2) is also characteristic of low thrombin concentrations. Such a situation would result in continued slow increase in turbidity but this is not observed with albumin. It is highly likely that albumin alters fibre diameter because of its oncotic effect. In a mixed solution of two components, e.g. a linear polymer and a protein, the chemical potentials of the components are higher than when the substances are present individually (Ogston, 1962; Laurent and Ogston, 1963; Edmond and Ogston, 1968; Laurent, 1971). The effect is interpreted from a mutual exclusion of macromolecules from part of the solution and has been termed the steric exclusion effect. Since albumin captures some of the water, the concentration of fibrinogen and thrombin is effectively increased. Reference to Chapter 4 will show that when thrombin and

fibrinogen concentrations are both increased, fibres are rendered thinner and permeability of network is reduced. These changes are similar to those induced by albumin.

9.4.2 γ-Globulins.

γ-globulins are a group of proteins which are divided into five classes (IgG, IgA, IgM, IgD, and IgE). The addition of γ-globulins to fibrinogen solution enhances fibrin fibre diameter. It has already been mentioned that addition of γ-globulins to fibrinogen solution causes fibrinogen solution to gel without the help of thrombin in a manner similar to paracoagulation. Whether the mode of action of γ-globulins is similar is unknown. Paracoagulation would, however, introduce a small number of nuclei early in the lag phase and around these fibrin monomer, as it forms, polymerizes rapidly.

The accretion of monomers around limited numbers of nuclei would be expected to result in the formation of thicker fibres and these would render the network as a whole more permeable. The addition of γ-globulins, in fact, decreases the lag phase of turbidity curves (Figure 9.4). It is not known whether thrombin activity is affected by γ-globulins. However, these curves are

similar to those observed when high and low concentrations of thrombin were used in plasma.

9.4.3 Fibronectin.

Fibronectins are large glycoproteins that have been implicated in a wide variety of cellular properties, particularly those involving interactions of cells with extracellular materials (Hynes and Yamada, 1982). Plasma fibronectin has been shown to be incorporated into fibrin clots (Chow *et al*, 1983). Fibronectin binds to fibrin by either non-covalent attachment, or covalent cross-binding. The covalent cross-binding of fibronectin to the fibrin α-chain is via an ε-(γ-glutamyl) lysine linkage mediated by Factor XIIIa. Mosher (1976) and Kamykowski *et al* (1981) have shown that fibronectin cross-linked to fibrin alters properties of the clot. Such cross-linkages may act as nuclei around which protofibril aggregation and polymerization is promoted, resulting in thicker fibres. Grinnell and Feld (1981) found that cross-linking of fibronectin to fibrin on a fibrin coated substratum enhances the attachment and spreading of cells Mosher *et al*, (1976) believe that covalent binding of fibronectin to fibrin may be important for adhesion and integration of cells proliferating into a wound area.

9.4.4 Antithrombin III.

ATIII affects network structure differently from the other three proteins tested. Although physiological concentrations of ATIII dramatically reduce fibre thickness and network permeability, it is interesting that 5u/ml ATIII reduces only μ_T whilst not affecting permeability or μ_P. Shah *et al* (1982) showed that a fibrin network is composed of a major network of thick fibres and a minor network of thinner fibres. As previously mentioned (Chapter 6) methodological studies have shown that whilst μ_T is sensitive to the major network, μ_P is influenced by the minor network. From this study it is apparent that 5u/ml ATIII has a specific effect of inhibiting lateral polymerization of major network fibres. It is particularly interesting that this effect is independent of any potentiation by heparin of ATIII activity. ATIII also has effects on the kinetics of thrombin action. However, apart from causing the turbidity curves in fibrinogen systems (Figure9.6) to plateau earlier (i.e. similar to the plasma curve) ATIII does not seem to affect μ_P or τ.

9.5 CONCLUSIONS.

1. Albumin induces fibrin fibres to become thinner and network permeability to decrease.

2. γ -globulin induces thicker fibres and increases network permeability.

3. Although physiological concentrations of ATIII dramatically reduce fibre thickness and permeability, 5u/ml ATIII only reduces μ_T fibres while not affecting permeability or μ_P.

4. Fibres are rendered thicker and networks more permeable by fibronectin.

5. Although all proteins tested alter network structure, the direction of alteration is not the same.

Figure 9.1 The Effect Of 4mg/ml Albumin On Networks Developed In Fibrinogen Solution (pH 7.35, Ionic Strength 0.153).

Fibrinogen Concentration was 2.5mg/ml. Results are mean of three experiments ± SEM and expressed as percentage of control. Black columns are results for control.

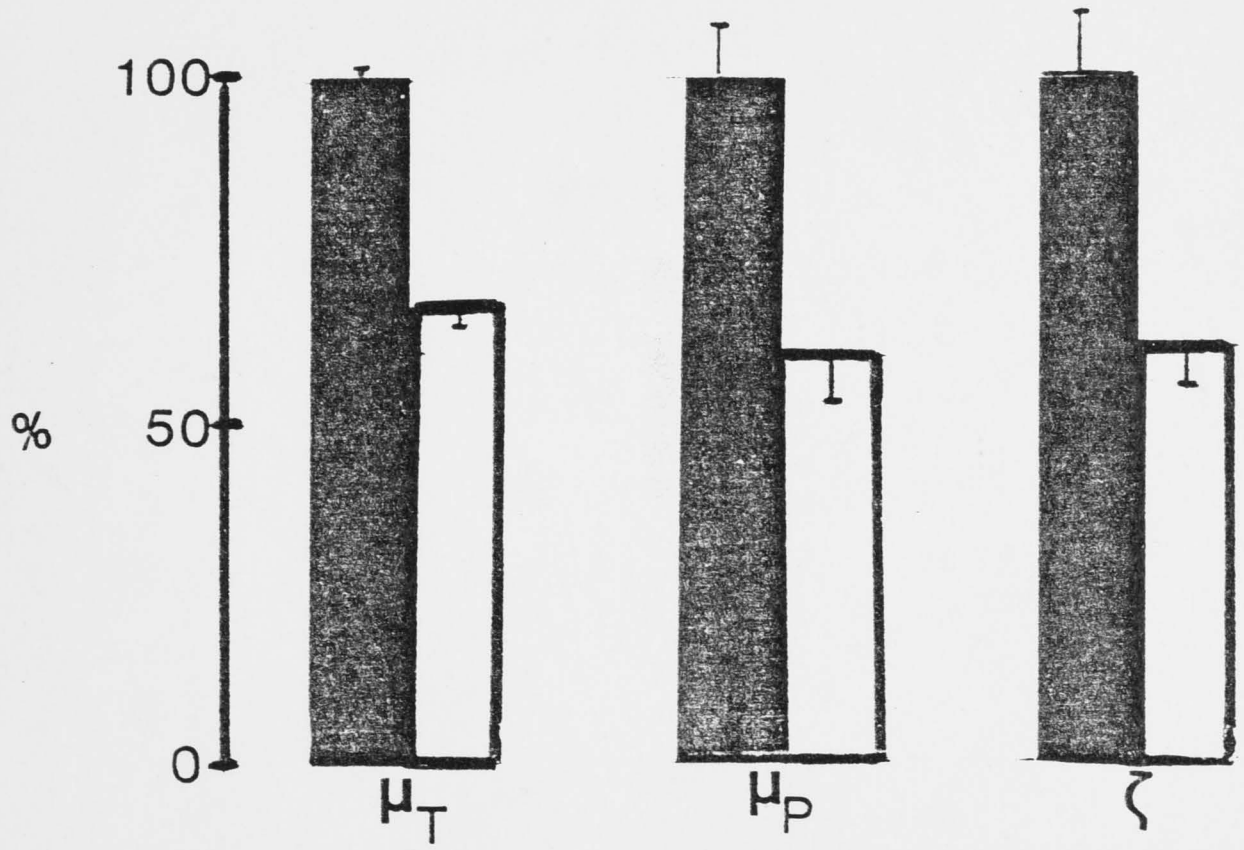

100
%
50
0
μ_T
μ_P
ζ

Figure 9.2 Turbidity At 800nm In Networks Developed In Pure Fibrinogen Solution With and Without 4mg/ml Albumin.

Fibrinogen concentration 2.5mg/ml.
Thrombin concentration 0.5u/ml.
pH 7.35, ionic strength 0.153.

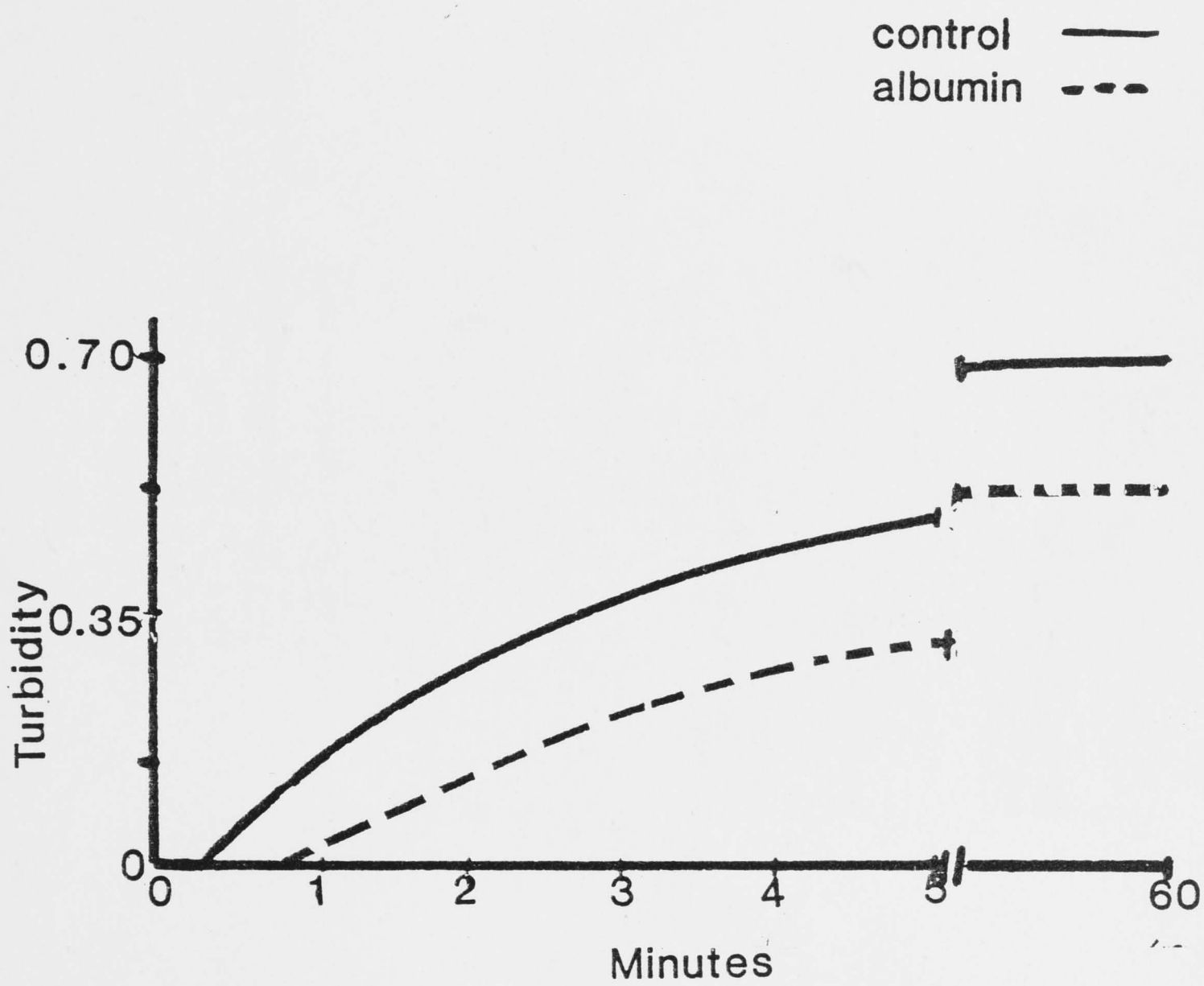

control
albumin
0.70
0.35
0
Turbidity
0
1
2
3
4
5
60
Minutes

Figure 9.3 Effect Of 0.6mg/ml γ-Globulin On Networks Developed In Fibrinogen Solution.

Fibrinogen concentration 2.5mg/ml.
Thrombin concentration 0.5u/ml.
pH 7.35, ionic strength 0.153.
Results are expressed as a mean of three experiments ± SEM and expressed as percentage of control.
Black columns show results in control experiments.

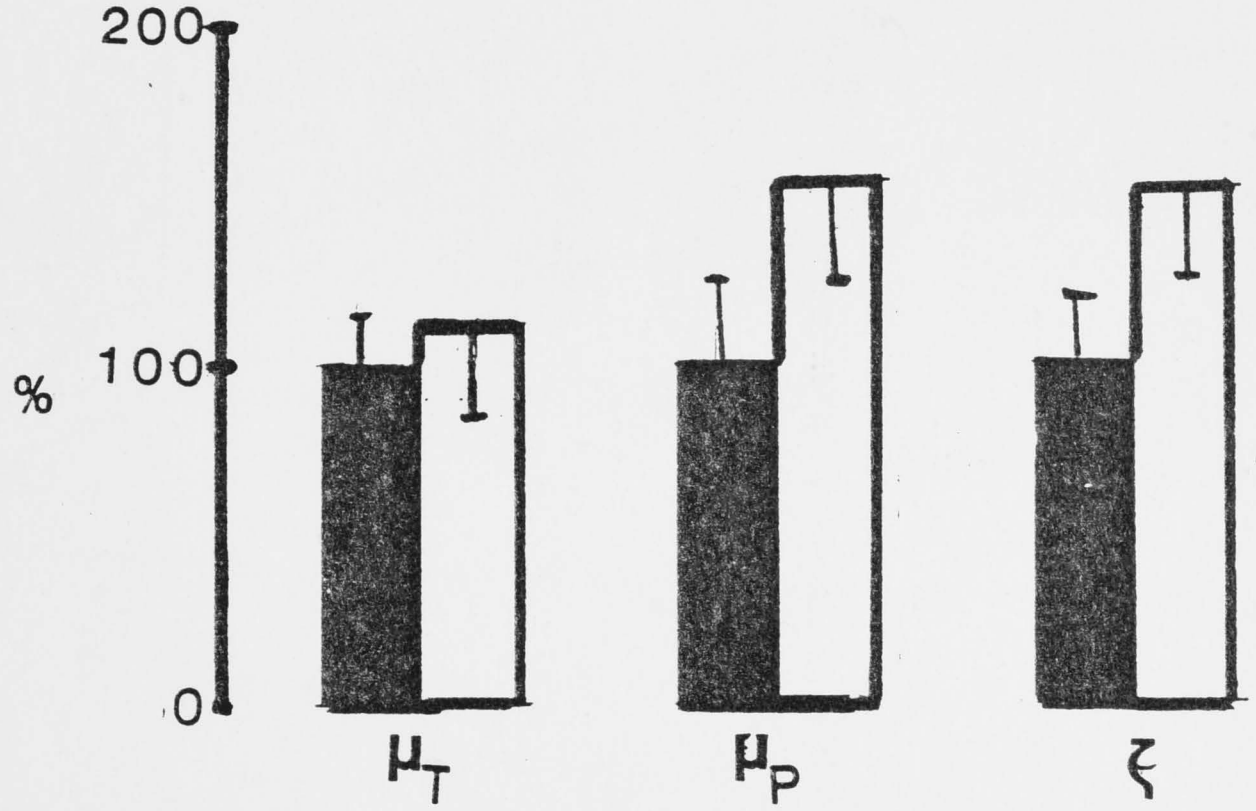

200
%
100
0
μ_T
μ_P
ζ

Figure 9.4 Turbidity At 800nm In Networks Developed In Fibrinogen Solution With and Without 0.6mg/ml γ-Globulin.

Conditions of clotting as stated in legend to Figure 9.3.

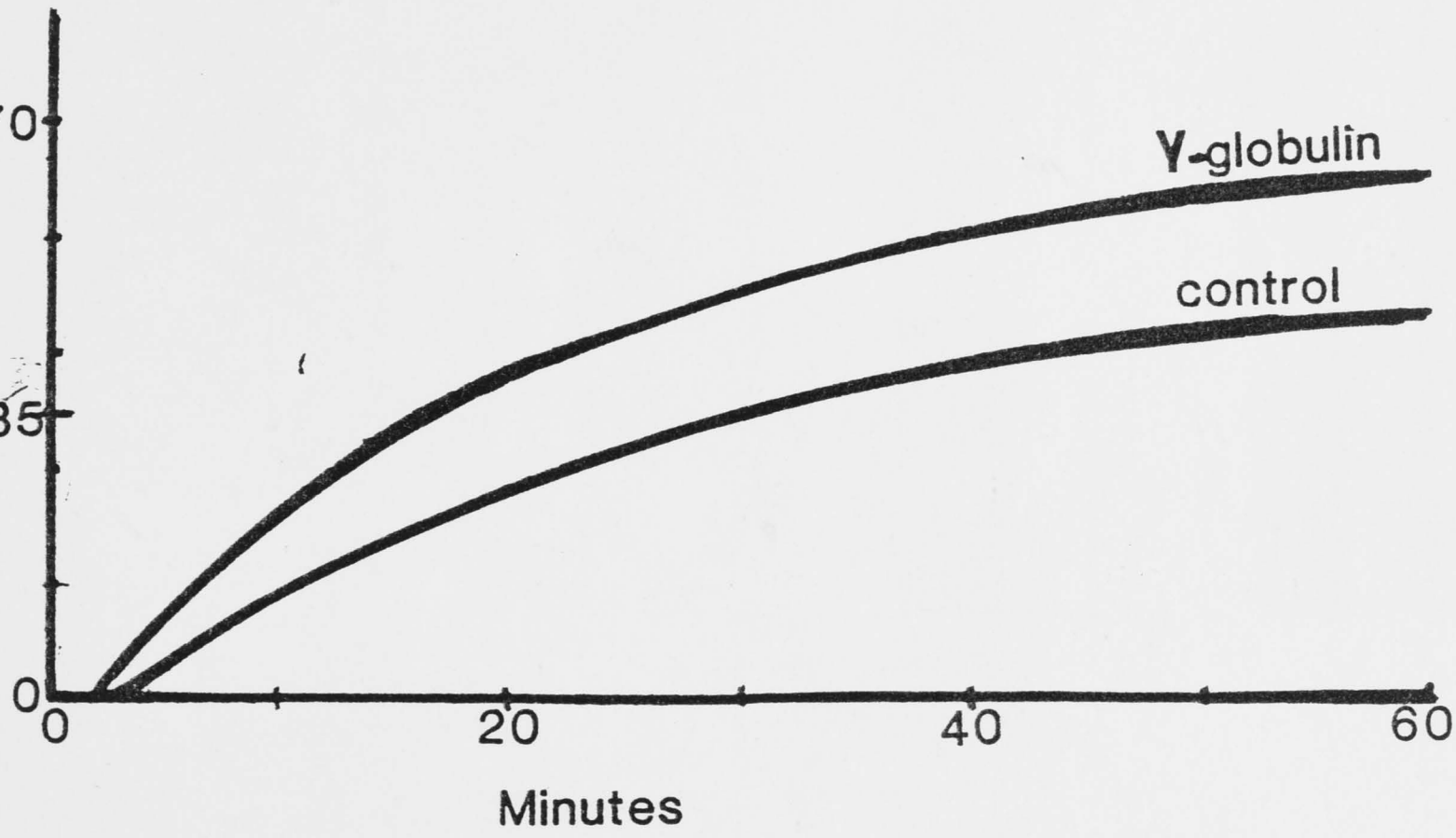

0.70
0.35
0
Turbidity
γ-globulin
control
0
20
40
60
Minutes

Figure 9.5 Effect Of 5u/ml Antithrombin On Networks Developed In Fibrinogen Solution.

Fibrinogen concentration 2.5mg/ml and thrombin concentration 0.5u/ml. pH 7.35, Ionic Strength 0.153. Results are mean of three experiments ± SEM and expressed as percentage of control.
Black columns represent results in control experiment.

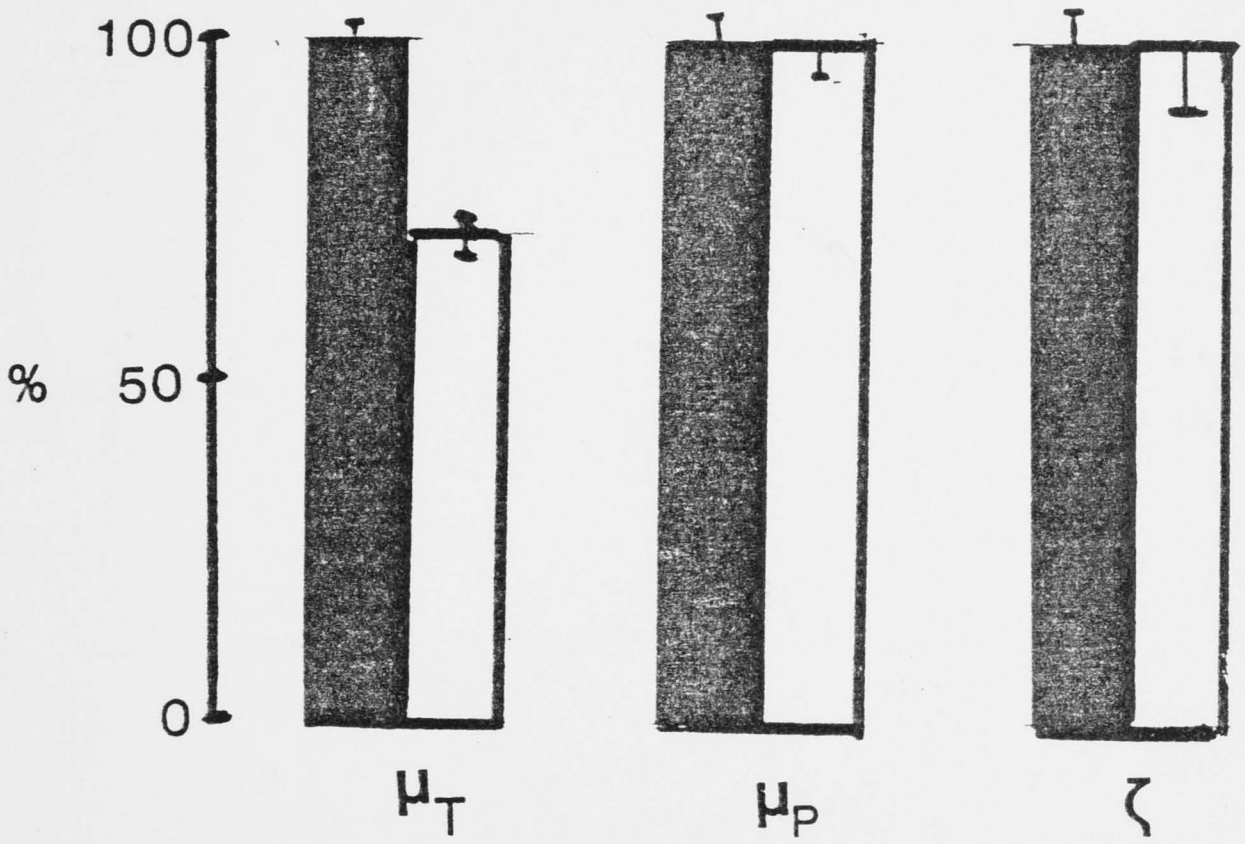

100
%
50
0
μ_T
μ_P
ζ

Figure 9.6 Effect Of ATIII On Turbidity During Network Formation Using Two Thrombin Concentrations.

Fibrinogen concentration 2.5mg/ml.
pH 7.35, ionic strength 0.153.
Thrombin concentrations 0.25 and 1.50u/ml.
ATIII concentration 5u/ml.

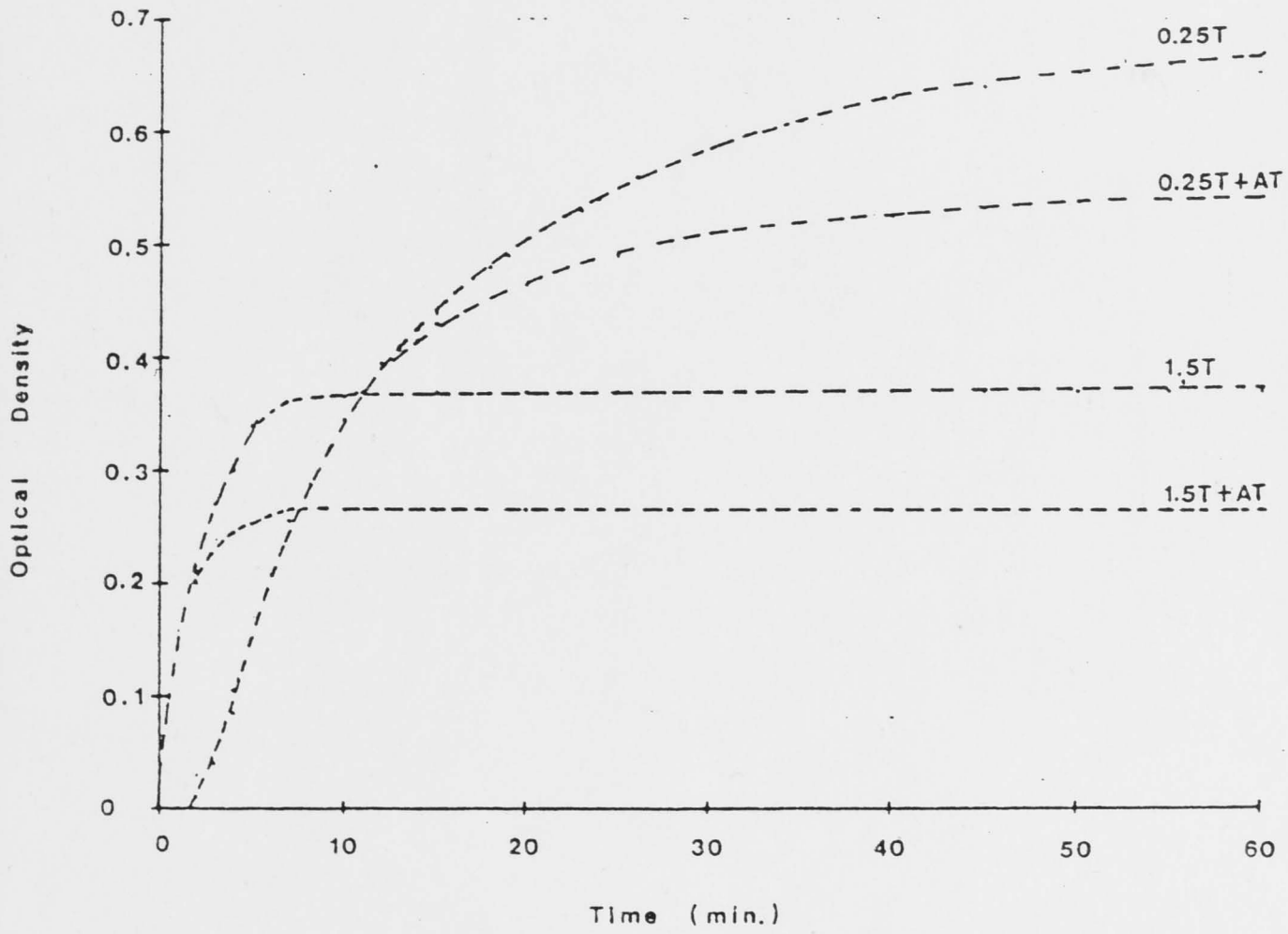

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Optical Density
0
10
20
30
40
50
60
Time (min.)
0.25T
0.25T+AT
1.5T
1.5T+AT

Figure 9.7 Effect Of 300mg/ml Fibronectin On μ_P, μ_T and τ In Networks Developed In Fibrinogen Solution.

Fibrinogen concentration 2.5mg/ml and thrombin concentration 0.5u/ml. Results are mean of three experiments ± SEM and expressed as percentage of control. Black columns represent results in control experiments.

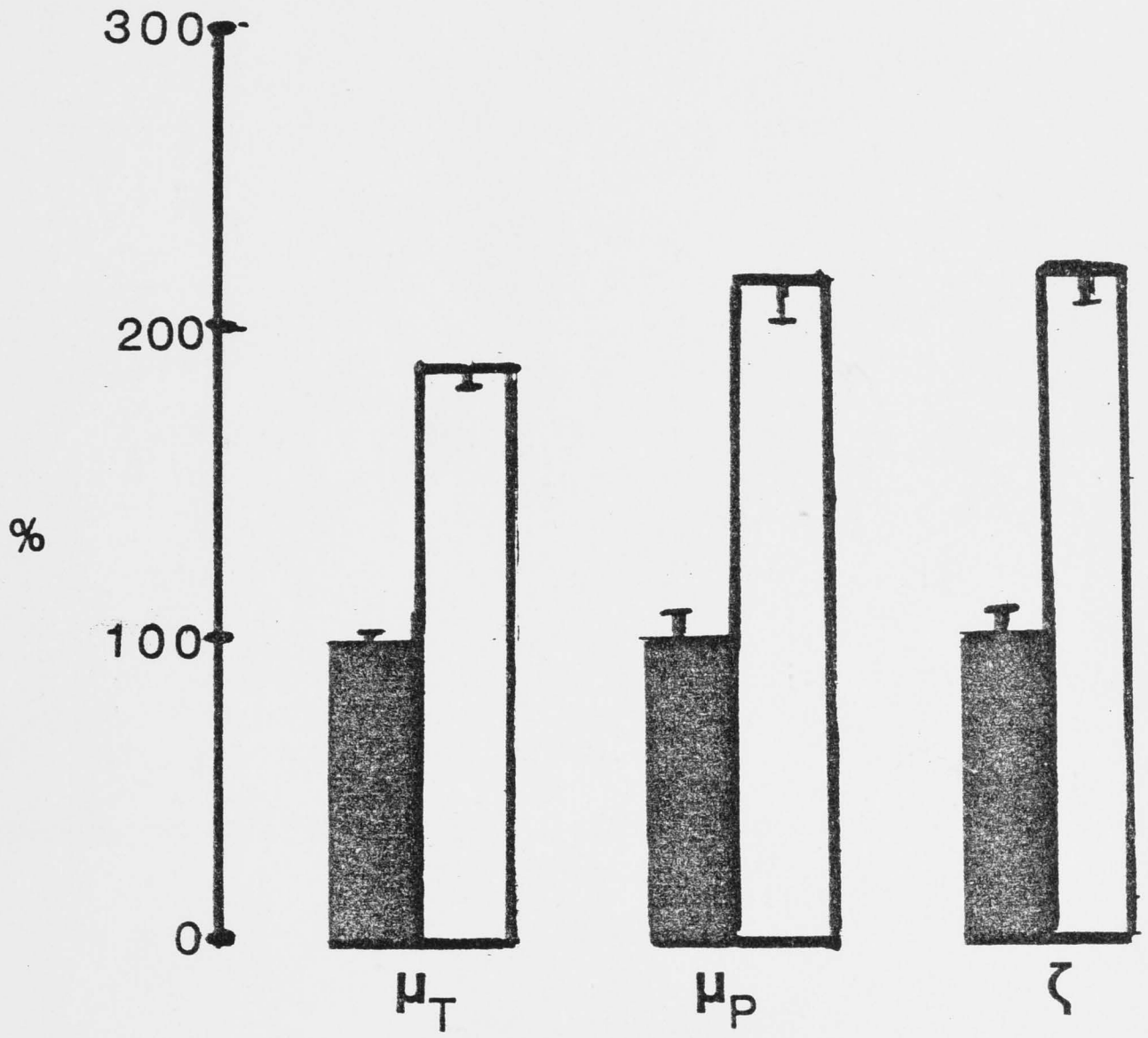

300
200
100
0
%
μ_T
μ_P
ζ

Figure 9.8 Turbidity At 800nm In Networks Developed In Fibrinogen Solution With and Without 300mg/ml Fibronectin.

Conditions of clotting as stated in legend to Figure 9.7.

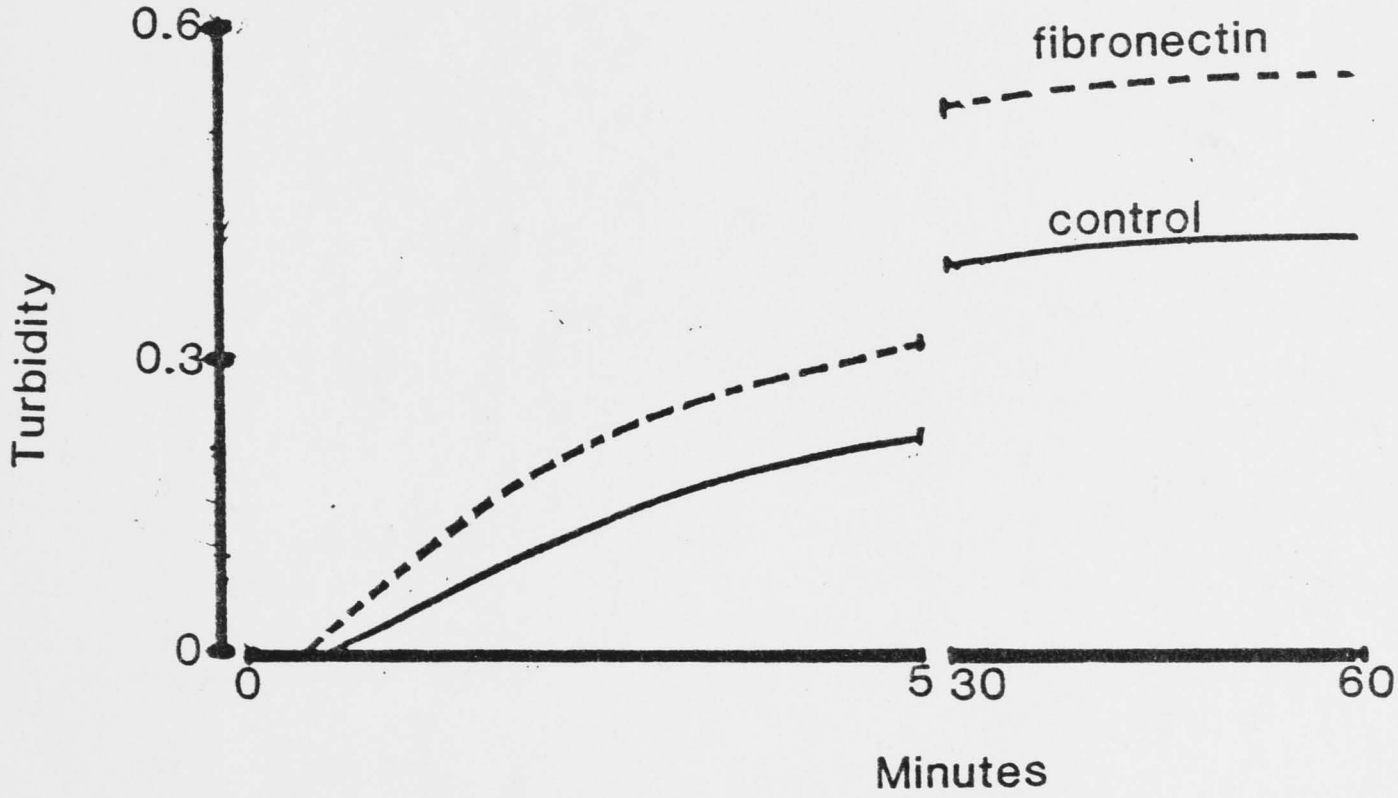
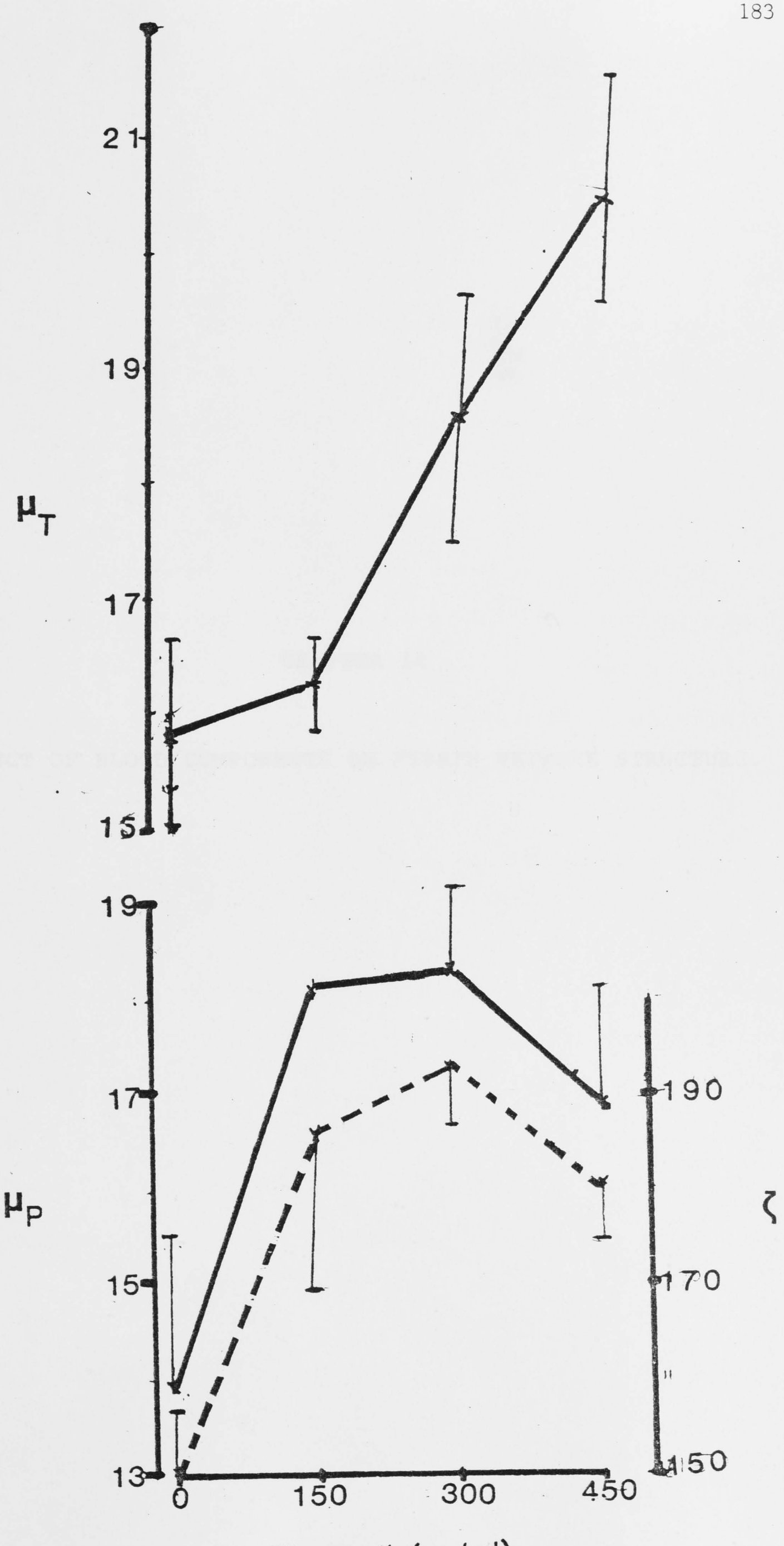

0.6
0.3
0
Turbidity
fibronectin
control
0
5
30
60
Minutes

Figure 9.9 Effect Of Concentration Of Fibronectin On μ_T In Networks Developed In Pure Fibrinogen Solution.

Fibrinogen concentration 2.5mg/ml and thrombin concentration 0.5u/ml. pH 7.35, Ionic Strength 0.153. Results are mean of three experiments ± SEM.

Figure 9.10 Effect Of Concentration Of Fibronectin On μ_P and τ In Networks Developed In Pure Fibrinogen Solution.

Conditions of clotting as stated in Figure 9.9.


21
19
17
15
μ_T
19
17
15
13
μ_P
190
170
150
ζ
0
150
300
450
Fibronectin (μg/ml)

CHAPTER 10

EFFECT OF BLOOD COMPONENTS ON FIBRIN NETWORK STRUCTURE.

10.1 INTRODUCTION.

Complex interaction occur between fibrin formation and the cellular elements of blood within a developing thrombus. A thrombus comprises masses of platelets, erythrocytes and leucocytes entrapped in a fibrin network. In the preceding chapters it was shown that final network structure is not only determined by pH, ionic strength and temperature, but also by plasma proteins, albumin, γ-globulins, fibronectin, and Antithrombin III. The role of cellular elements in determining properties of the networks remains unexamined and are investigated in this chapter.

10.2 MATERIALS AND METHODS.

General methods have been described in Chapter 2. When fibrinogen solution was used, fibrinogen concentration was 2.5mg/ml, pH was 7.35 and ionic strength was 0.153. Thrombin concentration was 0.5u/ml when networks were developed in fibrinogen solution, and 1.5u/ml when networks were developed in plasma.

10.2.1. Preparation of Platelet Extract.

Platelet concentrate prepared by the Australian Capital Territory (ACT) Blood Bank for transfusion was centrifuged at 2400g for 15 minutes to obtain a platelet button. The platelets were washed by resuspending them in a solution containing: 8.00mg/ml NaCl, 0.20mg/ml KCl, 1.00mg/ml $NaHCO_3$, 3.50mg/ml albumin, 1.00mg/ml glucose, pH 6.5 (Ardlie and Han, 1974). In addition, 1.0ml of 2% EDTA was added to each 10 ml of the first platelet suspension just prior to centrifugation. Low speed centrifugation (150g for 5 minutes) was used to sediment contaminating red cells. The platelet concentrate was then centrifuged to give a platelet button. After a second washing, the platelets were resuspended in 0.9% NaCl and washed twice in 0.9% NaCl. The first platelet button was resuspended in 2ml of 0.9% NaCl and platelet counts made using a Coulter S Plus Phase 4 (Higleah, Fl.,USA). This machine also gave an indication of white cell and red cell contamination, which was found to be minimal. The suspension was then frozen in an ethanol-dry ice mixture and thawed. Freezing and thawing was repeated five times. The homogenate was centrifuged at

20,000g for 30 minutes and the supernatant platelet extract separated.

10.2.2. Preparation of Platelet Extract before and after Aggregation of Platelets.

Effects of platelet extracts prepared from platelets before and after they had undergone aggregation were investigated as follows: Platelets obtained from platelet concentrate centrifuged at 100g for 15 minutes at 4°C were washed twice, as described above, and then suspended in a solution containing 8.00mg/ml NaCl, 0.20mg/ml KCl, 1.00mg/ml $NaHCO_3$, 0.20mg/ml $MgCl_2.6H_2O$, 0.44mg/ml $CaCl_2.6H_2O$, 3.50g/ml albumin, 1.00mg/ml glucose, pH 7.35 (Ardlie and Han, 1974). The suspension was divided into two aliquots and ADP (90.9µM final concentration) made up in Tyrode's solution was added to one aliquot. A magnetic stirrer was used to stir it slowly for ten minutes. The resulting platelet aggregates were separated, washed, suspended in saline, and platelet extract prepared by freeze-thawing. To the second aliquot an appropriate volume of Tyrodes' solution alone was added and the platelets stirred for ten minutes after which platelet extract was prepared from these unaggregated platelets as described above.

10.2.3 Effect of Platelet Extract on Networks Developed in Fibrinogen Solution and Plasma.

Platelet extract was added to fibrinogen solution or plasma in a final concentration equal to that of extract from 300,000 plts/ml. The mixture made in fibrinogen solution was clotted with 0.5u/ml thrombin and that in plasma with 1.5u/ml thrombin. μ_T, μ_P and τ and compaction (only in networks developed in plasma) were determined.

10.2.4 Preparation of Red Cell Ghosts.

Red cell concentrate from the A.C.T. Blood Bank was centrifuged at 1500g for ten minutes. The plasma supernatant was removed and the red cells suspended in 0.9% NaCl, and recentrifuged at 1500g for ten minutes. This procedure was repeated four times. The red cells were finally suspended in saline and the red cells quantified using a Coulter S-Plus Phase 4 (Hialeah, Fl., USA). This suspension had less than 3000 platelets/ml - there were however 4000 white cells ± 200 per ml. 1ml red cell concentrate was added to distilled water in a ratio of 1:10 and left for fifteen minutes. The solution was

then centrifuged at 27,000g for 30 minutes. This procedure was repeated five times, or until the red cell button was relatively clear of contaminating haemoglobin. The final button was resuspended in 2ml of saline.

10.2.5 Effect of Red Cell Ghosts on Networks in Fibrinogen Solution and Plasma.

The equivalent of 5000 red cells/ml (physiological concentration of red blood cells) were added to fibrinogen solution and to plasma, and clotted with 0.5u/ml and 1.5u/ml thrombin respectively. In each case, preliminary experiments had shown that red cell ghosts do not increase turbidity appreciably. Reference cuvettes had red cell ghosts in the same concentration. μ_T, μ_P, τ and compaction (latter only in plasma) were determined.

10.3 RESULTS.

10.3.1 Effect of Platelet Extract on Networks Developed in Fibrinogen Solution.

When platelet extract was added to fibrinogen solution, μ_T, μ_P and τ were significantly reduced. Fibrinogen conversion was similar (Figure 10.1).Networks, therefore, became less permeable and had thinner fibres.

10.3.2 Effect of Platelet Extract on Networks Developed in Plasma before and after Aggregation.

Extracts from unaggregated platelets, as well as previously aggregated, induced significant decrease in each of μ_T, μ_P, τ and compaction (Figures 10.2 and 10.3). Fibrinogen conversion remained unaffected. Networks developed with platelet extract from unaggregated platelets were not significantly different from those developed with platelet extract from aggregated platelets. Compaction was, however, higher in networks developed without extract from unaggregated or aggregated platelets (Figure 10.3).

10.3.3 Effect of Red Cell Ghosts on Networks Developed in Fibrinogen solution and Plasma.

A significant reduction in μ_T, μ_P and τ was observed when red cell ghosts were added to fibrinogen solution (Figure 10.4).

Fibrinogen conversion was similar when red cell ghosts were added to plasma. μ_T was significantly higher in networks developed in the presence of red cell , although

significant differences were not induced in μ_P, τ or compaction (Figure 10.6).

10.4 DISCUSSION.

Thrombi are composed of platelets, erythrocytes and leucocytes and consist of a white head composed of platelets and a red tail composed of fibrin and red blood cells(Poole, 1979). The prominence of red blood cells and platelets begs the question whether these cells or their contents alter fibrin network structure. The studies undertaken in this chapter show clearly that networks developed in fibrinogen and plasma are affected by platelets as well as red cells.

10.4.1 Platelets.

Blood platelets play a varied and complex role in haemostasis and coagulation. Primary haemostasis involves interaction of platelets with blood vessels to form a haemostatic plug (Sixma, 1981). During these processes, platelets undergo changes in their morphology. At a thrombin concentration which results in the conversion of fibrinogen to fibrin, platelets undergo not only aggregation and release reaction, but are completely disrupted (Wester *et al*, 1979) shedding their contents

into supernatant. Using opacity ratio, syneresis and permeation techniques Dhall *et al* (1983) showed that network structure is altered by soluble platelet components. Studies in this chapter confirm their observations. However, in the present study μ_P and μ_T were used to investigate network structure. Advantages of these techniques over the previously used opacity ratio and μ_T from single wavelength of incident light have already been elaborated in preceding chapters.

Dhall *et al* (1983) also showed that extract prepared from platelets which had been previously aggregated with ADP (but not adrenaline) were less effective in inducing network alterations when compared with extract from unaggregated platelets. They concluded that aggregation of platelets in plasma, followed by network formation in such plasma, induced subtle alterations in the fibre diameter of fibrin. Studies described in this chapter show that platelet extract from both aggregated and unaggregated platelets reduced fibre diameter and network permeability (Figure 10.1). However, significant differences could not be demonstrated between the alterations induced by the two extracts.

Networks which are less porous may be less susceptible to lysis on account of poor penetration by fibrinolytic enzymes into the clot interior. The lack of porosity in such clots may be physiologically advantageous, as the major role of fibrin is to form a clot to prevent blood loss.

10.4.2 Red Cell Ghosts.

In networks developed in fibrinogen solution (Figure 10.4) μ_T , μ_P and τ are significantly reduced by red cell ghosts. However, in networks formed in plasma, although μ_T is slightly decreased, significant differences were not observed in μ_P and τ. Conversion of fibrinogen remained unaltered. Furthermore, compaction in networks made with and without red cell ghosts did not change. This intriguing observation highlights the caution that observations made using purified fibrinogen may not necessarily have meaningful application to plasma.

Thick fibrin fibres have been found, associated with platelets in thrombi (Gottlieb, 1975; Hattori *et al*, 1978; and Hisano, 1978), and in haemostatic plugs (Sixma and Wester, 1977). Ferguson and Sander (1979) have shown that red cell ghosts accelerate fibrin cross-

linking in plasma, but not when prepared by hypotonic lysis. Thus, although red cells may cause the formation of thicker fibres, because hypotonic lysis was used to prepare them in this study, fibrin cross-link accelerating activity may have been inactivated. This area requires further study using different methods of preparation of red cell ghosts.

10.5 CONCLUSION.

Every component of plasma and blood tested has shown some effect. It is clear that participation and interaction of substances in the formation of fibrin and fibrin network structure is complex and at several levels. It would, therefore, not be unreasonable to postulate that several factors in combination ultimately orchestrate the final network structure in plasma, possibly reflecting physiological and varying demands placed on the networks.

Figure 10.1 Effect Of Platelet Extract On Networks Developed In Fibrinogen Solution.

Thrombin concentration was 0.5u/ml, pH 7.35 and ionic strength 0.153. Fibrinogen concentration was 2.5mg/ml.
Results are mean of three experiments ± SEM.
Black columns represent results in control experiments.

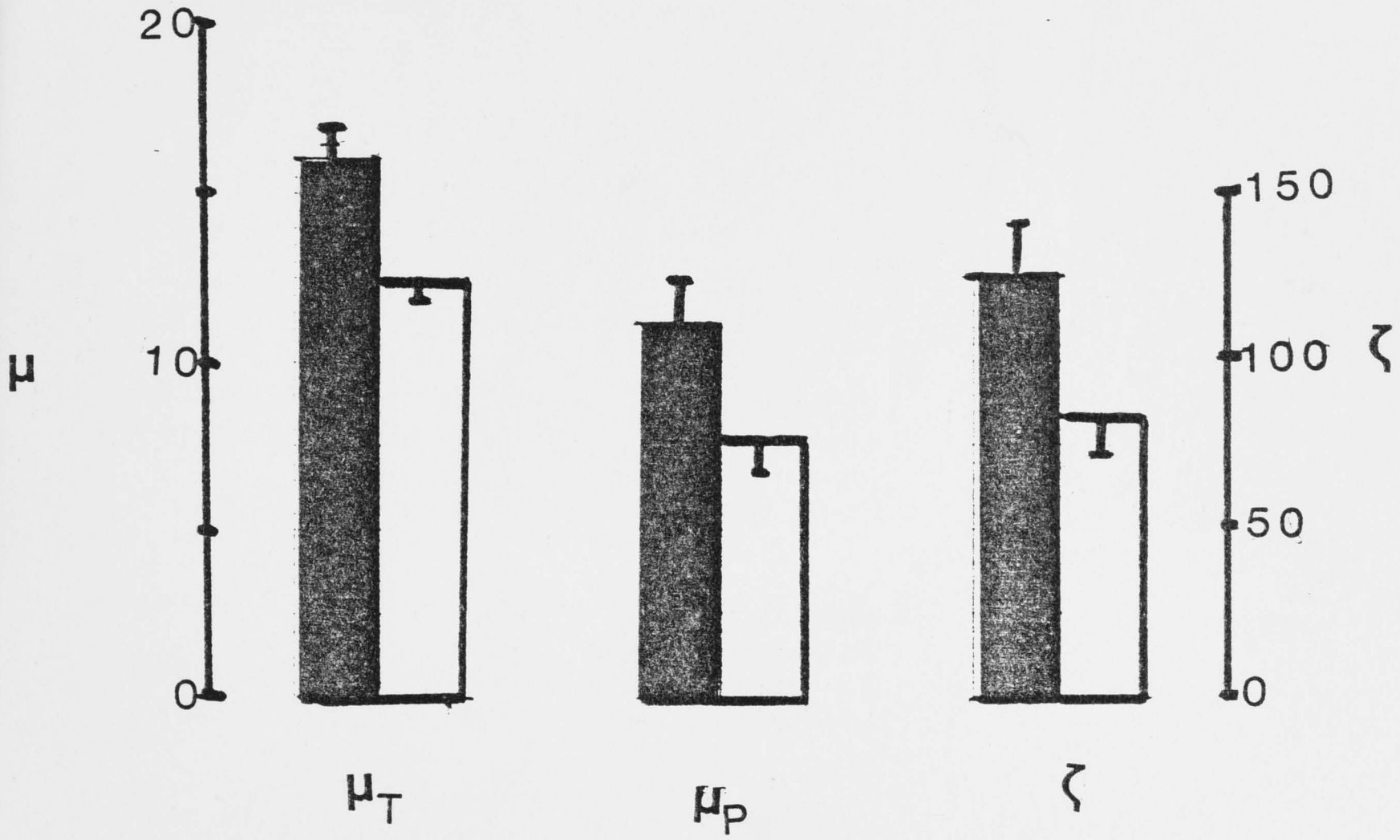
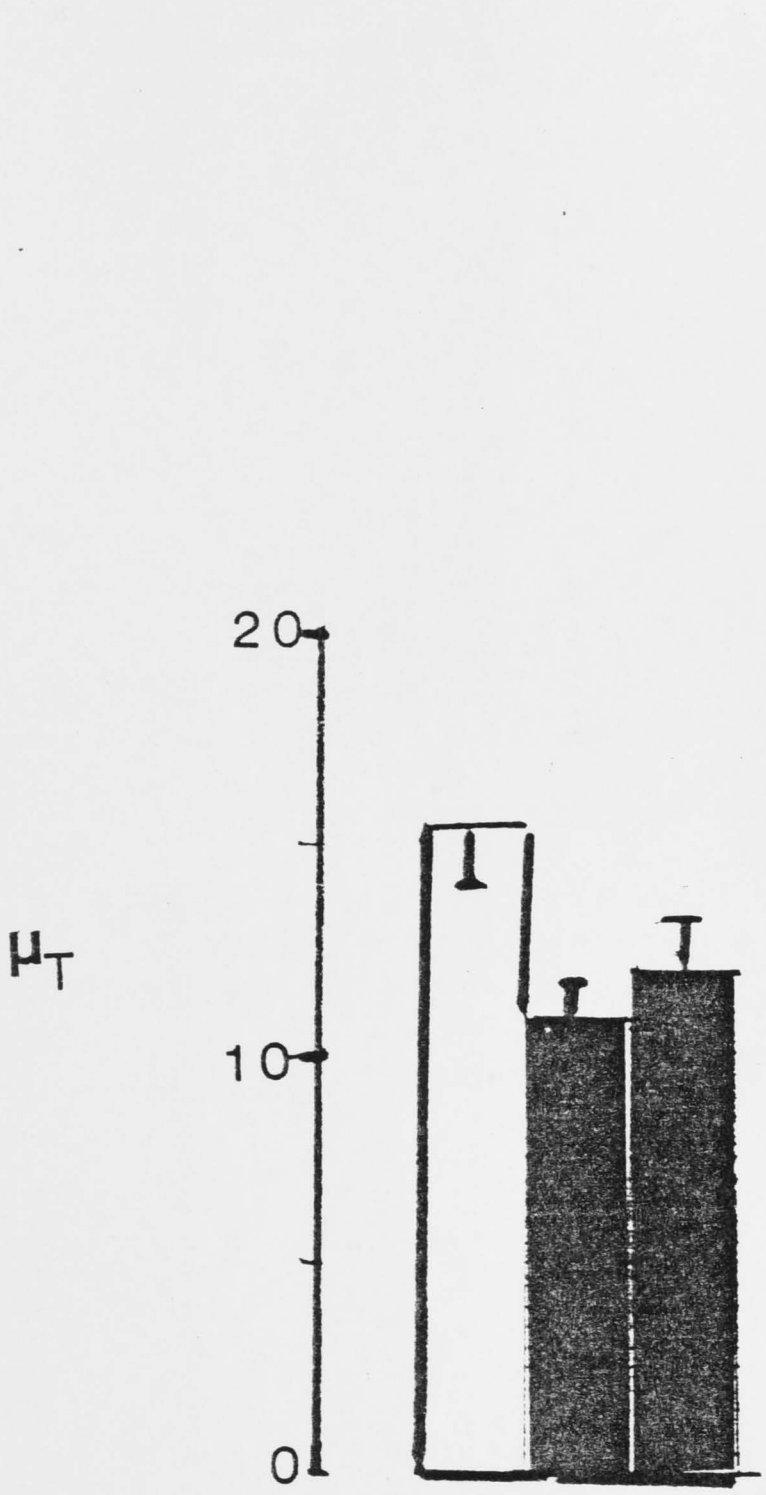

P< 0.001
P < 0.001
P < 0.001
20
μ
10
0
150
100 ζ
50
0
μ_T
μ_P
ζ

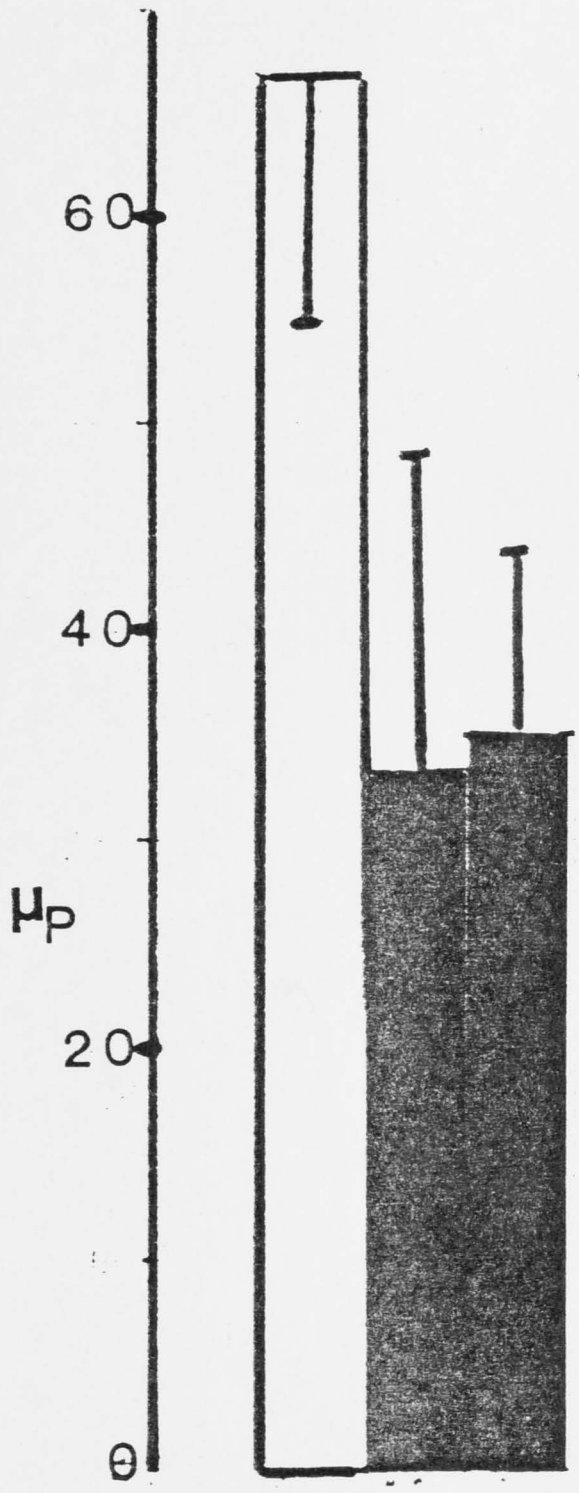
Figure 10.2 Effect Of Platelet Extract On μ_T and μ_P Of Networks Developed In Platelet Poor Plasma.

Conditions of clotting were stated in Figure 10.1.
Results are mean of three experiment ± SEM.

Figure 10.3 Effect Of Platelet Extract Prepared From Aggregated and Unaggregated Platelets On Compaction In Networks Developed In Plasma.

Conditions of clotting were stated in Figure 10.1.
Results are mean of three experiments ± SEM.


μ_T
20
10
0
Control Unagg Agg


μ_P
60
40
20
0
Control Unagg Agg

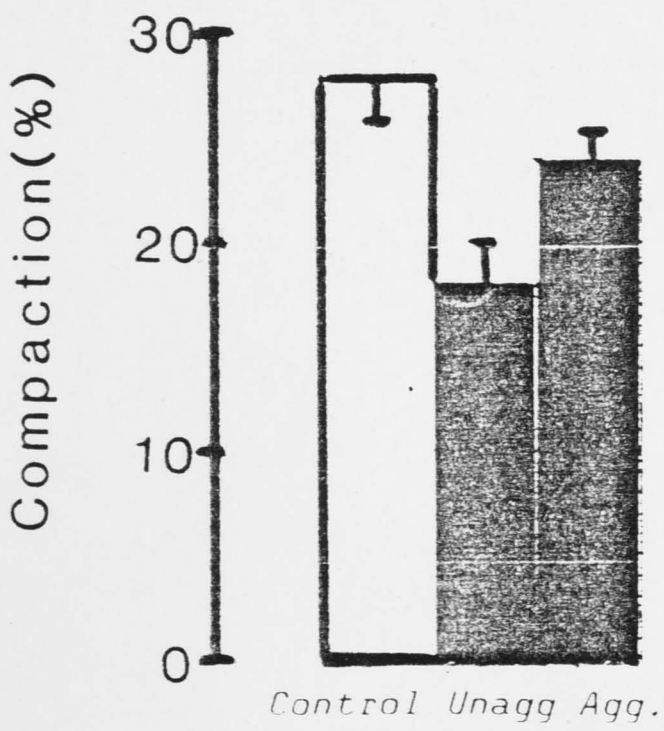

Compaction(%)
30
20
10
0
Control Unagg Agg.

Figure 10.4 Effect Of Red Cell Ghosts On μ_T, μ_P and τ Of Networks Developed In Fibrinogen Solution.

Red cell ghosts equivalent to 5000rbc/ml.
Thrombin concentration was 0.5u/ml, pH 7.35, ionic strength 0.153.
Results are mean of three experiments ± SEM.
Black columns represent results in control experiments.

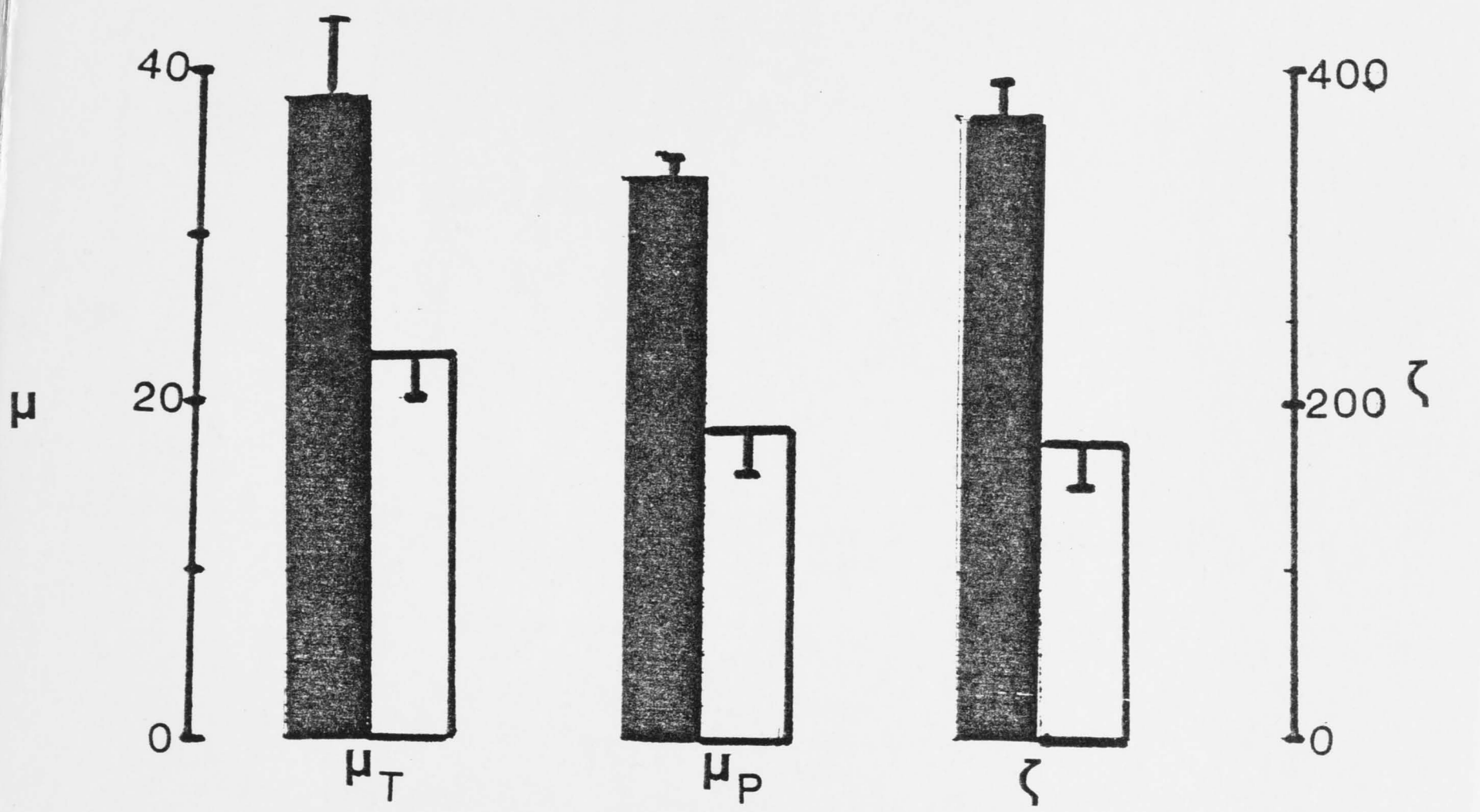
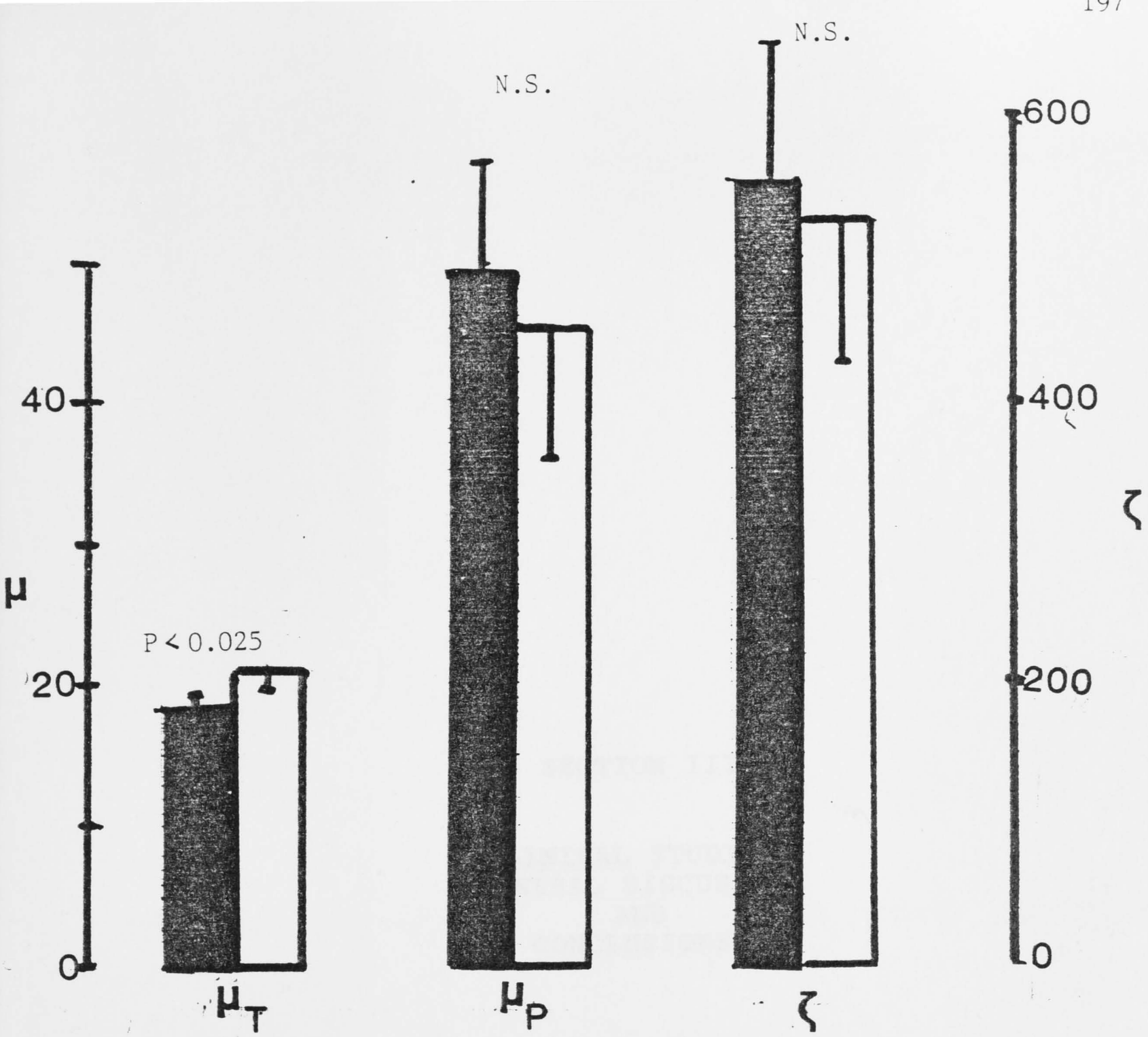

P < 0.001
P < 0.001
P < 0.001
40
20
0
μ
400
200
0
ζ
μ_T
μ_P
ζ

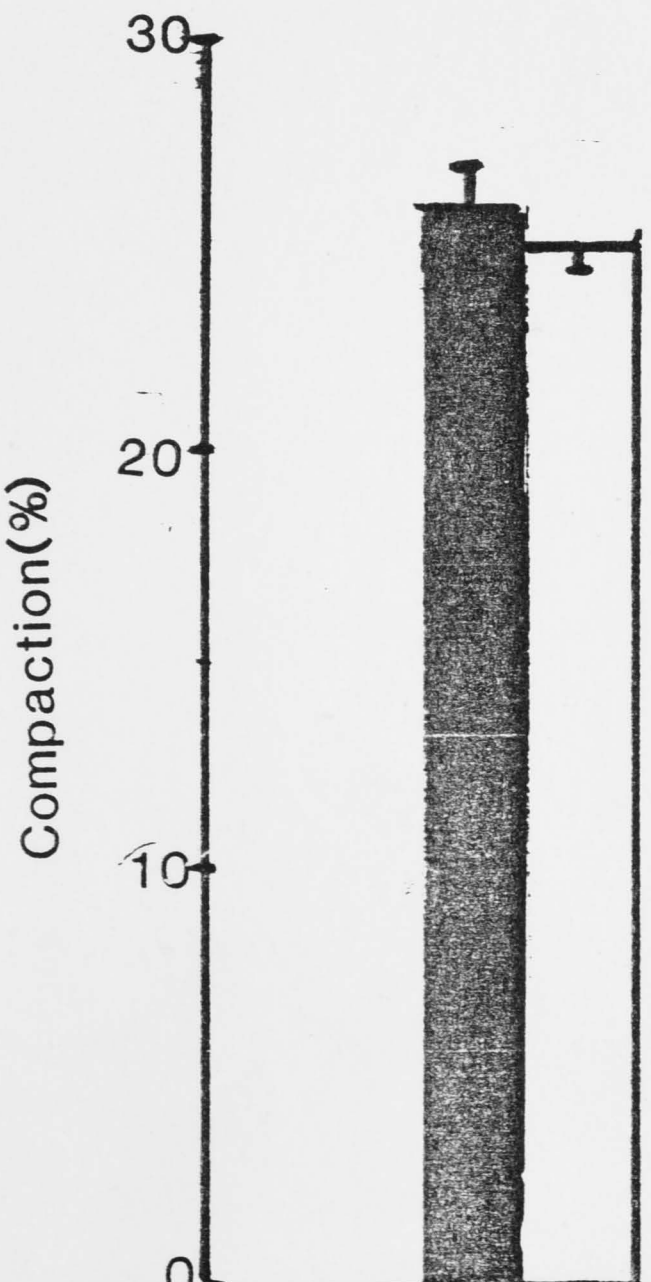
Figure 10.5 Effect Of Red Cell Ghosts On μ_T, μ_P and τ On Networks Developed In Plasma.

Red cell ghosts equivalent of 5000rbc/ml.
Thrombin concentration was 1.5u/ml.
Results are mean of three experiments ± SEM.
Black columns represent results in control experiments.
NS = not significant.

Figure 10.6 Effect Of Red Cells Ghosts On Compaction Networks Developed in Plasma.

Red cell ghosts equivalent of 5000rbc/ml.
Thrombin concentration was 1.5u/ml.
Results are mean of three experiments ± SEM.
Black column represents results in control experiments.
NS = not significant


N.S.
N.S.
600
400
200
0
ζ
40
20
0
μ
P < 0.025
μT
μP
ζ


30
20
10
0
Compaction(%)

SECTION III

CLINICAL STUDIES, GENERAL DISCUSSION AND CONCLUSIONS.

CHAPTER 11

EFFECT OF SURGERY AND DEXTRAN ON FIBRIN NETWORK STRUCTURE

11.1 INTRODUCTION.

From the foregoing chapters it is clear that mean fibrin fibre thickness can be measured reproducibly in plasma. In this chapter the techniques have been applied in a sequential study to assess applicability of the methods to clinical situations. Effects of infusion of dextran and postoperative changes in networks have been investigated.

Muzaffar *et al* (1972) showed that dextran alters the tensile behaviour of fibrin by modifying fibrin polymerization and networks characteristics. However, an examination of effects of dextran on fibrin network structure using the techniques described in this thesis have not been hitherto undertaken.

Post-operative period is characterized by a number of fairly consistent changes in the haemostatic mechanisms. Whether surgical trauma induces alterations in network structure was not known.

11.2 MATERIALS AND METHODS.

General methods have been fully described in Chapter 2.

11.2.1 The Effect of Dextran on Fibrin Network Structure: *in vitro* Study.

Networks were developed in citrated platelet-poor plasma in the presence and absence of dextran fractions, with a mean molecular weight (Mw) of 40,000 and 70,000. μ_T, τ and μ_P were determined. Dextran concentration was varied between 0.225% and 1.2%

11.2.2 The Effect of Surgery and of Dextran Infusion on Fibrin Network Structure.

Informed consent was obtained from 14 patients aged between 25 and 50 years undergoing major abdominal surgery. Six were infused with dextran 70, in a dosage of 3.5ml/kg over a four-hour period commencing during the operation. Eight patients were not given dextran. Blood was collected in both groups before the operation and then on days 1 and 3 after the operation. In the dextran group blood was also obtained four hours after the end of the infusion. In both the groups care was taken to exclude patients on Heparin therapy.

11.3 RESULTS.

11.3.1 The Effect of Dextran Concentration on Fibrin Network Structure: *in vitro* Study.

Figure 11.1 shows the effect of dextran on fibrin network structure developed in citrated, platelet-free plasma (PFP). Both dextran 40 and dextran 70 significantly increased permeability (τ), μ_P and μ_T. These effects were concentration-dependent but were independent of the molecular weight of the fraction.

11.3.2 The Effect of Surgery and of Dextran Infusion on Fibrin Network Structure.

Figure 11.2 shows the effect of surgery and dextran 70 infusion on fibrin network structure. μ_T increased after surgery and at day 3 was significantly higher than preoperative values. After the third day, μ_T began reverting to normal. In patients given dextran, however, μ_T was significantly higher than preoperative values four hours after infusion, and on days 1 and 3. μ_T in patients given dextran was higher for up to up to 24 hours than

in patients not given dextran. After this time values began returning towards normal.

Figure 11.3 shows the effect of surgery and dextran 70 on permeability. Permeability (τ) of the networks did not change after surgery. When dextran was given, however, permeability increased dramatically four hours after infusion and began returning to control values thereafter.

Fibre thickness derived from permeability (μ_P) increased in postoperative patients not given dextran (Figure 11.4). In the group of patients given dextran, μ_P was further increased significantly at four hours and on day 1. However, after four hours μ_P values began returning towards control.

Network fibrin content (Cn) (Figure 11.5) increased in patients after surgery in both groups. However, after dextran infusion Cn is lower at four hours than at preoperative measurements. This was probably from dextran-induced haemodilution. In both groups fibrinogen concentration increased after operation (Figure 11.6) although, as already stated, in patients given dextran plasma fibrinogen concentration was lower at four hours than preoperative values.

11.4 DISCUSSION

The discussion is divided into two sections. The first deals with the effect of surgery on fibrin network structure, and the second with the pharmacological effects of dextran in vitro and in vivo.

11.4.1 Effect of Surgery on Network Structure.

The effect of surgery on coagulation has been the subject of many studies (Warren *et al*,1950; Crane, 1952; Sharnoff *et al*, 1960; Egeberg, 1962; Innes and Sevitt, 1964; Ardlie *et al*, 1967; Ygge, 1970). Generally, these studies have shown that immediately after operation platelet count decreases, fibrinogen concentration and Factor VIII activity increases. There is also a decrease in both plasminogen and spontaneous fibrinolytic activity.

The present study has further added to these observations by demonstrating that network fibres become thicker twenty four hours after operation (Figures 11.2 and 11.4). Networks, however, do not show a change in permeability (Figure 11.5) despite a marked increase in

plasma fibrinogen concentration and network fibrin content (Figures 11.5 and 11.6).

Changes in fibrin network structure could have biological implications in the post-operative period when the physiological system would be primed for repair mechanisms to be the most effective. Any system which helps the repair processes would therefore be advantageous. Thicker and more numerous fibres would tend to support more easily the growth of repair tissue. Walter and Israel (1974) have suggested that fibrin assists by forming a union between severed tissues, thus limiting the exudative process and providing a haemostatic barrier. This mesh of fibrin may also act as a barrier to bacterial invasion.

11.4.2 Effect of Dextran on Fibrin Network Structure.

Fibrin fibres are rendered thicker and the network becomes more permeable when made in the presence of dextran. This effect is dependent on dextran concentration but is independent of the molecular weight. The increase in μ_T and reduction in μ_P indicate that network fibres are rendered thicker and the numerical density of minor network fibres is reduced.

It is interesting to note that the direction of changes induced by dextran when infused perioperatively is similar to those induced by operation, although the effect in magnitude is spectacular. But in addition dextran also enhances the permeability of the network.

Muzaffar *et al* (1972) and Dhall *et al* (1976),while investigating changes in fibrin network structure induced by dextran, associated these effects with altered biophysical properties of fibrin clots. The use of more sophisticated techniques in this present study allows further comment. Because conversion of fibrinogen to fibrin is not altered despite fairly significant changes in network characteristics, it is suggested that both operation and dextran cause a redistribution of fibrin within the network. The diameter of the major fibres is increased at the expense of numerical density of fibres in the minor network. Thus, the net result is a network which is more permeable and is composed of very thick fibres.

The mechanism underlying the influence of dextran on fibrin network structure is not fully understood, but is likely to arise from dextran enhancing the attractive

forces operating on the fibrin monomer such that their side to side alignment is promoted resulting in thicker chain bundles (Carlin *et al*, 1976; Dhall *et al*, 1976). It is likely that such a network is more amenable to fibrinolytic attack due to its increased porosity. The decreased tensile strength of the network also suggests that the clot is less able to withstand vascular shearing stress.

11.5 CONCLUSIONS

1. Fibrin fibres are rendered thicker and the network becomes more permeable when made in the presence of dextran. This effect is Mw independent.

2. Fibrin fibres in networks became thicker after operation.

3. Fibrin fibres are rendered thicker by dextran infused perioperatively.

4. Permeability of network does not change after operation but increases significantly when dextran is infused perioperatively.

Figure 11.1 Effect Of Increasing Concentrations Of Dextran 40 (dotted lines) and Dextran 70 (solid lines) On Fibrin Network Structure Developed In Citrated Platelet Poor Plasma.

Thrombin concentration 0.5u/ml. Results are expressed as percentage of control.

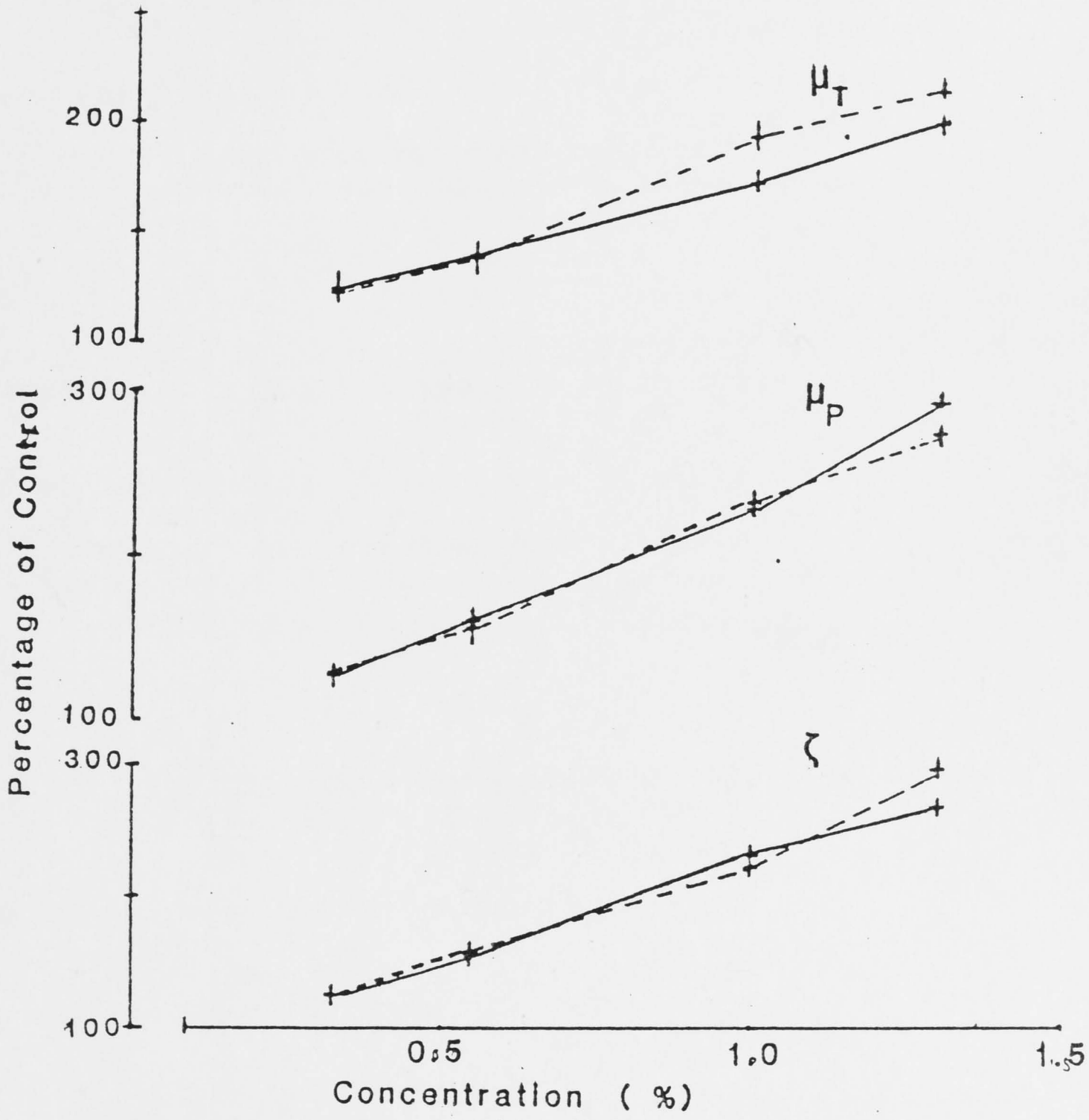

200
100
μ_T
300
100
μ_P
300
100
ζ
Percentage of Control
0.5
1.0
1.5
Concentration (%)

Figure 11.2 Effect Of Surgery Alone (■) and Effect Of Surgery and Infusion Of Dextran (X) On μ_T.

(Surgery alone n = 8, surgery and dextran n = 6).
Thrombin concentration was 1.5u/ml.
Results are expressed as percentage of control.

Figure 11.3 Effect Of Surgery Alone (■) and Effect Of Surgery and Infusion of Dextran (X) On μ_T.

(Surgery alone n = 8, surgery and dextran n = 6).
Thrombin concentration was 1.5u/ml.
Results are expressed as a percentage of control.

Figure 11.4 Effect Of Surgery Alone (■)and Effect of Surgery and Infusion Of Dextran (X) On τ.

(Surgery alone n = 6, surgery and dextran n = 6).
Thrombin concentration was 1.5u/ml.
Results are expressed as a percentage of control.

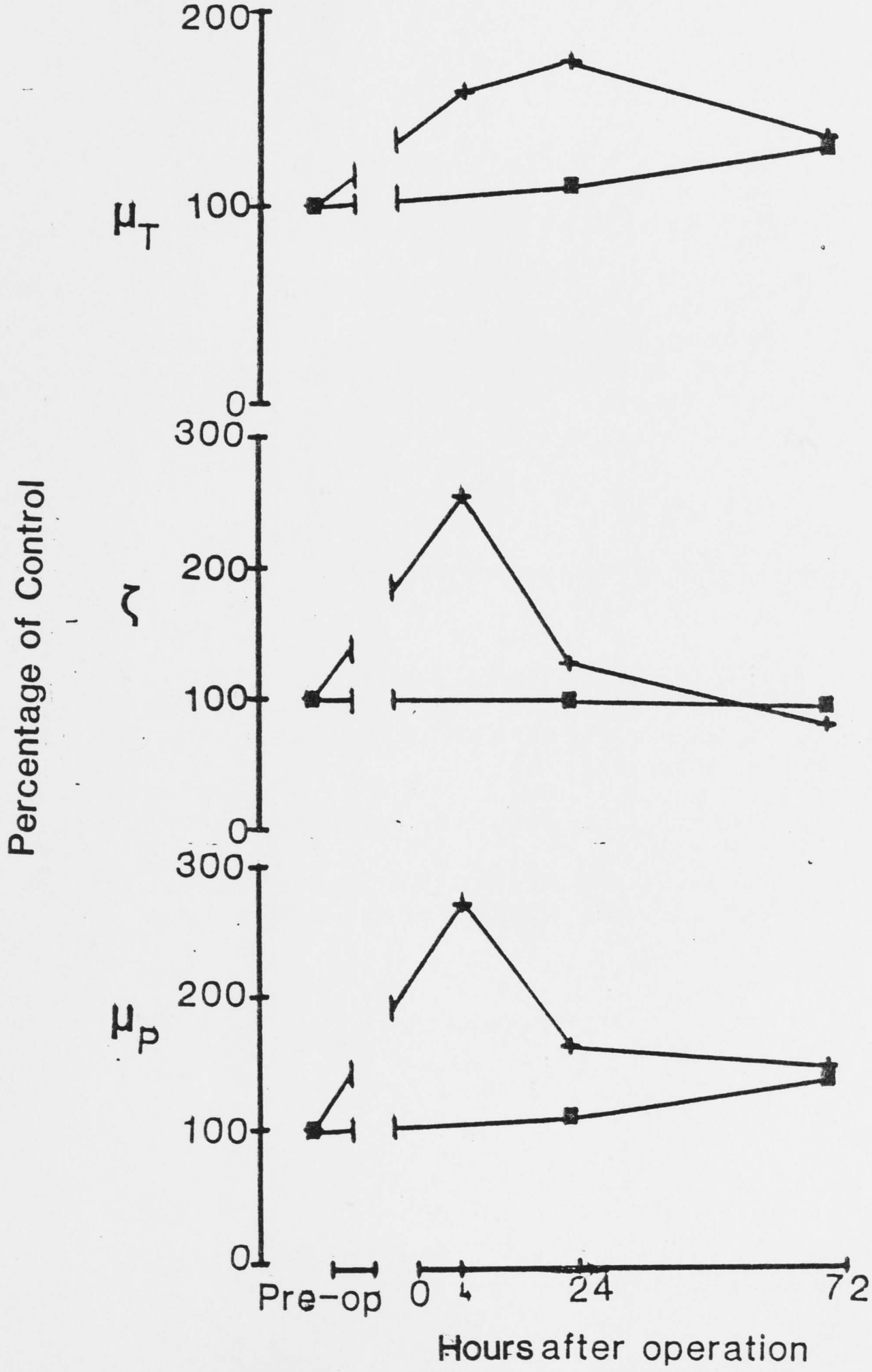
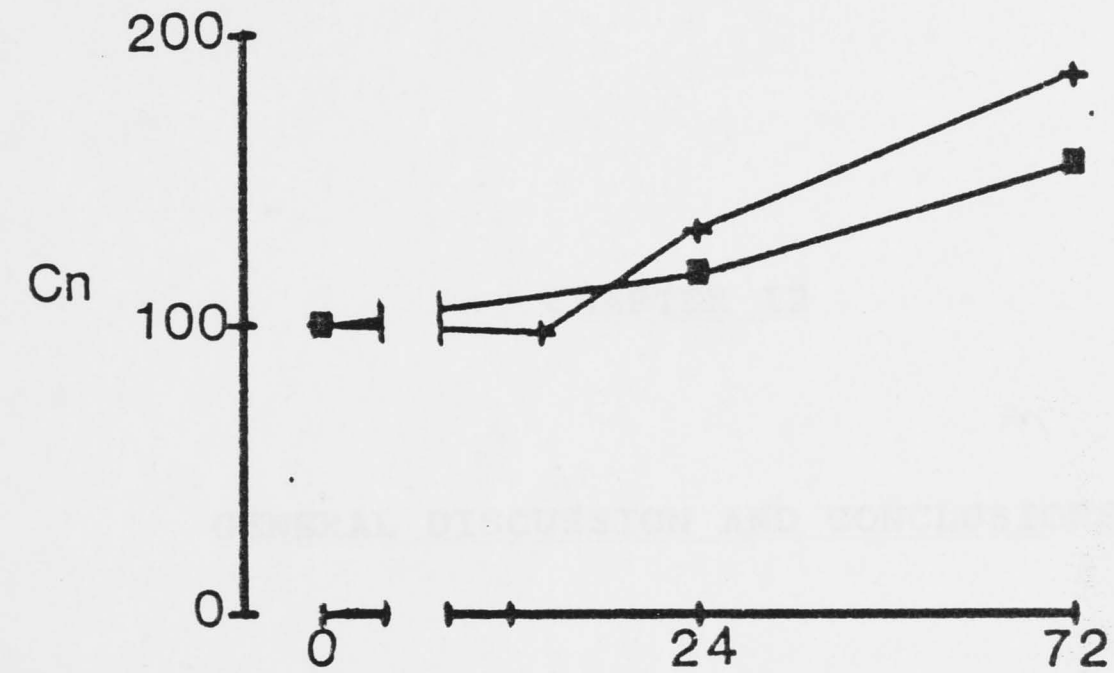

200
100
0
μ_T
300
200
100
0
ζ
300
200
100
0
μ_p
Pre-op
0
4
24
72
Hours after operation
Percentage of Control

Figure 11.5 Effect Of Surgery Alone (■) and Effect Of Surgery and Infusion Of Dextran On Network Fibrin Content (Cn).

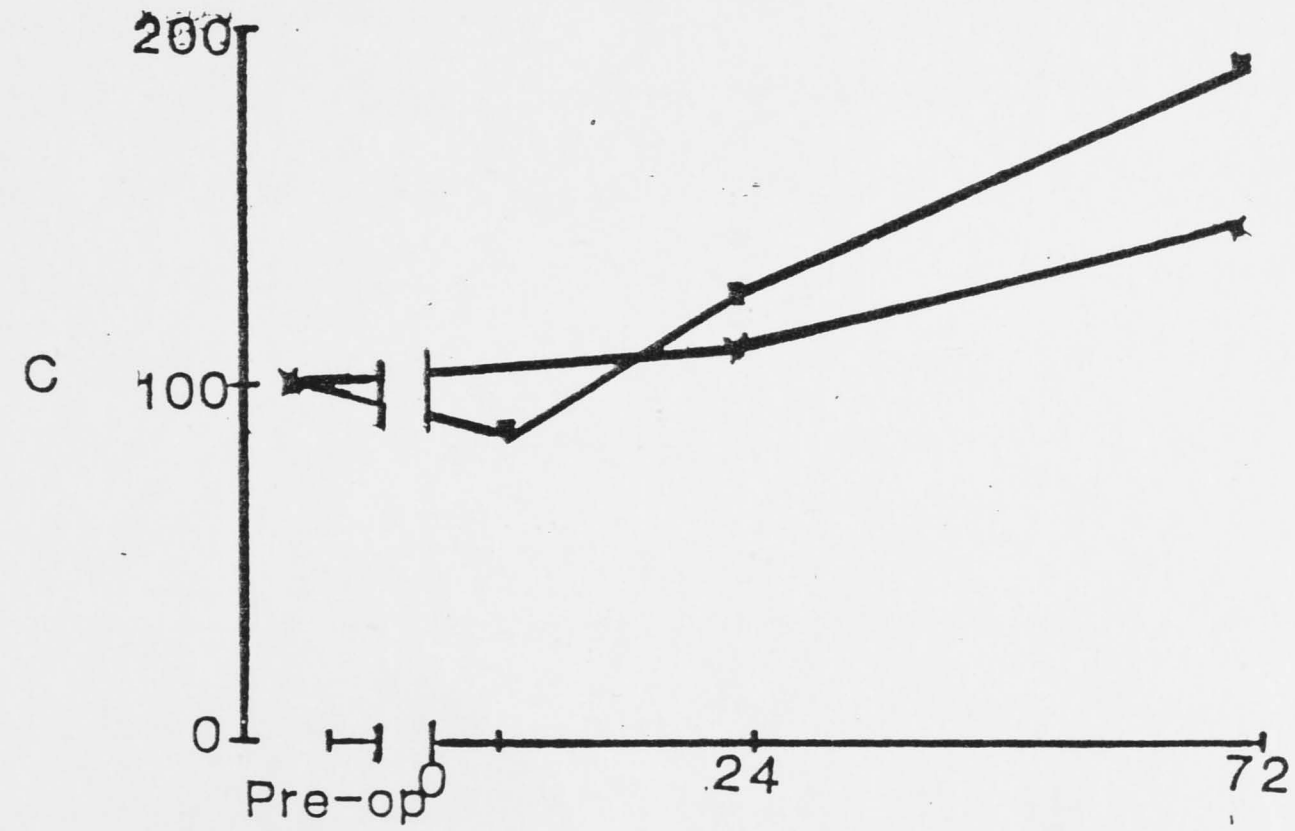
(Surgical alone n = 8, surgery and dextran n = 6).
Thrombin concentration was 1.5u/ml.
Results are expressed as percentage of control.

Figure 11.6 Effect Of Surgery Alone (■) and Effect Of Surgery and Dextran Infusion (X) On Concentration Of Fibrinogen In Plasma (C).

(Surgery alone = 8, surgery and dextran n = 6).
Thrombin concentration was 1.5u/ml.
Results are expressed as percentage of control.



Hours after operation



Hours after operation

CHAPTER 12

GENERAL DISCUSSION AND CONCLUSIONS.

Much of the work on coagulation has centered on the enzyme cascade which ultimately converts prothrombin to thrombin which in turn leads to the conversion of fibrinogen to fibrin. Until 1947, the properties of the end product of this series of reactions - the fibrin network - had not been studied. A major reason for this was a lack of quantitative methods to characterize networks.

It has become possible recently to measure mass-length ratios (i.e. μ_P and μ_T) to characterize fibrin networks. These methods have been advanced as providing quantitative measurements conveying greater information on network characteristics than the previously used opacity ratio. Why μ_T and μ_P measurements are considered more accurate has been fully elaborated elsewhere (Chapter 1). The question whether the newer technique actually provides more information about the network than the other methods needs to be examined.

Mass-length ratio is purported to provide an indication of fibre thickness. The methods are based on an assumption that fibrin fibres are made up of long, rod-

like particles (Hangten and Hermans, 1979). However, a fibre or chain bundle need not necessarily be of uniform radius all through its length. Furthermore it is known (Shah *et al*, 1982) that in any given network there is a range of fibre diameters differing by a factor of 10. A single value of mass length ratio thus is an average value. Therefore, in general terms both opacity ratio and the newer methods simply state whether fibres have become thicker or thinner. Nonetheless there are several advantages in using mass-length ratio as the preferred method and these have been discussed fully in Chapter 1.

The newer techniques used in this thesis have led to a better understanding of the mechanism of interaction during fibrin monomer polymerization and fibrin assembly. Possibly because initially methodology was directed to clarifying biophysics and biochemistry of monomers to polymer conversion, alterations in network characteristics induced by physiological variation in clotting conditions remained unexamined until recently. Ferguson (1983) made the first attempt to investigate this. However, as described in detail in Chapter 1, his investigations were unsatisfactory.

The investigations described in this thesis are concerned in the first place with examining the effect of changes in pH, ionic strength, ionic composition, fibrinogen concentration and temperature in the range compatible with physiology. A second major deficiency in previous studies has been the lack of critical appreciation of the differences between fibrin networks developed in pure fibrinogen systems and those in plasma. This area has been fully explored in this thesis and some of the differences between fibrin networks in the two systems have been ascribed to the presence of certain factors in plasma. In the third place theoretical reasoning underlying the methods have been critically examined to determine the applicability of the methods to a study of networks in plasma. This has been followed by experimental studies directed not only towards determining reproducibility and reliability but also towards standardization of the techniques when applied to plasma. Finally, the question of whether the methods are applicable to plasma in a clinical setting for sequential study has been examined by an investigation into the effect of operation and dextran infusion in patients undergoing abdominal operation. Detailed discussion follows.

12.1 Studies in Fibrinogen Solutions.

Three methods described in Chapter 2 have been extensively used to characterize fibrin networks formed in pure fibrinogen solutions using a range of thrombin and fibrinogen concentrations which would be regarded as pathophysiological (Chapter 4). Using these techniques, the effects of physiological variation in pH, ionic strength, ionic composition and temperature (Chapter 5) were investigated. It was shown that all these factors when varied within the physiological range are able to modulate the structure and behavioural characteristics of fibrin network.

Studies based on fibrin conversion and turbidimetric observations during formation of fibrin networks have provided important insights into the mechanism of network growth. These observations are especially significant in light of recent studies on fibrin polymerization (Carr *et al*,1972; Carr and Hermans, 1978; Hangten and Hermans, 1979) which have investigated networks formed under non-physiological conditions. Investigations described in Section 1 established that kinetics of network development determine the final structure. It was also

shown that kinetics of thrombin action and of fibrin assembly are separately regulated. Lowering thrombin or decreasing temperature, for example, decreases the amount of fibrin in the protofibril network formed during the lag phase. This leaves a larger amount of fibrin available for subsequent incorporation, thus resulting in thicker fibres. On the other hand when enzyme kinetics are not modified, for example when pH or ionic strength is increased (Chapter 5), but assembly of available monomer is slowed, a greater amount of monomer is incorporated into protofibril network leaving smaller amounts for subsequent incorporation. This results in thinner fibres.

Studies on networks developed in fibrinogen solution have been made using techniques which overcame deficiences in previous work. Investigations described in Chapter 1 showed that network continues to develop well after the gel point. Many of the previous studies (Ferguson, 1983) have ignored this and investigations have not been made on completely formed networks. In this investigation it was shown that time taken for complete development of networks depends on the concentration of thrombin used. Appropriate steps were taken to ensure that all

observations were made on completely formed networks. Methodological studies also established that mass-length ratio can be confidently measured to detect alterations in fibrin networks developed under physiological conditions of clotting.

12.2 A Comparison of Networks Formed in Fibrinogen Solution and Plasma.

Investigations described fully in Chapter 7 in this thesis show that methods used to determine mass-length ratio from turbidity and permeability in networks developed in fibrinogen solution are also applicable to plasma. However, comparative studies on network developed in fibrinogen solution and human plasma (Chapter 8) have shown important differences even when the net fibrinogen converted to fibrin is similar in both systems. When high concentrations of thrombin are used networks in plasma have significantly thicker fibres than those in fibrinogen solution. This difference was shown to arise from differing kinetics of fibrin assembly in the two systems (Chapter 8). When thrombin concentration is increased, fibrin fibres are rendered thinner in the fibrinogen solution but fibre thickness remains unchanged

in the plasma. From this and similar observations it is clear that conclusions derived from purified fibrinogen solutions should be extended to the plasma with considerable caution and for physiological studies, investigations should be made using plasma.

Since differences in network characteristics in plasma and in fibrinogen solutions could arise from some of the proteins, extensive studies were undertaken in Chapter 9 to explore this possibility. It was shown that albumin, γ-globulin, fibronectin and antithrombin singly (and presumably in combination) are capable of modifying fibrin network structure. Since developing thrombus contains cellular elements of blood and since little is known about the role of these in the regulation of network structure, a study was undertaken to examine the role of cellular elements in modulating fibrin network structure (Chapter 10) in plasma and in fibrinogen solution. It was shown that soluble components of platelets have spectacular effects in modifying the final behaviour and structural properties of fibrin networks developed in either plasma or pure fibrinogen solution. Red cell membranes were found to reduce fibre thickness and network permeability in fibrinogen solution. In

plasma fibre thickness derived from turbidity was found reduced but not μ_P or permeability. These results re-emphasize the conclusion that observations in fibrinogen solution need not necessarily extend to the plasma.

Investigations described in this thesis have identified several determinants of fibrin network characteristics - proteins in plasma and possibly cellular elements as well. The latter area needs further examination. Whether these various factors modulate networks in response to a variety of physiological demands made on it remains unknown. Equally it is unknown whether alterations in networks induced in pathological states themselves initiate haemostatic and thrombotic problems. These and related areas should be fully explored in the future.

12.3 Clinical Studies.

From the foregoing discussion it is clear that until recently methods were not available for investigating fibrin networks in artificial media at fibrinogen concentrations in the physiological range. Such methods have only just become available and a critical evaluation was undertaken in Chapters 8 and 9 (also Nair *et al*,

1986). It is evident that these techniques are theoretically and experimentally valid when applied to studies on human plasma. Detailed investigations showed that although healthy individuals vary in their network characteristics within a certain range, in any one individual under basal conditions fibre thickness is a reproducible measurement, not only at different times in one day, but also from one day to the next. It was also shown that blood samples may be stored for one hour without altering network characteristics. Anticoagulant used has also been shown to be of critical importance (Nair *et al*, 1986). A standardized technique of venepuncture, blood collection and handling of plasma developed in this thesis allowed sequential studies with considerable confidence using the methods described in Chapters 2 and 7.

Two investigations were undertaken to determine applicability of the methods. In patients undergoing elective general surgery (Chapter 11) the methods were used to show consistent changes in network structure in the post-operative period. In the study on the antithrombotic activity of dextran (Chapter 11) it was

demonstrated that dextran 70 has spectacular effects in increasing network fibre thickness and permeability immediately and for up to 24 hours after infusion in patients.

12.4 Bimodality and its Implications.

Experimental observations on μ_P and μ_T, both of which purport to measure fibre thickness, do not always alter in parallel under all experimental conditions (Chapters 4 and 11). Under certain conditions - for example variation in fibrinogen concentration at high thrombin (Chapter 4) - minute changes in one network can influence the bulk characteristics of the whole network. In other situations - for example the effect of increasing dextran concentration . (Chapter 11), the minor and major network change in a similar direction making the network as a whole more permeable. In patients undergoing abdominal surgery (Chapter 11) μ_P, μ_T and Cn were found increased postoperatively In this situation, fibrin fibres become thicker but despite this, permeability remains unaltered. Since it has been shown that μ_T is in the main a measurement of the major network fibres, and μ_P is greatly influenced by the magnitude and stability

of the minor network, it follows that the relationship between μ_T and μ_P is not fixed and invariant. It would seem that bimodality allows network characteristics to be altered flexibly to meet with varying physiological demands placed on the network.

A corollary of the bimodality of the size is the need for a dual approach to studies on network characteristics in which both μ_T and μ_P give valuable, independently derived information.

12.5 Future Studies on Fibrin Network Structure.

It is clear from the investigations in this study that these techniques which have been demonstrated to be applicable to plasma in sequential studies can be used to examine clinical areas in which interesting information on the pathogenesis of degenerative disease and thrombosis may be anticipated. Some areas which may be of interest include the role of fibrin network in diabetes and malignant diseases.

LIST OF PUBLICATIONS AND PRESENTATIONS TO LEARNED SOCIETIES DURING Ph.D SCHOLARSHIP.

PUBLICATIONS.

1. Shah, G.A., **Nair, C.H.** and Dhall, D.P.
"Physiological studies on fibrin network structure."
Thromb. Res. 40; 181 - 188, 1985.

2. **Nair, C.H.**, Shah, G.A. and Dhall, D.P.
"Effect of temperature, pH and ionic strength and composition on fibrin network structure and its development."
Thromb. Res. 42, 809- 816, 1986.

3. Shah, G.A., **Nair, C.H.** and Dhall, D.P.
"Comparison of fibrin networks in plasma and fibrinogen solution."
Thromb. Res. accepted for publication.

4. Nair, C.H., Shah, G.A. and Dhall D.P.
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In press, 1986.

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1. **Nair, C.H.**, Shah, G.A. and Dhall, D.P.
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"Influence of kinetics of network growth on fibrin network structure".
Thromb.Res. (Suppl.IV), 63, 1986

PAPERS GIVEN TO LEARNED SOCIETIES.

1. **Nair, C.H.,** Shah, G.A., Ferguson, I.A. and Dhall, D.P.
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2. **Nair, C.H.,** Shah, G.A. and Dhall, D.P.
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3. **Nair, C.H.,** Shah, G.A. and Dhall, D.P.
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4. Shah, G.A., **Nair, C.H.** and Dhall, D.P.
"Clinical relevance of fibrin network structure."
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5. **Nair, C.H.,** Shah, G.A. and Dhall, D.P.
"Fibrin network structure: bimodality and its implications."
Symposium and workshop on Recent Advances in Haemostasis and Thrombosis, Melbourne, 30 November - 1 December, 1984.

6. **Nair, C.H.,** Shah, G.A. and Dhall, D.P.
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8. **Nair, C.H.,** Shah, G.A. and Dhall, D.P.
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APPENDICES

APPENDIX 1

REFRACTIVE INDEX MEASUREMENTS.

Materials and Methods.

Refractive index measurements of fibrinogen solution or plasma were made at 22°C using a ABBE-refractometer Model A calibrated for 589.3nm wavelength. Refractive indices were measured at a wavelength of 615nm which was found not to be significantly different from those measured at 589.3nm (Ferguson, 1982). These latter values were thus assumed to be not significantly different from 608nm.

Results and Discussion.

The refractive index of Tris-saline was found to be 1.336 and increased slightly but significantly with fibrinogen solution. The refractive index of 10mg/ml fibrinogen in tris-saline was 1.338.

The refractive index of plasma from ten healthy individuals was 1.347 ± 0.004. Refractive index of plasma from patients undergoing surgery was not significantly different.

The refractive indices of defibrinated plasma enriched with fibrinogen (Chapter 8) did not vary significantly.

The refractive indices used to calculate μ_T in purified fibrin and in plasma clots were 1.336 and 1.347 respectively. Calculations show that a difference in refractive index of 0.002 affects μ_T by an insignificant 0.2% It is possible to calculate μ_T from plasma and fibrinogen, therefore from that same refractive index without much introduction of error in the final computation.

dn/dc used in calculating μ_T was a value of 1.001 calculated from data presented by Carr et al (1977).

APPENDIX 2

TABULATED DATA OF THE EFFECTS OF CHANGES IN CLOTTING CONDITIONS AND THE EFFECT OF VARIOUS SUBSTANCES ON FIBRIN NETWORK STRUCTURE

The following data details the effect on network structure of:

(1) Thrombin concentration

(2) Fibrinogen concentration

(3) Ionic strength

(4) pH

(5) Temperature

(6) Common ions

(7) Thrombin concentration on:
a) networks developed in fibrinogen solution
b) networks developed in plasma

(8) Fibrinogen concentration in defibrinated plasma

(9) Albumin

(10) γ-globulin

(11) ATIII

(12) Fibronectin (physiological conc)

(13) Fibronectin concentration

(14) Platelet extract on networks developed in:
a) fibrinogen solution
b) plasma

(15) Red cell ghosts on networks developed in:
a) fibrinogen solution
b) plasma

(16) Dextran

(17) Surgery

(18) Surgery and dextran

Networks were developed as described in Chapter 2 and in other chapters.
NA, or (-) indicate experiment not done.
Data is presented as mean of at least three experiments ± SEM.

APPENDIX 2

	$\mu_T \times 10^{12}$ (daltons/cm)	Permeability (T) $\times 10^{11}$ (cm^2)	$\mu_P \times 10^{12}$ (daltons/cm)	Compaction (%)	Cn (mg/ml)
Networks Developed In Fibrinogen solution					
1. Effect of Thrombin					
0.15	35.0 ± 2.7	186 ± 14	20.0 ± 1.8	NA	2.28 ± 0.01
0.25	29.0 ± 1.6	170 ± 8	18.7 ± 1.0	NA	2.30 ± 0.01
0.50	22.0 ± 2.5	125 ± 6	13.7 ± 0.8	NA	2.30 ± 0.01
1.00	16.7 ± 2.0	97 ± 6	10.5 ± 0.8	NA	2.30 ± 0.01
1.50	13.7 ± 1.1	85 ± 5	9.0 ± 0.5	NA	2.28 ± 0.002
2. Effect of Fibrinogen					
a) 0.15u/ml Thrombin					
1.5	24.5 ± 0.3	389 ± 14	24.9 ± 0.8	NA	1.35 ± 0.01
2.5	29.0 ± 0.9	222 ± 17	23.5 ± 1.8	NA	2.25 ± 0.08
3.5	22.0 ± 1.4	175 ± 8	25.6 ± 1.1	NA	3.12 ± 0.11
4.5	16.7 ± 1.2	143 ± 8	27.2 ± 1.2	NA	4.05 ± 0.15
5.4	13.7 ± 0.6	113 ± 9	24.5 ± 1.0	NA	4.86 ± 0.18

	$\mu_T \times 10^{12}$ (daltons/cm)	Permeability (T) $\times 10^{11}$ (cm^2)	$\mu_P \times 10^{12}$ (daltons/cm)	Compaction (%)	Cn (mg/ml)
b) 0.5u/ml Thrombin					
1.5	17.3 ± 0.9	269 ± 21	17.3 ± 1.3	NA	1.37 ± 0.01
2.5	20.8 ± 1.2	172 ± 13	18.4 ± 1.2	NA	2.28 ± 0.01
3.5	22.4 ± 0.8	116 ± 10	17.4 ± 1.4	NA	3.19 ± 0.01
4.5	25.9 ± 1.0	87 ± 6	16.9 ± 1.2	NA	4.10 ± 0.08
5.4	29.1 ± 0.5	71 ± 2	16.5 ± 0.5	NA	4.91 ± 0.02
c) 1.5u/ml Thrombin					
1.5	11.7 ± 0.2	188 ± 17	12.0 ± 1.2	NA	1.35 ± 0.01
2.5	12.5 ± 0.3	111 ± 6	11.5 ± 1.0	NA	2.25 ± 0.02
3.5	12.1 ± 0.4	64 ± 4	9.3 ± 0.5	NA	3.19 ± 0.01
4.5	11.0 ± 0.3	38 ± 6	7.3 ± 0.3	NA	4.01 ± 0.02
5.4	11.5 ± 0.6	33 ± 3	7.6 ± 0.7	NA	4.81 ± 0.03

	$\mu_T \times 10^{12}$ (daltons/cm)	Permeability (T) $\times 10^{11}$ (cm^2)	$\mu_P \times 10^{12}$ (daltons/cm)	Compaction (%)	Cn (mg/ml)
3. Ionic Strength					
0.133	26.3 ± 1.8	265 ± 12	27.2 ± 1.0	NA	2.23 ± 0.00
0.143	20.7 ± 1.8	211 ± 11	22.0 ± 0.8	NA	2.23 ± 0.02
0.153	17.5 ± 2.0	148 ± 7	15.1 ± 0.7	NA	2.18 ± 0.03
0.163	14.0 ± 2.0	107 ± 10	11.2 ± 1.0	NA	2.20 ± 0.02
0.173	9.1 ± 2.1	56 ± 16	5.8 ± 0.9	NA	2.23 ± 0.02
4. pH					
7.15	32.6 ± 2.0	274 ± 12	27.7 ± 1.0	NA	2.17 ± 0.01
7.25	31.4 ± 1.3	211 ± 15	21.1 ± 0.7	NA	2.16 ± 0.01
7.35	25.8 ± 1.8	162 ± 18	16.4 ± 1.0	NA	2.19 ± 0.02
7.45	23.9 ± 1.7	116 ± 13	13.2 ± 1.0	NA	2.22 ± 0.02
7.55	22.8 ± 1.3	110 ± 14	11.2 ± 0.7	NA	2.17 ± 0.01

	μ_T x 10^{12} (daltons/cm)	Permeability (T) x 10^{11} (cm^2)	μ_P x 10^{12} (daltons/cm)	Compaction (%)	Cn (mg/ml)
5. Temperature.					
15°C	26.8 ± 0.4	185 ± 1	19.6 ± 0.9	NA	2.23 ± 0.02
24°C	22.0 ± 0.5	159 ± 7	16.9 ± 0.6	NA	2.25 ± 0.02
30°C	23.7 ± 1.5	163 ± 8	17.2 ± 0.8	NA	2.20 ± 0.04
37°C	21.2 ± 1.6	171 ±24	17.8 ± 1.4	NA	2.19 ± 0.02
6. Common Ions					
Control	25.7 ± 0.9	450 ± 40	45.0 ± 4	NA	2.13 ± 0.02
4.30mM KCl	25.9 ± 0.6	413 ± 26	41.3 ± 3	NA	2.10 ± 0.02
0.35mM Na_2SO_4	19.3 ± 0.9	385 ± 22	28.8 ± 2	NA	2.13 ± 0.02
1.11mM NaH_2PO_4	24.4 ± 0.5	420 ± 22	41.3 ± 3	NA	2.12 ± 0.01
29.00mM $NaHCO_3$	24.7 ± 0.6	272 ± 21	38.3 ± 2	NA	2.11 ± 0.01

	$\mu_T \times 10^{12}$ (daltons/cm)	Permeability (T) $\times 10^{11}$ (cm^2)	$\mu_P \times 10^{12}$ (daltons/cm)	Compaction (%)	Cn (mg/ml)
Comparison Of Networks.					
7. Effect Of Thrombin Variation.					
a) Networks Developed In Fibrinogen.					
0.1875	19.46 ± 0.90	127 ± 18	12.9 ± 1.8	NA	2.13 ± 0.01
0.375	12.69 ± 1.50	85 ± 14	8.8 ± 1.4	NA	2.16 ± 0.02
0.75	7.96 ± 1.00	39 ± 10	4.1 ± 1.0	NA	2.15 ± 0.01
1.50	5.65 ± 0.46	20 ± 5	2.0 ± 0.5	NA	2.15 ± 0.01
b) Networks Developed In Plasma.					
0.1875	19.87 ± 0.87	1866 ± 505	111.4 ± 25	65 ± 1	1.29 ± 0.01
0.375	18.83 ± 0.68	745 ± 106	47.0 ± 9	51 ± 1	1.63 ± 0.02
0.75	18.03 ± 0.69	469 ± 39	41.2 ± 4	40 ± 1	1.87 ± 0.01
1.50	18.89 ± 0.23	361 ± 25	35.2 ± 2	31 ± 2	2.10 ± 0.01

	$\mu_T \times 10^{12}$ (daltons/cm)	Permeability (T) $\times 10^{11}$ (cm^2)	$\mu_P \times 10^{12}$ (daltons/cm)	Compaction (%)	Cn (mg/ml)
8. Effect Of Fibrinogen In Defibrinated Plasma					
2mg/ml	12.93 ± 0.20	365 ± 38	23.8 ± 0.8	27 ± 1	1.54 ± 0.9
3mg/ml	12.55 ± 0.50	231 ± 18	22.9 ± 1.0	20 ± 1	1.98 ± 0.01
4mg/ml	13.27 ± 0.60	179 ± 7	20.5 ± 0.5	16 ± 1	2.36 ± 0.01
5mg/ml	13.33 ± 0.50	96 ± 5	14.8 ± 1.0	8 ± 1	2.75 ± 0.01
9. Effect Of Albumin.					
Control	17.74 ± 0.15	271 ± 28	28.6 ± 2.6	NA	2.23 ± 0.04
Albumin (4mg/ml)	11.68 ± 0.41	162 ± 19	16.7 ± 1.9	NA	2.18 ± 0.03

	μ_T x 10^{12} (daltons/cm)	Permeability (T) x 10^{11} (cm^2)	μ_P x 10^{12} (daltons/cm)	Compaction (%)	Cn (mg/ml)
10. Effect Of γ-globulin					
Control	26.61 ± 1.45	18.5 ± 1.2	206 ± 12	NA	2.19 ± 0.02
γ-globulin (6mg/ml)	30.08 ± 1,28	28.7 ± 1.5	312 ± 16	NA	2.18 ± 0.02
11. Antithrombin III					
Control	30.04 ± 1.50	258 ± 27	26.9 ± 2.3	NA	2.13 ± 0.01
ATIII (5u/ml)	21.60 ± 0.42	268 ± 55	26.6 ± 3.2	NA	2.10 ± 0.02
12. Fibronectin					
Control	16.86 ± 1.43	116 ± 15	12.0 ± 1.4	NA	2.20 ± 0.01
Fibronectin (300ug/ml)	31.89 ± 3.25	256 ± 31	26.1 ± 3.2	NA	2.15 ± 0.02

	$\mu_T \times 10^{12}$ (daltons/cm)	Permeability (T) $\times 10^{11}$ (cm^2)	$\mu_P \times 10^{12}$ (daltons/cm)	Compaction (%)	Cn (mg/ml)
13. Dose Response Curve For Fibronectin					
0ug/ml	16.66 ± 0.91	150 ± 16	13.9 ± 1.5	NA	1.99 ± 0.07
150ug/ml	17.27 ± 0.48	186 ± 29	18.1 ± 3.1	NA	2.05 ± 0.03
300ug/ml	20.05 ± 1.35	192 ± 10	18.3 ± 1.4	NA	2.01 ± 0.05
450ug/ml	22.39 ± 1.14	181 ± 13	16.8 ± 1.2	NA	1.98 ± 0.05
14. Platelet Extract. Fibrin.Solution.					
Control	16.25 ± 1.18	126 ± 16	12.2 ± 1.6	NA	2.05 ± 0.05
Platelet Extract	12.48 ± 0.63	84 ± 13	7.9 ± 1.2	NA	1.98 ± 0.03

	$\mu_T \times 10^{12}$ (daltons/cm)	Permeability (T) $\times 10^{11}$ (cm^2)	$\mu_P \times 10^{12}$ (daltons/cm)	Compaction (%)	Cn (mg/ml)
b) Platelet Free Plasma.					
Control	15.51 ± 1.86	406 ± 73	66.9 ± 14	28 ± 0.5	1.89 ± 0.2
Platelet Extract	10.83 ± 0.45	380 ± 50	33.5 ± 8.6	19 ± 0.5	1.92 ± 0.2
Platelet Aggregate	12.08 ± 1.48	691 ±140	69.1 ± 1.41	22 ± 0.3	1.75 ± 0.35
15. Effect Of Red Cell Ghosts.					
a) Fibrinogen.					
Control	38.63 ± 2.64	375 ± 9	33.9 ± 0.5	NA	1.917 ± 0.04
Red Cell Ghosts	23.26 ± 1.84	197 ± 14	18.5 ± 1.17	NA	1.98 ± 0.03

	$\mu_T \times 10^{12}$ (daltons/cm)	Permeability (T) $\times 10^{11}$ (cm^2)	$\mu_P \times 10^{12}$ (daltons/cm)	Compaction (%)	Cn (mg/ml)
b) Plasma					
Control	18.73 ± 0.5	558 ± 6	49.6 ± 8.8	26 ± 0.5	1.83 ± 0.02
Red Cell Ghosts	21.15 ± 1.17	526 ± 4	45.1 ± 6.1	25 ± 0.2	1.80 ± 0.03
16. Effect Of Dextran.					
Control	16.85 ± 0.98	460 ± 6	33.5 ± 0.6	NA	2.25 ± 0.01
Dextran 40, 0.225%	20.54 ± 0.34	582 ± 17	43.1 ± 0.7	NA	2.25 ± 0.01
0.45%	23.33 ± 0.79	718 ± 30	52.3 ± 1.6	NA	2.25 ± 0.01
0.9%	32.44 ± 1.10	1063 ± 67	77.8 ± 6.1	NA	2.25 ± 0.01
1.20%	36.01 ± 1.86	1361 ± 77	98.1 ± 7.8	NA	2.25 ± 0.01
Control	18.40 ± 1.25	464 ± 23	34.2 ± 2.2	NA	2.25 ± 0.01
Dextran 70, 0.225%	22.83 ± 1.51	579 ± 18	43.6 ± 0.4	NA	2.25 ± 0.02
0.45%	25.57 ± 1.38	735 ± 12	55.4 ± 2.1	NA	2.25 ± 0.01
0.98%	31.67 ± 1.89	1032 ± 51	77.9 ± 5.2	NA	2.25 ± 0.02
1.20%	36.63 ± 2.94	1239 ±128	93.3 ±10.1	NA	2.25 ± 0.01

	$\mu_T \times 10^{12}$ (daltons/cm)	Permeability (T) $\times 10^{11}$ (cm^2)	$\mu_P \times 10^{12}$ (daltons/cm)	Compaction (%)	Cn (mg/ml)
17. Effect Of Surgery					
Pre-op	22.68 ± 3.16	441 ± 53	44.8 ± 3.3	NA	2.25 ± 0.20
Post-op 24hrs	24.37 ± 1.84	431 ± 53	50.0 ±.4.6	NA	2.48 ± 0.20
72hrs	30.21 ± 4.59	411 ± 49	64.3 ± 5.0	NA	3.03 ± 0.20
18. Effect Of Surgery And Dextran					
Pre-op	22.26 ± 3.00	348 ± 36	27.41 ± 22.9	23 ± 1	1.74 ± 0.25
Post-op 4hrs	34.12 ± 5.39	883 ±153	74.04 ± 15.9	41 ± 3	1.65 ± 0.17
24hrs	34.16 ± 4.47	418 ± 26	43.92 ± 2.1	34 ± 2	2.21 ± 0.13
72hrs	30.00 ± 6.49	305 ± 44	42.79 ± 6.6	31 ± 3	3.03 ± 0.21